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LYMPHOCYTE STIMULATION

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LYMPHOCYTE STIMULATION

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LYMPHOCYTE STIMULATION

Completely revised edition

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Preface to second edition

The tremendous expansion of interest in the lymphocyte, in the seven years since the first edition was prepared, has been at once encouraging and bewildering. The rewriting has taken over three years and much more time and effort than we had anticipated. Ch. 1 remains as in the first edition but the rest of the book has been completely rewritten. It was originally intended to include a chapter on 'Lymphocyte activation in relation to disease' but this was finally omitted to avoid delay in publication. Although all the chapters have been revised during the last few months there are limits to the number of alterations which can be made and we are well aware of many remaining imperfections and omissions. References to the literature could not possibly be comprehensive and if many pioneers in the various fields feel that they have not been given proper recognition it is because we have concentrated on providing the reader with good leads into the literature by selecting recent important papers, particularly those which contain a good bibliography. The flood of publications, often in new and inaccessible journals, has unfortunately increased the difficulties of the researcher not attached to a big centre. It is one of the many undesirable side-effects of rapid progress in any scientific field.

We have tried to be objective without being aloof, airing our views but restraining our prejudices. This has not been easy because we have both found that we are most likely to differ from the majority opinion when we know most about the particular subject reviewed. We do not pretend that we are ourselves always in agreement and readers may like to know that chs. 12, 13 and 14 were written by J. K., ch. 6 jointly and the remainder by N. L.

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April 1974

N. R. Ling
J. E. Kay

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The transformation phenomenon

1.1. The survival and growth of leucocytes in vitro

When leucocytes from the peripheral blood, suspended in their own serum and a simple culture medium, are left at 37 °C for several days in an airtight container, some of the leucocytes degenerate but others remain viable and morphologically little changed. The cells which degenerate are the polymorphonuclear leucocytes (PMN). The eosinophils survive with little morphological change. The monocytes, while still recognisable from their nuclei, have increased in size and a cytoplasm, containing large vacuoles and frequently ingested debris, is bounded by an ill-defined cell membrane. They have matured into cells indistinguishable from tissue macrophages or histiocytes. The lymphocytes, which are now the most numerous of the viable cells, are also largely unchanged. No dividing cells are present unless the cultures are continued for a long period (say 7 days or more) and even then are few in number. If the whole procedure is now repeated, but with the addition of a small quantity of an extract of red kidney bean, *Phaseolus vulgaris*, to the culture fluid, marked changes occur in the morphology of some of the cultured cells. Large, active cells appear, with large nuclei and prominent nucleoli and, on the third or fourth day of culture, mitotic cells are to be seen. From this superficially simple phenomenon, first clearly described by Hungerford et al. in 1959 and Nowell (1960) stems most of the work described in this volume. The large, active cells are variously referred to in the literature as blast cells, transformed cells or stimulated cells. These terms will be used throughout the book. The use of the term 'transformed' cell may be misleading since it is used in a rather different sense by virologists; but it was used before the more accurate terms 'activated' or 'stimulated' were commonly adopted and shows no sign of disappearing from the

literature. Occasionally the term 'blastoid cell' is also used and the more general term 'large pyroninophilic cell', which is usually applied to all blast cells found in lymphoid tissue regardless of origin, also includes cells of this type.

1.1.1. The lymphocyte origin of the blast cells

The recognition that mitotic cells could be regularly obtained from peripheral blood created tremendous interest among workers in the field of human genetics. Further technical advances followed rapidly. Low concentrations of colchicine were added to accumulate mitoses arrested in metaphase and, after Moorhead et al. (1960) had devised a fixing and spreading technique for the display of chromosomes, cytological analysis became practicable and reliable. The method has been in general use throughout the world since 1960 and many important contributions in the field of human genetics have been made with it. The investigation of the nature of the cell which is mitotically activated by substances in extracts of *Phaseolus vulgaris* (phytohaemagglutinin, hereafter referred to as PHA) was not, at first, a matter which was pursued with any urgency and the result does not seem to have been awaited with great interest. The responsive cell was generally thought to be a monocyte or a large lymphocyte. Carstairs (1961, 1962), working at St. George's Hospital, London, was the first to show clearly that the small lymphocytes were the source of the dividing cells. At this time, he noted, two contradictory views on the nature of the small lymphocyte were held. The first view was: 'The small lymphocyte is a poor sort of cell, characterised by mostly negative attributes; small in size with especially little cytoplasm, unable to multiply, dying on the least provocation, surviving in vitro for only a few days, living in vivo for perhaps a few weeks'. The other view, expressed by Yoffey and Courtice (1956) was: '... the small lymphocyte is a specialised form of mesenchymal cell in a resting, relatively inactive state and reduced to the smallest possible size for the purpose of easy mobilisation and transport through the blood stream'. The demonstration by Carstairs, that the small lymphocyte could be activated to a dividing state was thought to be evidence in favour of a pluripotential role for it, as may be judged from the title of one of his papers.

Carstairs treated blood with iron-carbonyl particles to remove phagocytes and with PHA to sediment erythrocytes and provide stimulant. The initial nucleated cell content of his cultures was $0.56 \times 10^6/\text{ml}$, of which 83% were small lymphocytes, 12% medium and large lymphocytes and less than 0.1%

monocytes. Within 24 hr of setting up the cultures there were many cells which resembled medium lymphocytes. At 36 hr many cells contained deeply basophilic cytoplasm and the nuclei possessed prominent nucleoli. At 48 hr mitotic figures were common and many cells which superficially resembled reticulum cells, plasmablasts or proerythroblasts from a megaloblastic marrow were observed to be present. Nucleoli were prominent and sometimes irregular in outline. In the cytoplasm a few cells had a sprinkling of granules similar to the non-specific granules of promyelocytes. Vacuoles were common in the cytoplasm. By the end of the third day these cells formed almost the entire population. During the time taken for the small lymphocytes to drop from 83 to 25% of the nucleated cells (about 36 hr) there was a decrease in total count of approximately 10%. After the first 38 hr the population of small lymphocytes decreased still further, but in cultures continued beyond the period of maximum mitotic activity (68 to 86 hr) the number of small lymphocytes began to increase. These morphological observations fitted well with the observations of Cooper et al. (1961) on the uptake of tritiated thymidine ($^3\text{H-TdR}$) into deoxyribonucleic acid (DNA) in PHA-stimulated leucocyte cultures. At 24 hr they found a sharp increase in $^3\text{H-TdR}$ -positive cells and at 70 hr 50% of the mononuclear cells were labelled.

1.1.2. Early observations of blast cells in leucocyte cultures

It might be presumed, from the accounts given in the preceding sections, and in the other chapters, that lymphocytes had not been cultured and observed to undergo morphological changes until the late 1950s. This presumption would be erroneous. The movements of rabbit blood lymphocytes kept at 37 °C were examined microscopically by Ranzier in 1875 and, as early as 1888 Renant was suggesting that lymphocytes circulated in vivo and that some transformed and differentiated into connective tissue cells (both authors quoted by Coulson 1966). To a certain extent what an investigator finds is influenced by what he is looking for. During periods when controversy concerned the relative importance of cellular and humoral immunity many experiments were designed to discover whether or not leucocytes (usually without attempt to subdivide them) produced active protective substances. It was established in the 1890s that bacterial agglutinins found in the blood of a patient were preformed and that the titre was unaffected by the removal of the leucocytes, although it was considered by Metchnikoff that some bactericidal substances in serum might arise from the destruction or injury of

phagocytes. When the opposing ‘end-cell’ and ‘multipotent cell’ theories became the principle subject of argument, the emphasis, in leucocyte culture experiments, shifted to morphological observations of the relationships between lymphocytes and other cells. Maximow (1902) found blast cells (he called them polyblasts) in inflammatory exudates. He suggested that some of these blast cells arose from the mobilisation of local fixed histiocytes, others from lymphocytes and monocytes. Maximow strongly supported the contention that blood lymphocytes transform morphologically and functionally under an appropriate stimulus. He later observed that if tissue extracts were added to plasma clot cultures of rabbit lymph node cells, the lymphocytes hypertrophied into blast cells.

Scientific advances are sometimes divided into those which would not have been possible without the advent of a new technique (e.g. cellular ultra-structure could not have been examined before the development of the electron microscope) and those which have not required any new technique or equipment, or even knowledge, and could have been made much earlier. The phenomenon of lymphocyte transformation (consider, in particular, the mixed lymphocyte reaction, ch. 7) must be considered to be in the second category. Discoveries not married closely to technological advance may meet the charge that they are not new discoveries at all, but have already been reported in papers which have escaped attention. Although often factually correct these charges may be misleading. It could truthfully be claimed that Maximow and others observed lymphocyte transformation during the early part of this century. But it would have been difficult to have distilled this simple fact from the early literature, or even with confidence, from Maximow’s own papers. The blame for this, if it is not to rest with the original investigator, must rest with his contemporaries rather than with a later generation. Much of the early confusion arose from the complexity of the media used and the arbitrary inclusion of sera and tissue extracts of diverse origin. More arose from the conviction that macrophages and many other cell types could be derived from blood lymphocytes and the ease with which this conclusion was accepted. Some idea of the confused interpretations often placed upon experimental research which, by modern standards, was exceptionally painstaking and detailed may be obtained by reading the review by Bloom (1938): ‘... In normal blood in the first thirty-six hours great numbers of amoeboid cells were found which became more or less rounded up and resting at forty eight hours. Scattered among the numerous lymphocytes on the second day of culture many hypertrophic cells of mono-

cytic type were found which were arranged in groups and showed no mitoses. The cytoplasm of these cells seemed homogeneous and slightly basophile, the nucleus filled with coarse chromatin particles. In the protoplasm of these cells there were a few deep-blue nuclei of leucocytes. On the third and fourth days the number of lymphocytes continuously decreased and only occasionally is a normal lymphocyte found. On the fifth and sixth days the picture is dominated by cells with a basophile protoplasm and an eccentric, slightly indented nucleus which are (considered to be) hypertrophic monocytes. From these there are transitions to large, rounded cells, with darker, foamy basophile cytoplasm. They develop processes and some of them merge into fibroblast-like cells which have a weak basophilia and occasionally a slightly eosinophile cytoplasm ...' The following could be interpreted to be an early example of antigen-specific transformation: 'When rabbit lymphocytes were injected into living connective tissue and the connective tissue explanted with embryonic extract, the lymphocytes turned very rapidly into macrophages while they remained unchanged for many days if bone-marrow extract was used. The addition of whole *Ascaris* extract (1 in 20 in Tyrode solution) did not affect the lymphocytes. But when the lymph of *Ascaris*-immunised rabbits was injected into rabbit connective tissue with bone-marrow extract and *Ascaris* extract and cultured, myelocytes developed in 6 days in 3 out of 5 sets of cultures from the explanted small and large lymphocytes ...' Maximow deserves the credit for insisting that lymphocyte transformation was possible. He observed: '... Occasionally some of the lymphocytes in chick leucocyte cultures develop large amounts of deeply basophilic cytoplasm, the nucleus becomes vesicular and the nucleolus very large. Such cells are obviously undergoing a transformation into haemocytoblasts. In the course of the first ten to fifteen hours in vitro the lymphocytes and monocytes hypertrophy into small macrophages, usually with very few mitoses accompanying this process ...' Christschoff (1935) also impresses one as being ahead of his time in developing a leucocyte culture technique (using a medium containing embryo extract) suitable for the study of the karotype of dividing cells. Timofejewsky and Benewolenskaja (1926, 1928) appear to have been the only other authors who drew attention to the fact that mitotic activity might sometimes be found in leucocyte cultures and they almost certainly observed a leucocyte activation induced by tubercle antigens. When optimal concentrations of virulent mycobacteria tuberculosis were added to leucocyte cultures, lymphocytes and monocytes, they found, transformed into polyblasts and, eventually, into epithelioid cells. The selection, for com-

mendation, of those authors who obtained positive results, does not reflect discredit on those who, probably through failing to introduce foreign proteins in sufficient quantity into their media, obtained negative results and truthfully reported them. Veratti (1928) considered that lymphocytes did not undergo change in culture and that the macrophage-like cells which appeared were derived from monocytes. Lady Jean Medawar (1940) also found no evidence of transformation of lymphocytes into macrophages in cultures but was able to correlate the number of mononuclear phagocytes which appeared with the number of monocytes present in the original cell population.

1.1.3. PHA-induced transformation

The survival and reactivity of cells in vitro is liable to be affected by alteration of any of a large number of environmental factors such as pH, O₂ and CO₂ tensions, temperature, the nature and shape of the culture vessel, serum concentration, cell concentration, the culture medium and whether the cells are allowed to settle at the base of the container, or are mixed by continuous agitation. Conditions which are thought to be optimal are outlined in ch. 3. Lymphocytes may be spun down from serum or plasma and resuspended in fresh serum plus medium without damaging the cells, provided that mild centrifugation conditions are used. Repeated washing of peripheral leucocytes has been shown by Caron (1967) to cause an inconstant reduction in their ability to respond to PHA. The effectiveness of PHA in cultures is dependent, principally, on using a suitable quantity of an active preparation. The importance of the 'dose variable' was realised by Newsome (1963). In cultures containing 1% PHA, 4–13% of the lymphocytes underwent morphological change compared with 36–76% using 10% PHA. Dose-response curves for one of the varieties of PHA in common use, PHA-P (see ch. 11 for definition), were prepared by MacKinney (1964). A maximal response was obtained with 8.5 µg PHA-P/ml of culture fluid and a 50% response with 3.0 µg/ml. Approximately constant mitotic activity was obtained in the range 8.5 to 100 µg/ml. Very high concentrations of PHA-P were found by Wilson (1966) to be inhibitory and the same is true of PHA-M. PHA may be regarded as a universal stimulant for lymphocytes. It will stimulate a high proportion of the lymphocytes of most individuals and it will stimulate the lymphocytes of many animal species. Unresponsiveness of a lymphocyte population, when it is encountered, may be due to an abnormality of the cell population of that individual or to inhibitory substances in the serum.

Relatively pure populations of lymphocytes, from human thoracic duct lymph, undergo transformation in the same manner as blood lymphocytes (Lindahl-Kiessling et al. 1963). Cord blood lymphocytes are also responsive to PHA. In fact Lindahl-Kiessling and Böök (1964) found them to be more responsive than lymphocytes from the blood of adults. They also obtained a good PHA response using thymus lymphocytes from foetuses 17–21 weeks of age. The reactivity of lymphocytes in relation to tissue source is discussed in more detail in ch. 11. Many other substances, in addition to PHA, are now known to activate lymphocytes. Staphylococcal filtrate (SF) is another very potent stimulant. Other stimulants, e.g. tuberculin and foreign lymphocytes, are especially interesting from an immunological standpoint and have received attention from immunologists. Curiosity about the way in which the stimulants act, whether they be immunospecific in nature or not, has motivated other investigators.

Several reviews of lymphocyte transformation have appeared during the last few years (Robbins 1964; Cooper and Amiel 1965; Mellman 1965; Gowans 1966; Heath 1966).

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The properties of lymphocytes from various lymphoid tissues

2.1. What is a lymphocyte?

Most of us first encounter the lymphocyte in blood smears as a small round cell with a characteristic pachychromatic nucleus and a thin rim of cytoplasm lacking distinct granules and staining a pale blue. Cells of similar morphology are found in the spleen, bone marrow, lymph nodes, thymus and in other areas, e.g. Peyer's patches, tonsil, throughout the body constituting in toto, a considerable mass of tissue. The human body contains 1300 g of lymphocytes outside the blood, lymph nodes and bone marrow, 70 g in the bone marrow, 3 g in the circulating blood and 100 g in lymphatic tissue (Pulvertaft, 1959).

The lymphocyte is defined by haematologists as a round cell (diam. 7–12 μ) with scanty cytoplasm, which in stained preparations lacks basophilia but may contain azurophilic granules. The nucleus is round, sometimes indented, with chromatin arranged in coarse masses and without visible nucleoli. Small, intermediate or large forms are recognised. Immunologists, uneasy about the known functional heterogeneity of cells of this morphology, would prefer a definition indicative of the immunological role of the typical lymphocyte e.g. a cell which undergoes clonal expansion on encounter with antigen. Even this definition would cover two distinct cell types of different migratory properties and destined for different pathways of differentiation. But the real objection to attempting to redefine a term rooted in classical haematology is the continuing need for a descriptive term in the routine pathology laboratory. As yet no simple cytochemical tests are available for sub-dividing small round cells. It is obvious from the gross morphological changes which occur on stimulation of small lymphocytes that their size, the prominence of their nucleoli and the degree of basophilia of their cytoplasm

may simply reflect the state of activity of the cells. Thus, morphologically different lymphocytes may be part of a functionally similar population in different states of activation. Conversely, metabolically quiescent small lymphocytes which appear morphologically similar may be functionally dissimilar. In short, if the lymphocyte is to be defined purely on morphological grounds the term may cover cells with quite different biological properties. Lymphocytes may be sub-divided according to their surface antigens, their reactions to stimuli, their immunological potential, their migratory habits and their life-span.

2.1.1. Migratory properties, immunological role and life-span of lymphocytes

A large pool of lymphocytes is continuously circulating through the bone marrow, spleen, lymph nodes, Peyer's patches and other lymphoid foci in the body via the blood and lymphatics. After intravenous infusion of RNA-labelled thoracic duct lymphocytes into syngeneic rats, Gowans and Knight (1964) found that the many lymph nodes examined 24 hr later all contained labelled cells. Similar concentrations were found in lymphoid tissue at the root of the lungs. The labelled cells were observed, in sections, in the mid and deep zones of the cortex, but they did not penetrate into the germinal centres nor into the cuff of small lymphocytes surrounding them. Under the electron microscope it was shown that the walls of the post-capillary venules in the mid and deep cortical zones contained many small lymphocytes some of which appeared to be penetrating the endothelium while most lay under the endothelium and in the layers of the periendothelial sheath of connective tissue. In the spleen, localisation of labelled lymphocytes was found around the central arteriole. No labelled lymphocytes were found in the adult thymus but some entered the thymus of newborn rats. Labelled cells were present in Peyer's patches as well as in the nodes; they were found in the marrow but no labelled erythroid or myelocyte precursors were seen. The output of lymphocytes in the circuit, as judged from the numbers drained from the thoracic duct, was about 10^9 per day.

Evidence for an equilibrating pool of circulating lymphocytes has also been provided by the experiments of Hall and Morris (1965) on popliteal nodes of intact sheep. When cells in the nodes were labelled by continuous perfusion with $^{3}\text{H-TdR}$, it could be shown that in unstimulated nodes no more than 4% of the lymphocytes in the efferent lymph were actually formed in the node. Since it was shown that the afferent lymph conveyed few cells to the node, it was

clear that most of the lymphocytes in the efferent lymph must have come from the blood. Hall (1967) calculated that 30×10^6 lymphocytes per hour appear in the efferent lymph from a sheep lymph node weighing 1 g; 12% of the lymphocytes in blood flowing through the node migrated into the node. This evidence is important since it confirms that the recirculation of lymphocytes which several groups of workers have shown to occur in the rat, also occurs in other species. Most of the recirculating population is now known to be composed of thymus-derived lymphocytes. Non-thymus-derived lymphocytes are also found in rat thoracic duct lymph and have been shown to recirculate from blood to lymph but their migratory patterns have yet to be defined (Strober, 1972). Cells of the major recirculating population are readily removed by lymphatic drainage and mix readily with transfused lymphocytes. Another population is penetrated with difficulty by blood lymphocytes and releases cells into the thoracic duct lymph at a slow and constant rate. A sub-population of cytotoxic lymphocyte-like mononuclear cells appears to be confined mainly to the intravascular compartment. Some segregation of the recirculating population into lymph node-seeking and spleen-seeking lymphocytes is another recent achievement (Diamond and Lance, 1974).

Lymphocytes are so difficult to keep alive for very long in vitro that one views with some suspicion the reports that lymphocytes have a life-span of months or years in vivo. There is very good evidence, however, to support these claims. It is based upon the proportion of cells labelled at various times after continuous infusion of $^3\text{H-TdR}$ (tritiated thymidine) and the time taken for the disappearance of these labelled cells.

When $^3\text{H-TdR}$ is infused into rats over long periods it will label all newly formed cells. It was found that 50% of rat lymphocytes were replaced by labelled cells by the end of a month but 10% were still unlabelled after 3 months of infusion (Little et al., 1962). In another series of experiments of similar type (Robinson et al., 1965) rats were infused with $^3\text{H-TdR}$ for 6–9 months and the disappearance of label followed for 2–3 weeks after the infusion had ceased. Significantly lower grain counts were observed in 30% of the small lymphocytes suggesting the presence of two populations, the more heavily labelled having the fastest turnover. The median survival of small lymphocytes was about one month; 5–8% had a life-span of more than 9 months. Caffrey et al. (1962), after labelling rat lymphocytes by $^3\text{H-TdR}$ infusion, found a relatively rapid decrease in the proportion of labelled cells during the first and second weeks, again suggesting that two

populations of circulating small lymphocytes exist. Everitt et al. (1964) injected mice with ^3H -TdR every 6 hours for 1–11 days. One animal was sacrificed daily after the last injection. The percent of labelled small lymphocytes increased rapidly until about the 4th or 5th day, by which time labelling of short-lived cells approached 100%. Thereafter increase in per cent labelled cells was thought to be indicative of the rate of formation of the long-lived population. In blood about 66%, and in thoracic duct lymph about 90%, of the lymphocytes were judged to be long-lived, the remainder short-lived. The proportion of lymphocytes labelled was very high in the thymus, lower in the spleen, lower again in the blood and lowest of all in the thoracic duct lymph. Newly formed thoracic duct small lymphocytes with a short life-span have been shown to be the cells which characteristically accumulate in acutely inflamed tissue (Koster and MacGregor, 1970). All the measurements of life-span therefore indicate that the circulating lymphocytes are by no means a single uniform population. Even the θ -bearing cells in mice can be divided into long and short-lived varieties (Olsson and Claesson, 1973).

In humans the evidence is based on the occurrence of chromosome abnormalities in blood lymphocyte cultures many months or years after the patient had received X-irradiation or after the administration of radio-mimetic drugs (Fitzgerald, 1964). The most important studies were those of Buckton and Pike (1962) on patients treated with spinal X-radiotherapy for ankylosing spondylitis. The proportion of cells with unstable chromosome abnormalities rose to a peak value at 25 times the control level 2–3 weeks after the end of the treatment and then fell, but they were present at twice the control level as long as 5 years after treatment and continued near this level of frequency during the following years. The stable abnormalities rose to 12 times the control level immediately after the completion of treatment and thereafter did not change significantly for up to 20 years. The most plausible explanation of these findings is that the cells in which the unstable abnormalities occur are normally long-lived and that there have been few or no divisions since the abnormalities were induced.

Animals which for one reason or another (e.g. irradiation, chronic lymphatic drainage or neonatal thymectomy) have a low level of circulating lymphocytes are less capable of responding to immunological stimuli than normal animals (Gowans and McGregor, 1965). The lymphoid tissues of rats or mice subjected to neonatal thymectomy or chronic lymphatic drainage have been shown to be severely affected with the cortical areas of lymph nodes and the periarteriolar sheaths of the spleen denuded of lymphocytes.

The depleted areas have been called thymus-dependent areas and most of the cells seen in sections in this paracortical zone in normal animals are thymus-derived T lymphocytes. Germinal centres and medullary regions of nodes of T-deficient animals are much less affected. Impaired delayed hypersensitivity reactions and impaired capacity to reject grafts have been found in such animals but the capacity to form antibody has been less consistently affected depending on whether or not the antigen concerned is thymus-dependent (see Roitt, 1972).

The depletion of circulating cells produced by lymphatic drainage is probably more complete than that produced by neonatal thymectomy since it has been shown that some lymphoid cells (about 1% of the number obtained from normal mice) may be drained off from neonatally thymectomised mice. The immunological unresponsiveness produced by either of these procedures may be corrected by the injection of syngeneic (strain-compatible) cell suspensions consisting, in some cases, almost entirely of small lymphocytes. By employing different donor/recipient combinations it has been shown that survival of the injected cells is essential. By transferring lymphoid cells from normal mice or mice primed with antigen into syngeneic irradiated mice and testing their response to antigenic challenge (adoptive immunity) it has been possible to test the relative potency of cells from various sources. In their capacity to restore a primary or a secondary antibody response blood lymphocytes were approximately as good as lymph node or spleen lymphoid cells (Makela and Mitchison, 1965). Experiments of this kind have established that circulating lymphocytes, or a substantial portion of them, are cells which 'recognise' foreign antigens and initiate reactions against them. Such cells are sometimes referred to as 'immunologically competent cells'. They may be 'committed' or 'uncommitted' to particular antigens. 'Committed' lymphocytes are responsible, at least in part, for long-term immunological memory.

2.1.2. Sub-populations of lymphocytes

Before 1960 it was not uncommon to hear it suggested that lymphocytes had no known function. Between 1960 and 1965 many immunologists believed that all immunological potential was contained in the recirculating population of lymphocytes and that every small lymphocyte had the same capacity to develop into an antibody-producing cell or an effector cell active in graft rejection or in a delayed hypersensitivity reaction. Lymphocytes

were assumed to be heterogeneous only with regard to the specificity of their receptors for antigen and calculations based on the commonly accepted one-antigen, one-receptor, one-cell selective theory assumed that all the body lymphocytes were potential antibody-producers. Also the dividing-line between the morphology of antibody-forming cells and the typical circulating small lymphocyte became progressively thinner when it was shown that some small, round lymphocyte-like cells could manufacture antibody as well as plasma cells and plasmablasts. They could, for example, be found at the centre of some haemolytic plaques formed by lymphoid cells of animals immunised with sheep red cells.

At the same time it was also becoming a possibility, at least in the chicken, that the lymphoid system could be roughly divided into two separate morphological and functional units viz. the thymus, from which derived the cells thought to be principally involved in homograft and delayed hypersensitivity reactions and the Bursa of Fabricius, thought to control the development of germinal centres and the production of plasma cells making immunoglobulins. The results of neonatal thymectomy or chronic lymph drainage in mice and rats suggested that a functional division between thymus-dependent and independent reactions might be also present in mammalian tissues. It was shown that bone marrow cells of CBA mice bearing a T6 marker migrated both to the peripheral lymphoid tissues and to the thymus and that cells bearing the marker later migrated from the thymus to the peripheral tissues. The basis was thus provided for the now widely accepted view that one population of lymphocytes in peripheral tissue, B lymphocytes, having the capacity to differentiate into plasma cells after an antigenic stimulus, is derived from a bone marrow precursor which migrates to the peripheral lymphoid tissues without passing through the thymus while another population, the T lymphocytes, not capable of differentiating into antibody-forming cells and mainly concerned with 'cellular immunity' derives from a precursor which, although originally from the bone marrow, and possibly identical with the B stem cell, has been thymus-processed and has, as a result, undergone characteristic differentiation. One reason for the slow acceptance of this view by some was the difficulty in demonstrating, in the normal animal, that lymphocytes ever migrated in reasonable numbers from the thymus. Because of this and because of the restorative effects in thymectomised mice, of thymus fragments encased in millipore chambers thought to be cell excluding, the effects of thymectomy were attributed principally to deprivation of a thymic hormone factor rather

than of a population of cells which 'seeded' the peripheral lymphoid tissues. Another difficulty was that there appeared to be no obvious morphological distinction between the two types of lymphocyte. Since antibody-forming capacity was demonstrably present in relatively homogeneous rat thoracic duct lymphocyte preparations there seemed to be no reason to believe that all the lymphocytes present did not have this capacity. A crucial experiment by A. J. S. Davies established that lymphocytes of thymus origin (derived in his experiments from a T6-marked thymus implanted under the renal capsule of thymectomised CBA mice) were activated into division by the introduction of antigen but did not develop into antibody-forming cells. Antibody was formed by the host's own cells. Later experiments of Claman and Miller and their co-workers showed that lymphocytes from the thymus have an indirect but important role in antibody formation. Without their presence antibody to some antigens (thymus-dependent antigens) is markedly sub-normal but thymocytes, or the more mature thymus-derived peripheral lymphocytes, never develop into antibody-forming cells. An adequate level of T lymphocytes is necessary for the expression of 'cellular immunity', cells of this category having an important role in homograft and delayed hypersensitivity reactions (Davies et al., 1971; Miller et al., 1971; Owen, 1972).

In vitro tests to distinguish the two main populations of lymphocytes have been based upon the detection of distinctive antigenic determinants on one or both populations and upon their capacity to be mitotically activated in culture by stimulants thought to be specific for one or other of the populations. They have been separated on the basis of physical characteristics e.g. size, charge or density.

2.1.3. Presence of distinctive determinants on T and B lymphocytes

It is the intensive study of mouse lymphocytes which has shown that there are many distinguishing markers on the surface of T and B lymphocyte populations (see reviews by Raff and Cantor, 1971 and Waksman et al., 1972). The presence of relatively high concentrations of immunoglobulin on the surface of B lymphocytes, detectable by a membrane immunofluorescence test on live cells using fluorescein-conjugated anti-immunoglobulin, readily distinguishes them from T cells. Other markers which have been reported to be specific for B cells are mouse B lymphocyte antigen (MBLA) and a receptor for antigen-antibody-complement complexes (Eden et al., 1971). It would perhaps be better to regard some of the many markers now

being described as non-T markers as their specificity for plasma cell precursors has not been established. An appreciable number (3–14%) of the lymphocytes in mouse spleen lack both T (θ antigen) and B (immunoglobulin) cell markers (Stobo et al., 1973). Another problem is that the immunoglobulin on the surface of B lymphocytes is not, as might be expected, of the class which the cell progeny are destined to manufacture after they have differentiated into plasma cells. The immunoglobulin labelled by mild iodination of the surface proteins of the lymphocytes has been shown to be almost exclusively 8S IgM at a time when the cells can be shown, by simultaneous radio-amino acid incorporation studies, to be synthesising a similar amount of IgG (Vitetta et al., 1972). The immunoglobulin molecules are not rigidly fixed on the cell surface, as shown by the 'capping' and endocytosis which occurs after attachment of anti-immunoglobulin (see ch. 8), but fixation of complement occurs in the presence of antiserum and results in cell lysis. When mouse thoracic duct lymphocytes are treated with rabbit anti-mouse kappa chain serum (more than 95% of mouse L chains are of kappa type) and complement a selective killing of B lymphocytes results (Miller et al., 1972).

Antibodies directed against specific surface antigens have proved to be very useful reagents in identifying T lymphocytes in peripheral tissues and in studying thymocyte sub-populations (Raff and Cantor, 1971; Schlesinger, 1972). The antigens most studied have been the Thymus Leukaemia (TL) antigen which exists in four allelic forms and the θ antigen of which two alleles are known, one of which, θ AKR, is present on cells of only a few strains of mice. Antisera against the commoner (θ C3H) allele may be raised by immunisation of AKR mice with C3H thymus cells. The TL antigen is present on the cells of some (TL positive) leukaemias and is also present on thymocytes (but not mature peripheral T cells) of some (TL positive) strains of mice. The θ antigen is expressed on thymus cells and, in lower amount, on peripheral T cells. An antiserum to the θ antigen, in the presence of complement, is cytotoxic to θ -bearing cells and has been used for the detection and elimination of θ -bearing lymphocytes. The validity of θ as a T-cell marker has been confirmed by the finding of a very marked deficiency in the number of θ -positive lymphocytes in neonatally thymectomised mice and in 'nude' mice congenitally lacking a thymus. Brain cells also express the θ antigen and anti- θ sera have been prepared across species by immunising rabbits with mouse brain emulsified in complete Freund's adjuvant. Non-specific and species antibodies are removed by absorption with spleen cells

of a nude mouse. Mouse allo-antisera to the AKR θ allele will also react with rat T cells (Micheel et al., 1973).

2.1.4. Surface markers on human T and B lymphocytes

Human thymocytes and peripheral T cells have the unexpected property of forming rosettes with sheep red cells (Jondal et al., 1972; Papamichail, 1972; Wybran et al., 1972). Only human and pig lymphocytes appear to have this property although a related phenomenon has been described in mice (Dardenne and Bach, 1973). Guinea pig lymphocytes will form similar rosettes with rabbit red cells and rabbit thymocytes will rosette with red cells of some other rabbits (Binns et al., 1972; Wilson and Coombs, 1973). Antisera for the detection of thymic antigens have been prepared by immunising animals with human thymus or brain and absorbing with chronic lymphocytic leukaemic cells.

As in the mouse the presence of surface immunoglobulin is the hallmark of the B lymphocyte, but several other markers have also been discovered. A commonly used test, the formation of rosettes with EAC (sheep erythrocyte-IgM-antibody-complement) depends on the presence of receptors for C3 on B cells or a sub-population (Bianco et al., 1970; Stjernsward et al., 1973). Receptors for aggregated IgG (Fc determinant) are present on B lymphocytes (Dickler and Kunkel, 1972) but although absent from resting T lymphocytes may be present on activated T cells. Sheep erythrocytes coated with IgG antibody (EA) will bind K cells (see ch. 10) and monocytes.

The exact nature of the sub-populations detected by the empirical tests described is uncertain. Approximately 52–83% of human peripheral lymphocytes form E rosettes with sheep red cells and, at its best, this test appears to detect all the T lymphocytes present. About 20–30% of the peripheral cells are positive by the immunofluorescence test for surface immunoglobulin but a lower figure is usually obtained by the EAC rosette test. Sheep red cells, alone and complexed with antibody and complement, have been applied to human lymphoid tissue sections to distinguish T and B lymphocytes (Silveira et al., 1972).

2.1.5. Thymocytes

The thymus in mammals is principally a central lymphoid tissue not taking part directly in immune responses. It is the first tissue in the embryo to

become lymphoidal, the lymphocytes arising from blood-borne immigrant cells. Throughout embryonic and adult life the thymus is continuously receiving stem cells from the bone marrow. The stem cells proliferate on the matrix provided by the thymic epithelial reticular cells and the thymus of the young animal is a site of intense lymphoid cell proliferation unrelated to antigenic stimulation. The mitotic activity is associated with a maturation of a portion of the population to an almost mature T cell which migrates from the thymus never to return and completes its maturation in the peripheral tissue. It is then fully antigen-sensitive (for reviews see Raff and Cantor, 1971; Owen, 1972 and Davies et al., 1971; Schlesinger, 1972). Thymocytes differ from peripheral lymphocytes in their size, their mitotic activity, their fragility, their density, their surface antigens and their responses to stimulation.

The mouse thymus begins to receive lymphoid stem cell precursors at about the 12th day of gestation. Lymphocytes have been shown to appear in thymus tissue removed after this time and cultured in isolation. In the period from about the 14th day until birth (21 days gestation) lymphocytes are seen in increasing numbers at first consisting of large lymphocytes and lymphoblasts but later becoming predominantly small lymphocytes. The small lymphocytes, however, are very different from peripheral small lymphocytes. A size-distribution analysis (by Coulter counter) of the thymocytes of young rats has shown that there is a major population with a mean volume of 116–128 cubic microns compared with 167–179 cubic microns for thoracic duct lymphocytes. Thus the small lymphocytes present in the thymus in such large numbers are significantly smaller than peripheral small lymphocytes. They are also denser (Aisenberg and Murray, 1971). In the absence of antigenic stimulation the peripheral T population is comparatively quiescent but this is not true of the thymocyte population which has a high rate of autonomous mitotic activity. Most of the cells in DNA synthesis are to be found in the cortical zone. It has generally been assumed that the progeny of dividing cortical lymphocytes migrate to the medulla and mature for some time before leaving the thymus. However, it has been shown that cells labelled by injection of ^3H -thymidine migrate from the thymus within 3 days of labelling (Ernstrom and Larssen, 1969), and the general picture is one of rapid cell production and export. The turnover time of lymphocytes in the mouse thymus has been shown to be six days, the daily production of new cells 16.7% and the daily emigration $7.4 \pm 2.1\%$ (Claesson et al., 1972). In the guinea pig thymocytes differ from peripheral T cells in having a

high content of alkaline phosphatase (Runshanen and Kouvalainen, 1972).

The thymus of a heavily irradiated mouse is markedly depleted of cells when examined 4–6 days later. Some spontaneous repopulation then occurs with a second cycle of depletion around day 20 (Jacobsson and Blomgren, 1972). Normal repopulation of the thymus requires the provision of bone marrow stem cells; spleen or lymph node cells are ineffective. Treatment of rats or mice with cortisone causes a dramatic reduction in the number of lymphocytes in the thymus with a marked increase in the proportion of cells responsive to PHA and con A. This is due to destruction of the major unreactive population and retention of the minor mitogen-responsive population (Claman, 1972). The mitogen-responsive population is also relatively radioresistant.

The expression of various surface antigens on cells in the thymus changes during the ontogenetic development of the tissue. Even in the adult the sub-populations of lymphocytes in the thymus bear different concentrations of surface antigens and there are also differences between thymocytes and peripheral T cells. The several allelic forms of the TL antigen which have been detected on mouse thymocytes are characteristic for certain mouse strains. TL antigens are not present on stem cells freshly arrived from the bone marrow but are expressed on progeny produced in the thymus. The TL antigens are not detectable on peripheral T cells. Other antigens, such as the θ antigen, are present on a 'mature' sub-population of thymocytes and in lower concentration on peripheral T cells and there are some antigens, such as H-2 antigens, which are present on peripheral T cells but in lower concentrations on thymocytes. It would be unwise to assume that differences of surface antigens within the thymocyte population are explicable solely in terms of stage of maturation of a single line of cells. It is thought that the daily production of new cells in the thymus is almost twice the daily emigration and the many cells which on this view must die in the thymus may represent a distinct sub-population. Another suggestion is that there is a thymocyte 'dump' outside the thymus, perhaps in the spleen. It is certain that the peripheral-like thymocytes which constitute 5% of the thymic lymphocytes must have arisen in the thymus from a stem cell lacking detectable TL or θ antigen which arrived from the bone-marrow and acquired these antigens within a few days and it is certain that cells of this sub-population migrate from the thymus to become the peripheral T population. But it is conceivable that a second distinct population arising from a bone-marrow stem cell also develops and that these cells never leave the thymus.

There is more certainty about the final stages of maturation. It is known that the small portion of the thymocyte population responsive to stimulation with PHA, con A or allogeneic lymphocytes is composed of medium to large cells which sediment more rapidly than the inactive small thymocytes (Knight et al., 1973). The active cells are also known to be less dense than those of the major population (Wu et al., 1972). In the intact animal thymocytes appear to migrate from the thymus while still in a mitotically active phase. However, when thymus tissue is removed and placed under the renal capsule of a mouse of the same strain PHA-reactive cells of the indigenous thymus population are found after mitotic activity of the thymus cells has ceased (Elliott, 1973). It is probable that under these conditions the mitogen-responsive cells gradually become smaller and more closely comparable to peripheral T cells. The final stages of maturation preceding antigenic activation appear to take place outside the thymus. This appears likely from observations that 'mature' thymocytes from a young animal are stimulated *in vitro* by syngeneic cells found in the peripheral lymphoid tissues. These 'stimulating' cells are not T lymphocytes but are B lymphocytes or glass-adherent splenic cells (Von Boehmer and Byrd, 1972; Mosier and Pierce, 1972). It is likely that after this peripheral syngeneic stimulation the thymus-derived cells are capable of efficient antigen-reactivity and of becoming cytotoxic killer cells after activation. Almost all this information has been derived from studies of mouse and rat thymuses. There are indications that in some other species there may be less resemblance of thymocytes to peripheral cells, probably indicating that the late stages of differentiation are not always completed in the thymus. Rabbit thymocytes, for instance, respond to PHA, con A and SF but do not respond to allogeneic lymphocytes and differ from peripheral blood lymphocytes in not responding to antibody to allotypic gamma globulin determinants. Guinea pig thymocytes usually have a low level of reactivity to common mitogens. Human thymocytes respond to PHA, con A and SF but only weakly to allogeneic lymphocytes and they do not acquire cytotoxic capacity even after incubation in mixed cell culture for one week. If there is an end cell of the T series corresponding to the plasma cell of the B series it has not yet been characterised but it is not at all unlikely that antigenic stimulation induces further differentiation of the 'mature' T cell leading to the expression of new antigens as new functional capacity is acquired. Stimulation of T cells extends the expression of HL-A antigens.

The 'activated thymocytes' from the thoracic duct of lethally irradiated

mice injected with syngeneic thymocytes appear to contain immunoglobulin-like substances on their surface (Miller et al., 1971). These cells arise exclusively from the 'mature' sub-population of thymocytes which has presumably undergone considerable expansion in the lethally irradiated host. In terms of differentiation these cells may well be different to peripheral T cells. Small differences in the path and extent of differentiation are very difficult to define. Capacity to be mitotically activated by mitogens is probably a relatively late property of thymocytes and may be dissociated from responsiveness to allogeneic lymphocytes. One of the characteristics of the T cell which is late to develop is cytotoxic capacity. In view of the uncertain specificity of T cell killing and the fact that autologous cells may be damaged, the failure of lymphoblasts within the thymus to assert cytotoxicity may be a protective mechanism designed to avoid damage to thymic epithelial cells.

2.1.6. Summary

Most of the lymphocytes in the blood of peripheral lymphoid tissues appear in stained smears as small round cells with little cytoplasm. In the living state they are amoeboid cells which move at intervals with a vigorous and characteristic 'hand-mirror' motion. They may enter other cells in culture ('emperipoleisis') and *in vivo* they migrate in large numbers through the post-capillary venules of lymph nodes. They do not attach to glass or plastic surfaces in the way that many other types of cell (fibroblasts, macrophages, epithelial cells) do. The principal population showing these characteristics is a recirculating thymus-derived (T) lymphocyte population consisting of predominantly long-lived cells which are constantly circulating through the peripheral lymphoid tissues (not the thymus) via blood and lymphatics. These T lymphocytes are concerned directly in 'cellular immunity' and indirectly in antibody formation. The next largest lymphocyte population is composed of immunoglobulin-bearing B lymphocytes which are precursors of plasma cells. Other cells of lymphocyte-like morphology may be promonocytes and have a purely effector role. Others may be stem cells. The thymus contains a mitotically active population of large and small lymphocytes only a minority of which resemble peripheral T cells in properties. The remainder may be T cell precursors in an early stage of differentiation. Distinctive surface markers on mouse and human lymphocytes enable them to be subdivided into sub-populations roughly corresponding to T and B cell types.

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CHAPTER 3

Methods of culturing lymphocytes

The media and methods used for culturing lymphocytes are not substantially different from those used for culturing other cells and cell lines. Lymphocytes do not adhere to glass or plastic surfaces like most cultured cells. They may be grown, a) in suspension with agitation to disperse the cells; b) without agitation, the cells being allowed to settle at the bottom of the chamber. This is the most commonly used technique; c) as intact nodes or as fragments of lymphoid tissue (from lymph node, spleen, thymus) by means of an explant or raft technique; d) as cell dispersions in certain specialised techniques e.g. the study of primary antibody responses in vitro (Mishell and Dutton, 1967) or of diffusible products of lymphocyte cultures using the apparatus of Marbrook (1967).

3.1. Growth media

A simple balanced salt solution containing 20% serum will permit some lymphocyte growth to occur as will the simple medium of Trowell (1963) supplemented with serum, but when activation occurs the nutritional needs of the cells increase and if maximal activity is to be realised a full medium must be used. Most conventional synthetic media (see Paul, 1965), when supplemented with 5–20% serum, will support the growth of lymphocytes. Eagle's medium, 199 medium, and, more recently, RPMI 1640 medium have been used. Eagle's medium, which has been the most systematically investigated, is commercially available (e.g. from Flow Laboratories Ltd., or Grand Island Biologicals) as a simple 'Basal' medium or as a 'Minimum Essential Medium' (MEM) which contains higher concentrations of essential amino acids. Several different balanced salt solutions have been used as the basis of the media. All are satisfactory but the culture conditions may be affected by the choice made, e.g. MEM (Hank's salts) contains 0.35 g of

sodium bicarbonate per litre, MEM (Earle's) 1.68 g, and MEM (Glasgow modification) 2.75 g per litre. A high bicarbonate medium will tolerate a relatively high cell concentration without becoming acid but will quickly become alkaline if there is insufficient carbon dioxide in the gas phase, whereas a low-bicarbonate medium may often be used without gassing if an air-tight vessel of total volume not more than 4 or 5 times that of the fluid is used. Medium RPMI 1640, which was developed at Roswell Park for the initiation and maintenance of human lymphoid cell lines, is recognised as an excellent medium in which to grow rat or mouse lymphocytes and has also been recommended for short-term culture of human lymphocytes (Junge et al., 1970). Eagle's and 199 media are available as concentrates ($\times 10$) which require addition of antibiotics (usually penicillin to 200 units/ml and streptomycin to 100 units/ml), glutamine and bicarbonate before use. Powdered media (Flow Laboratories Ltd., or GIBCO), which are the most economical to use, are converted to liquid media by dissolving the powder in good quality distilled water, and, after the addition of antibiotics, glutamine and bicarbonate, are filtered through a filtration pad of pore size 0.22 μ to sterilise. Cell growth is very satisfactory in these easily prepared media but occasionally, over longer growth periods, growth of lymphoid cell line cells has not, in our experience, been quite as good as in media purchased ready for use.

Bicarbonate-buffered media require a gas phase containing carbon dioxide to maintain a physiological pH. Gassing is unnecessary when the bicarbonate is replaced by Hepes (N-2-hydroxymethyl-piperazine-N'-2-ethane-sulphonic acid) as the buffering ion (Williamson and Cox, 1968; Darzynkiewicz and Jacobson, 1971). A 20 mM concentration in Eagle's medium at pH 7.4 is prepared by adding 4.67 g of Hepes and 9.2 ml of N NaOH to the dissolved constituents of the medium before adding glutamine, antibiotics and making up to one litre with distilled water. If the Hepes-buffered cultures are not set up in an air-tight container precautions should be taken against loss of water by evaporation at 37 °C e.g. the cultures should be placed in a moist chamber. 'Tris' (tris (hydroxymethyl) methyl ammonium chloride) has also been found to be as good as bicarbonate as a buffer in human lymphocyte cultures (Schellekens and Eijsvogel, 1968).

3.1.1. The gas phase

Cultures containing bicarbonate-based media which have been set up in

tubes with lightly fitting caps or in bottles with caps unscrewed should be placed in an air-tight moist chamber (an ordinary desiccator is often convenient) and gassed with 5% CO₂ in air. Bijou bottles (total volume 7 ml, fluid volume 3 ml) may alternatively be tightly capped (ordinary black rubber liners are adequate) without gassing. Lymphocytes, in common with other animal cells, require some oxygen for survival. Low oxygen tensions favour transformation but growth will not continue for long in completely anaerobic conditions. Incorporation of reducing agents, such as sulphite, L-cysteine, or reduced glutathione into the medium enhances the response to PHA (Fanger et al., 1970). The oxygen tension to which the cells are exposed in stationary cultures will depend on the depth of fluid and other factors, e.g. the number of red cells present. Empirically it has been found that when gassing with 5% CO₂ in air a depth of 2 cm is about right. High O₂ tensions are inhibitory and less transformation is obtained if 5% CO₂ in O₂ is used, particularly in shallow cultures. When gassing is not required precautions should be taken to avoid evaporation of medium which may be considerable in cultures left unsealed at 37 °C for long periods.

3.1.2. *Nature and quantity of serum*

There are five factors which must be taken into account in the choice of serum as a medium supplement: a) macromolecules which are protective or promote growth in some ill-defined manner, b) small molecules, e.g. nucleosides, vitamins, hormones, co-enzymes, which supply essential trace nutrients not present in the medium, c) factors (including antibodies) which neutralise or combine with the stimulant, d) the presence of antibodies to antigenic sites on the surface of the lymphocytes. These may be stimulatory or cytotoxic, e) foreign antigens. Heterologous sera will obviously contain a whole battery of potential antigens.

Foetal calf serum is an excellent source of nutritive value but also produces with human cells a considerable background stimulation which, over 7 days of culture, affected from 5 to 48% of the lymphocytes (Johnson and Russell, 1965). The level of background transformation with agammaglobulin serum, although less than with foetal calf serum, was still far higher than with autologous serum. That these results could not be accounted for by the inferior nutritional value of autologous serum was shown by the fact that, under the influence of PHA, the % of cells transformed at 3 days was consistently greater when the medium contained human

than when it contained foetal calf serum.

The growth promoting property of human serum bears no relation to whether it is of autologous or homologous origin, and it appears to be immaterial whether it is from an ABO compatible or incompatible donor although this will determine, of course, whether or not the red cells agglutinate. There appear to be no lymphocyte-activating agents present in normal serum of the same species. The background activity in human lymphocyte cultures containing homologous serum is not significantly higher than with autologous serum in our experience but higher values have been reported over long periods of culture (Bredt and Mardiney, 1969). Although not in themselves stimulatory, individual sera vary greatly in their capacity to support growth (McIntyre and Cole, 1969). This is true in cultures of lymphocytes stimulated by leucocyte antibody (table 3.1), staphylococcal filtrate or PPD (Heilman and McFarland, 1966) and it is probable that much of the inhibition is stimulant-specific being due to the presence of macromolecules, some of them antibodies, some of them glycoproteins, which combine with the stimulant and block its activity. The optimal concentration of PHA increases as the quantity of serum in a culture increases (Forsdyke, 1967). At fixed levels of stimulant Alford (1970a) found 1–10% autologous plasma optimal for PHA transformation whereas higher levels were optimal with a *Candida* stimulant.

Table 3.1

Influence of human serum from different donors on the level of DNA synthesis of peripheral lymphocytes activated with antiserum.

Cell source	Serum (inact.) source	Antiserum 501 dilution				Antiserum 502 solution		
		1/100	1/200	1/500	1/2000	1/30	1/100	1/200
K	K	4,200	3,300	340	16	17,000	184	179
K	S	39,100	18,600	330	33	33,200	9,200	2,000
S	S	26,300	5,300	550	51	24,700	11,000	1,650
S	K	9,000	6,360	690	129	9,500	135	80

The figures for ^{14}C -thymidine incorporation are in counts/min.

K = patient with ankylosing spondylitis.

S = patient with rheumatoid arthritis.

The quantity of serum optimal for growth depends upon the quality of the serum and the activity of the cells cultured but it is probable that with human serum 15–20% is adequate for near maximal synthetic activity in a good medium such as Eagle's medium. Transformation and reasonable cell survival can be obtained at low serum concentrations (of the order of 1%) but, after complete removal of serum by washing, lymphocyte survival is usually grossly impaired. For these reasons lymphocytes are best cultured in medium containing homologous serum at a concentration of 20% for those species of which the serum is nutritionally adequate. This appears to be the case for human, rabbit or pig lymphocytes. Survival of mouse lymphocytes is poor in media containing homologous serum and a good quality foetal calf serum should be substituted. The background stimulation is minimised by restricting the concentration to 5%. Rat lymphocytes also grow well in media supplemented with foetal calf serum although some authors prefer to use fresh (not frozen) rat serum (Wilson, 1967) or a mixture of foetal calf serum and rat plasma (Main et al., 1971). In our experience poor results are obtained with rat serum in rodent cultures. This discrepant result may be due to variations in the diet of laboratory animals in different centres. Pools of sera of the type preferred should be sterilised by Seitz filtration and stored in small aliquots at —20 °C or preferably at —70 °C.

3.1.3. The cell concentration and the culture chamber

In common with other cells, lymphocytes do not readily survive and grow at low cell concentrations (less than $10^5/\text{ml}$). It is more difficult to state what is the maximal cell concentration for optimal growth. In general the best growth conditions are those with the highest cell concentrations which the medium used will support. The most obvious indicator of medium exhaustion is a low pH (phenol red indicator turns yellow) which, in turn, is an indication of the rate of glycolysis. The rate of glycolysis of the various cells present in peripheral and lymphoid cell suspensions is often considerably higher than that of unstimulated lymphocytes (e.g. PMN neutrophils produce about 50 times as much acid as lymphocytes). The depth of fluid and the O_2 tension are related variables. Very high cell concentrations may be achieved without loss of viability if shallow cultures gassed with high O_2 tensions (5% CO_2 , 95% O_2) are used but these conditions may be disadvantageous at lower cell concentrations. With human peripheral leucocytes from defibrinated blood a nucleated cell concentration of $1\text{--}2 \times 10^6/\text{ml}$

is routinely used but Eagle's medium (Glasgow modification) will tolerate cell concentrations of 5 to 10×10^6 per ml for two or three days.

Many competing factors are influenced by the shape of the base of the container (see § 3.1.11). Table 3.2 shows the results of an experiment in which rabbit peripheral cells were cultured at various cell concentrations in either conventional convex-based 75 × 12 mm glass test tubes or in flat-bottom glass specimen tubes (area of base = 50 sq. mm). Each tube contained 1 ml of fluid and the approximate depth of fluid in each tube was 2 cm. The unstimulated cells were cultured in 20% autologous serum in Eagle's medium and the stimulated cells in the same medium containing SF to a final concentration of 10%. The culture period was 67 hr and ^3H -TdR (0.5 μCi) was present during the last 16 hr of this period. Instead of glass tubes plastic disposable tubes are now widely used for lymphocyte cultures and are usually monitored for the inhibitory substances found in some products (Bain and Lowenstein, 1965). Good growth also occurs in bijou bottles and McCartney bottles, both of which have a convex base. All cultures should be disturbed as little as possible. Continuous agitation markedly inhibits PHA-induced activation.

Table 3.2

The effect of surface area of the base of the culture tube on culture activity.

Culture tube	Cell count	^3H /TdR incorporation into DNA (dpm)		Disintegrations per 10^6 cells (stimulated)
		Unstimulated	Stimulated	
Round bottom	0.5×10^6	9	6,700	13,400
Round bottom	1.0×10^6	54	10,100	10,100
Round bottom	2.0×10^6	25	18,000	9,000
Round bottom	4.0×10^6	27	5,400	1,350
Flat bottom	0.5×10^6	7	3,500	7,000
Flat bottom	1.0×10^6	26	4,400	4,400
Flat bottom	2.0×10^6	15	2,900	1,450
Flat bottom	4.0×10^6	22	310	75

3.1.4. Collection of blood and separation of lymphocytes

Blood collected aseptically may be either defibrinated or heparinised. Pre-

servative-free heparin (20 units per ml of blood) is preferable to the product routinely used, which contains 0.15% chlorocresol. Other anticoagulants have rarely been used. Oxalate is unsuitable as it is toxic to cells. Acid citrate dextrose (16 g of hydrated sodium citrate + 4.7 g of citric acid + 25 g of glucose per litre; 0.2 ml per ml of blood) is satisfactory. Lymphocytes survive well in transfusion blood collected in this solution (Petrakis and Politis, 1962; Polesky and Helgeson, 1970). There is no need for rigorous washing to remove citrate before setting up cultures from cells collected in this way. Only very high concentrations of citrate (24 mM or greater) will inhibit lymphocyte transformation by PHA and this effect is completely reversible by calcium and partially by magnesium ions, in contrast to the inhibition induced by EDTA which is not entirely reversible by restoration of free calcium ions (Alford, 1970b). Transformation of lymphocytes from blood collected in EDTA (1.0 mg of the neutral salt per ml of blood) does occur, however, provided the EDTA-plasma is thoroughly removed before the cells are cultured. Freshly drawn blood is preferable for most purposes, but lymphocytes from blood stored at 4 °C for 24 hr respond well to stimulation. Lymphocytes from blood collected up to 24 hr post-mortem will also respond to PHA (Mold, 1966).

Whenever possible it is best to avoid anticoagulants and defibrinate the blood. A satisfactory technique of defibrination is a simple rotation in the blood, by hand, of sterile cherry sticks (15 cm × 2 mm diam; 2 or 3 per 20 ml of blood). After slow stirring for some minutes a tight cylinder of fibrin forms around the sticks. It is important to avoid subjecting the blood cells to mechanical trauma during the defibrination otherwise damaged and misshapen red cells will appear which do not form rouleaux and are difficult to remove by the commonly used purification techniques. If glass beads (2 or 3 of diameter 5 mm) are used the blood should not be shaken or vigorously inverted. Gentle consistent rotation (not inversion) of the bottle just sufficient for the whirl of the beads to be audible, is recommended. A roughened glass rod or a glass rod fitted with glass spikes may be rotated in the blood to defibrinate. An automatic method which depends on a motor-driven rotation (without lateral movement) of a spiked glass rod at 250 revolutions per minute in the blood has been described by Wilson et al. (1969). Between 18 and 19.3 ml of blood was recovered from a 20 ml sample with little cell trauma. About 15% of the lymphocytes and 49% of the neutrophils were lost. A simple method, which has given good results in the hands of Dr. J. Rees in London and Dr. P. Wolf in Birmingham, is

Table 3.3
Separation of lymphocytes from human blood*.

Donor	Whole blood						Defibrinated blood						Gelatin supernate					
	Approx. vol. (ml)	WBC count, cells per c.mm	W.B.C. differential	Total L ($\times 10^6$)	Approx. vol. (ml)	W.B.C. differential	Total L ($\times 10^6$)	W.B.C. differential	Total L ($\times 10^6$)	%L	%N	%O	%L	%N	%O	Total L ($\times 10^6$)		
C.L.	20	6590	48.8	46.6	64	18	79.2	19.4	45	84.7	14.7	0.6	—	—	—	—		
C.L.	17	7370	47.0	51.4	1.6	59	15	68.2	30.2	1.6	39	69.6	29.4	1.0	19.5	—		
C.L.	16	6250	50.0	45.9	4.1	52	14	70.7	27.3	2.0	38	67.5	30.6	1.9	20.0	—		
H.R.	17	5240	48.0	48.2	3.8	43	14	84.2	14.6	4.8	27	92.0	7.0	1.0	15.0	—		
H.R.	20	4930	58.8	38.6	2.6	58	17	84.8	13.2	0.0	40	93.0	6.6	0.4	15.0	—		
T.B.	19	6620	41.8	54.4	3.8	53	14½	66.6	28.6	0.6	32	71.8	26.3	1.9	15.0	—		

* From Thomson et al. (1966).

W.B.C. = white blood cells, L = lymphocytes, N = Neutrophils, O = acidophils and monocytes.

simply to transfer freshly-drawn blood with minimal trauma to a plastic tube (half filled) or a clean bottle (two-thirds filled) which is then sealed with a plastic top or a cap with a silicone-rubber liner and placed on a Catherine-wheel (Matburn) mixer. Rotation at 28 revolutions per minute is continued for $\frac{1}{2}$ to 1 hr when either no clot or a small clot is formed. Cell yields are similar to those using the automatic defibrillator. Not everybody, however, has had satisfactory results with this technique and if slow clotting is not achieved large clots will form and cell yields will be markedly reduced.

Removal of red cells by simple sedimentation is an inefficient method unless the esr is high and for most purposes it is advisable to add an inert rouleaux-forming agent. This is commonly a protein or polysaccharide of high molecular weight. Dextran and gelatin have usually been preferred but fibrinogen (6 mg/ml), macroglobulins and polyvinyl pyrrolidone have occasionally been used. Cellulose derivates have been shown to be among the most effective compounds (Böyum, 1968) and the polysucrose Ficoll will also aggregate red cells. Methyl cellulose (2.5 mg per ml final concentration) has been frequently used (Coulson and Chalmers, 1967) and so have dextrans of molecular weight approx. 150,000 (final concentration 6 mg per ml). The efficiency of dextrans and other rouleaux-forming agents increases with increase in molecular weight. A disadvantage of methyl cellulose is that it tends to come out of solution at 37 °C and to interfere with the reading of dye-exclusion viability tests.

The best erythrocyte-sedimenting agent for human and animal bloods is gelatin. A gelatin of high molecular weight (e.g. 190,000) is dissolved in warm saline to a 3% solution and sterilised by Seitz filtration. One third to one half a volume is added to one volume of blood. The mixture is poured into a fresh sterile bottle taking care to avoid fouling the neck of the bottle with blood and not to produce surface foaming. The cells are left to sediment at 37 °C for 1-2 hr. About half the lymphocytes are recovered in the supernate (see table 3.3). If better recovery is required more gelatin may be added to the red cell layer and a second sedimentation performed. It is important to make up the gelatin in saline and not phosphate-buffered saline because commercial gelatins contain sufficient calcium to precipitate added phosphate at blood pH. The gelatin will dissolve more readily if allowed to soak in cold saline for 15 min or more before adding the hot saline. The ratio of lymphocytes to other leucocytes in supernates from gelatin-sedimented, defibrinated blood is much increased compared with that of whole blood.

Three quarters of the enrichment is due to the removal of other leucocytes during the defibrination (table 3.3). Lymphoblasts and other large cells, if present, are also likely to be preferentially reduced in numbers by separation procedures of this type and that is why, for some purposes, whole blood cultures may be preferable. The erythrocyte contamination of defibrinated blood-gelatin supernates varies from 2 to about 30 red cells per leucocyte. The number is closely related to the degree of mechanical trauma to which the blood has been subjected. An important advantage of using defibrinated blood, rather than heparinised or citrated, is that platelets are removed and cell suspensions may be centrifuged and the cell deposit readily dispersed in medium to give a clean even suspension without the clumping of cells and the platelet adherence which is so hard to avoid with anticoagulated blood. A disadvantage of gelatin is that it sets to a gel at room temperature but in other respects it is ideal.

Lymphocytes may be obtained more directly and quickly from heparinised or defibrinated blood by simply centrifuging the blood through a Ficoll-Triosil reagent of standard density (see § 3.3.1). The erythrocyte contamination of the lymphocyte band is much reduced if the blood is first diluted with one or two volumes of medium. For example: mix 5 ml of whole blood with 5 ml of Hepes-buffered medium in a 25 ml McCartney bottle or a 20 ml blood-collection bottle. Draw up 5 ml of Ficoll-Triosil in pipette, place the tip of the pipette at the bottom of the bottle containing the blood-medium mixture, run in the 5 ml and carefully remove the sealed pipette. Centrifuge for 30 min at 400 g and suck off the lymphocyte band, dilute with medium and wash the cells.

3.1.5. Typical leucocyte cultures

Venous blood (20 ml) drawn into a sterile syringe is transferred to a sterile screw-top universal container (vol. 25 ml) and defibrinated by rotating two cherry sticks in it. Sterile freshly prepared 3% gelatin (7 ml) is added and, after mixing, the blood is poured into a clean sterile bottle, the cap replaced, and the blood stood at 37 °C for one hour. The leucocyte-rich serum is removed with a Pasteur pipette and transferred to a screw-capped glass or plastic tube. A lymphocyte count is performed and the volume of fluid noted. The cell suspension is centrifuged at about 500 g for 5–10 min, the supernate removed with a Pasteur pipette (and checked to ensure absence of cells), the tube tapped to disperse the cell pellet and medium (complete with

serum) is added to yield a lymphocyte concentration of $2 \times 10^6/\text{ml}$. One of two procedures may then be adopted:

a) A simple culture followed by morphological examination. Aliquots (3 ml) are placed in bijou bottles (vol. 7 ml), stimulant is added to appropriate bottles. All bottles are screwed down tightly and left at 37 °C for 2–7 days. The cells are resuspended by swirling the bottles and the cell suspensions transferred to tubes which are centrifuged at approx. 400 g for 5 min. Most of the supernate is removed from each tube and, after allowing a minute or so for fluid to run down the side of the tube, the remainder of the supernate is removed as completely as possible with a fine-tipped Pasteur pipette. The cells are then lightly sucked up into the tip of another clean, fine Pasteur pipette. The tiny volume is transferred to several grease-free slides, smears are made (as for blood smears) and they are air-dried and fixed in methanol. They are stained (see § 3.1.6) and the number of blast cells expressed as a percentage of the total number of lymphocytes (see § 3.1.5).

b) Scintillation counting with viability and morphology check. Distribute one ml aliquots of the cell suspension into sterile capped tubes, setting up 4 tubes without stimulant and 4 tubes for each dose of stimulant under test. As soon as the setting up has been completed transfer all the tubes to a moist container fitted with inlet and outlet tubes. Gas with 5% CO₂ in air for 15–30 min. Seal off the container. After 1–6 days of culture add ³H-thymidine (0.5 µCi) of low specific activity (see § 3.1.10) to 3 tubes of each set of 4 and regas. Twenty-four hr later remove all tubes. Those which have received ³H-thymidine should be processed for scintillation counting (see § 3.1.10 and § 3.1.15). The cells in the remaining tube are resuspended, four drops are removed and added to one drop of 1% trypan blue. A sample is transferred to a counting chamber and the number of viable cells assessed (see § 3.1.7). The cells remaining in the tube are spun down and a smear is made which is fixed, stained and examined as above. When autoradiographs are required ³H-thymidine is added to all four tubes, the fourth tube is used to prepare smears which are processed as in § 3.1.8 and § 3.1.9.

Occasionally whole blood cultures may be preferred. This will be so if only very small volumes of blood are available or if an assessment of the level of DNA synthesis of fresh blood cells is required. Because about 1000 red cells are present for each leucocyte, whole blood cultures contain very low leucocyte concentrations. However, viability and mitogen-responsiveness are satisfactory. The following is typical: To 3 ml of culture medium in a bijou bottle add 0.1 ml of whole blood. Set up two bottles without

stimulant and two with stimulant. After culture for 1–3 days add 6 µCi of ^3H -thymidine (sp. act. 600 mCi/mM) to each bottle. Twenty four hr later measure out 2×1 ml aliquots from each bottle into tubes. Spin down the cells and process for scintillation counting as in § 3.1.10.

3.1.6. Staining of smears

Conventional Jenner–Giems α staining of smears gives excellent results, the small lymphocyte appearing, just as in blood smears, with a dark-blue (not black) nucleus in which the arrangement of chromatin is clearly visible and with a surrounding rim of pale-blue cytoplasm contrasting with the dark blue cytoplasm and paler nucleus of lymphoblasts. Macrophages present in smears from peripheral cell cultures are derived from monocytes. They appear large, flat, have light nuclei with homogeneous chromatin, sometimes large nucleoli and always have a relatively large amount of faintly-staining cytoplasm containing large and irregular vacuoles in contrast to the small, regular and usually grouped vacuoles seen in blast cells. Some authors add polystyrene latex particles (diameter 1.3 µ, 0.2 ml of the Dow product diluted 1 in 100 per 12 ml of culture). Phagocytic cells take up the particles which appear in the cytoplasm in smears as highly regular bunches of vacuoles. Darkly stained cells and precipitation of stain are seen when too large a volume has been smeared and a deposit of salts has formed on the slide. If all the blast cells are at the edges the smear has been made too slowly. Cells may be more evenly distributed by using a cyto-centrifuge (Shandon) but there is a danger of some small lymphocytes being absorbed by the filter paper while all the larger cells remain adherent to the slide, thus giving a false impression of the numbers of blast cells present.

3.1.7. Viability tests and cytotoxicity assay

Motility is the most reliable evidence of viability but even viable lymphocytes are not always on the move. A simple, practically useful dye-exclusion test for viability is as follows: to 4 drops of the cell suspension in 20% serum add one drop of 1% trypan blue. Count the unstained (viable) cells. Eosin Y is also satisfactory and many prefer nigrosin. Unstained cells with pycnotic nuclei which are obviously dead are occasionally seen, especially in mouse or rat lymphocyte cultures and the test is open to other errors (see Black and Berenbaum, 1964). Some workers prefer to rely on the morphological

changes seen under a phase-contrast microscope. If it is not convenient to read the dye-exclusion test immediately the preparation may be fixed by the addition of 2 to 5 volumes of 40% formalin (neutralized with CaCO_3) as described by Joysey (1968). A more positive test for cell viability is the active uptake of a fluorescent compound such as fluorescein diacetate (Rotman and Papermaster, 1966). Another useful technique is haemalum staining of Susa-fixed smears for detection of cells with pycnotic nuclei (Wilson and Thomson, 1968).

Radiochromium release (Sanderson, 1964) is the most satisfactory of several biochemical methods for assaying the cytotoxicity of antisera or effector cells. Target cells may be labelled by overnight incubation with $\text{Na}_2^{51}\text{CrO}_4$ at 1 μCi per ml. In the morning the cells are washed four times in medium containing serum, resuspended in medium and incubated at a final concentration of $0.4 \times 10^6/\text{ml}$ with the cytotoxic agent under test for 1–24 hr after which the tubes are centrifuged, the supernates removed and the radioactivity of supernates and deposits (dissolved in NaOH) measured in a gamma counter. The percent radioactivity released into the supernate is a measure of cell damage. Since only part of the $^{51}\text{chromate}$ is releasable on cell death it is advisable to determine the maximum possible release. Unfortunately there is no entirely satisfactory method of doing this, but repeated freeze-thawing, water shock or the use of cytotoxic antiserum are methods which have been used. There are sometimes advantages in labelling the target cells over a shorter period with higher doses of $^{51}\text{chromate}$, e.g. 0.4 ml of cell suspension containing 20×10^6 cells/ml is incubated with 40 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (sp. act. 100–400 $\mu\text{Ci}/\mu\text{g}$ of chromium) for 30 min at 37 °C and the cells washed five times before use (Brunner et al., 1968).

3.1.8. Autoradiographs

Three techniques may be used, a) the stripping film technique of Doniach and Pelc (1950), b) the 'dipping' technique, an excellent elementary account of which has been given by Baserga and Kisieleski (1963), c) autoradiography combined with electron microscopy.

The advantages of the stripping film technique are its simplicity, its sensitivity, its constancy from one batch of slides to another and in the even spread of emulsion over the slide. It is equally suitable for one or two slides or for large batches. Its disadvantages are the danger of lifting of the film, particularly if it has not been allowed to expand properly before placing on

the slide and staining difficulties. The 'dip' technique is generally more wasteful of emulsion and the results less consistent. For routine purposes the following variant of the stripping film technique is satisfactory: A box of Kodak AR-stripping film is opened in complete darkness or using an indirect safe-light containing a 15-watt bulb and fitted with a Wratten 2 (dark-red) filter. A plate coated with emulsion is held emulsion uppermost and scored with a scalpel with 3 lines parallel to the short edge and 2 parallel to the long edge. Squares of film are lifted with the scalpel and transferred face downwards onto a bowl of water at 26 °C. When the film is saturated with water (a few minutes, indicated by a shiny to dull change) a slide on which a smear has been made (previously methanol-fixed and washed for some hours in running water) is dipped into the water, smear uppermost, and lifted so that the film is squarely on it and wraps itself closely around the slide. The slides are dried in a rack in the dark in the air-stream from a rubber-bladed fan. The slides are now transferred to slide boxes which are placed inside another cardboard box containing desiccant (silica gel) and stored at 4 °C usually for 1–2 weeks. The slides are then put in a metal rack and placed in a pot containing Kodak D19B developer at 10 °C for 6 min. After a brief rinse in tap water the slides are transferred to acid-hard fixative at 19 °C for 12 min. They are then washed in running water (below 19 °C) for 30 min and dried. Under these conditions lymphoblasts exposed in culture to ^3H -thymidine (0.5 μCi per ml, sp. act. 150 mCi per mmole for 24 hr) are clearly labelled.

3.1.9. Staining of autoradiographs

The choice of stain to be used depends upon whether it is to be applied before or after developing the autoradiograph. Pre-stains avoid the gelatin background staining but are inferior in revealing morphological detail. Pre-stains: a) orcein. The dry methanol-fixed slides are immersed for 10–20 min in orcein (2% in 45% acetic acid) and rinsed in 45% acetic acid before drying. b) PAS air-dried smears fixed in 4% formalin in 90% methanol for 10 min are washed briefly in tap water. They are treated with 1% periodic acid for 10 min, washed and blotted dry. They are immersed in Schiff's reagent for 50 min and washed in tap water for 5–10 min.

A satisfactory post-staining technique is that of Gude et al. (1955). There is a danger of loss of silver grains when Romanowsky stains are applied to autoradiographs. This loss is avoided by making sure the developer is fresh

and above all, by drying the preparations thoroughly before staining: The dry autoradiographs are placed in a metal slide rack and immersed for 15 min in buffered water pH 6.8 followed by 15 min in buffered Giemsa (Giemsa stain 2.5 ml, methyl alcohol 3.0 ml, M/10 citric acid 11.0 ml, M/5 disodium phosphate 6.0 ml, distilled water 100 ml). The racks are drained and dried in a current of air.

3.1.10. Use of radioactive precursors

Thymidine-2-¹⁴C (¹⁴C-TdR) is approximately 50 times the price of tritiated thymidine (³H-TdR). On the other hand, only about one tenth as much ¹⁴C-TdR is normally used and ¹⁴C is easier to count. If a suitable liquid scintillation counter is available ³H-TdR is preferable for routine purposes. After adding ³H-TdR for an appropriate time (depending on its specific activity – see ch. 14 § 1 for a discussion of this point) the cells are spun down and washed successively in phosphate-buffered saline (once), 5% trichloracetic acid (twice) and methanol (once). The cell deposit is taken up in N NaOH (aqueous 0.1 ml) and the tube incubated at 56 °C for 30 min. Methanol (1 ml) is added and the solution transferred to a scintillation bottle with 10 ml of a scintillation fluid prepared by dissolving 0.6 g of 2,5-diphenyloxazole and 0.012 g of 1,4-bis-(2-(5-phenyloxazolyl)-benzene in xylene (100 ml) and adding 50 ml of Triton-X-100 (Patterson and Greene, 1965). Addition of Triton-X-100 to the scintillator reduces the counting efficiency by about 30% but this disadvantage is more than offset by the greater reproducibility of results and avoidance of the difficulties of bringing the cell deposits into solution which often occurs when organic bases like Hyamine are used and by the independence of the results of traces of water. The water present in the aq. NaOH does not affect the counting efficiency with Triton present. If very large numbers of red cells are present (or routinely) the phosphate-buffered saline used for the first wash may be replaced by saline containing 1% acetic acid. Incorporated thymidine is stable in trichloracetic acid. Preparations may be left at 4 °C at this stage for at least one week.

The method outlined is based on the assumption that acid-insoluble label is label present in newly synthesised DNA. This has been confirmed by isolation of the labelled material in a saline precipitable (DNA) fraction. While DNA is stable to strong acid at room temperature this is not true of RNA and when RNA is being estimated (e.g. after incubation of cells with

^3H -uridine) the trichloroacetic acid should be used cold and stored at 4 °C. In all cases counts obtained in a scintillation counter must be corrected for chemical and colour quenching to obtain the count per minute at 100% counting efficiency which should, but rarely does, give a figure for the disintegrations per minute (dpm), independent of the counting techniques. For a full discussion of the technique of scintillation counting the book of Schram (1963) is recommended.

Radioactive iododeoxyuridine is an alternative to thymidine as a specific precursor for the measurement of DNA synthesis (Craig et al., 1969). Since it emits a gamma rather than a soft beta radiation the counting of samples is easier. The washed pellet may even be counted directly in the tube without solubilisation. Disadvantages are the short half-lives of I-131 and I-125 and the inefficiency of incorporation of deoxyuridine compared with thymidine. Yet another alternative is to use a non-specific precursor such as ^{32}P -phosphate and to fractionate the labelled cells by a Schmidt-Tannhauser separation, but this technique is laborious and insensitive and has fallen out of favour.

3.1.11. Micro-methods of culture and automation

When large numbers of cultures are routinely set up, culturing in plastic trays can result in the saving of a great deal of time and material. Disposable polystyrene trays suitable for micro-cultures (containing 96 wells per tray) are commercially available (e.g. from Cooke Engineering). As with all plastic containers used in tissue culture work it is advisable to test the batch used to make sure that no toxic materials have been left on the plastic during their manufacture. Both flat-bottomed and round-bottomed wells have been successfully used for cultures. Factors which will affect comparative results are: a) the cell concentration – optimum higher in flat- than round-bottomed wells, b) the number of macrophages – their presence is essential in flat-bottomed trays, less so in round-bottomed, c) the bicarbonate reserve of the medium – less reserve needed with flat-bottomed than round-bottomed wells, d) accessibility of nutrient materials and O_2 to the cells, e) opportunities for cell-cell interactions – particularly important in mixed cell cultures.

The following method for the culture of rat thymocytes is typical: Aliquots (0.2 ml) of rat thymocytes in RPMI 1640 medium containing 5% foetal calf serum (2×10^6 per ml, i.e. 4×10^5 cells per well) are pipetted in triplicate in round-bottomed wells of a tray. Stimulant (0.01 ml of 40 µg/ml con A)

is added to appropriate wells. The completed tray is immediately placed in a chamber at 37 °C and gassed with 5% CO₂ in air. ³H-TdR (0.2 µCi, 150 mCi/mM, 0.03 ml) is added 24 hr before harvesting on day 4. Processing of the cultures and scintillation counting follow the procedure for macrocultures except that 0.2 ml volumes of reagents (measured with Eppendorf syringes) are used. The cells are deposited by centrifuging the plates on special swing-out aluminium carriers and the supernates removed by a single sharp throwing action.

Harvesting of cultures for scintillation counting is a laborious and time-consuming procedure. Many workers prefer to collect and wash the cell deposits on a sampling manifold containing glass fibre or Millipore membranes (pore size 0.8 µ). It is this part of the procedure which is most obviously open to automation (see Sanderson et al., 1974). Commercial automatic harvesters are also becoming available e.g. from Skatron, Norway.

3.1.12. Phase contrast observations

Since lymphocytes do not adhere to glass and settle at the base of any container in which they are placed an inverted phase-contrast microscope is required for long-term observations of living lymphocytes. If this requirement is met the culture chamber itself may be extremely simple. A Pulvertaft chamber consisting of a Teflon ring stuck to a slide on one side and a cover slip on the other with high-vacuum silicone grease may be used over short periods of culture. A conventional methacrylate chamber with a central platform and surrounding air space and sealed with paraffin wax is suitable for cultures of 7 days or more duration. In cloning experiments methacrylate slide chambers with a device for isolating cells have been used (Marshall and Roberts, 1965).

3.1.13. Chromosome preparations

In cultures containing lymphocytes from more than one donor the most reliable way of determining the source of the activated lymphocytes may be to examine the metaphases accumulated after the addition of colchicine or colcemid. If one lymphocyte donor is a female and the other male it is possible to identify the source of the metaphase by sexing the chromosomes. Another use of chromosome preparations is, in conjunction with auto-

radiography after a pulse label of ^3H -TdR, to determine whether the divisions are of first or second generation according to whether both or only one of a pair of chromatids are labelled. A distinctive chromosome marker (the T6 marker) has been used to identify the tissue source of blood lymphocytes of X-irradiated, reconstituted mice using PHA as a mitotic activator of the cultured cells.

Techniques currently in use are discussed by Yunis (1965). There are considerable variations in procedure in different laboratories, particularly in the concentration and time of exposure to colcemid. The following modification is suitable for most purposes: Colcemid (0.4 $\mu\text{g}/\text{ml}$) is added for 2–6 hr. The cells are spun down at 1000 rpm for 5 min and resuspended in 0.9% trisodium citrate (2 ml) for 20 min at 20 °C. The cells are again spun down and resuspended in 2 ml of ethanol:acetic acid (3:1) and left for 30 min. The fixed cells are resuspended by sucking and blowing through a Pasteur pipette. The cells are spun down and resuspended in a small volume (e.g. 0.2 ml) of 45% acetic acid. Drops of the suspension placed on scrupulously clean slides are air-dried on a hot-plate at 54 °C. They are then stained and examined.

3.1.14. Continuous culture of lymphocytes and related cells

It is common experience that very few lymphocytes are left in cultures after a few weeks and until recently continuous culture of lymphocytes was considered to be impossible although it has been known for a long time that permanent strains of glass-adherent cells of fibroblast-like morphology consisting of macrophages (from monocytes) could be established from blood cells. Lymphoblasts were first grown *in vitro* for long periods from tissue biopsies of patients with Burkitt's lymphoma (Pulvertaft, 1964) and other lines from Burkitt patients (e.g. the Epstein-Barr, EB lines) are now available (see Moore and McLimans, 1968). Many lines of mononuclear cells which do not stick to glass and are of lymphoid morphology have also been established from the peripheral leucocytes of patients suffering from leukaemia, infectious mononucleosis and other diseases, and even from the blood of normal individuals (Moore et al., 1966, 1967; Gerber and Monroe, 1968).

Due to improved techniques lymphoid lines may be initiated with a high success rate from comparatively small volumes (even as little as 20 ml) of the peripheral blood of normal individuals whereas previously this was only

possible with blood samples from patients with lymphoproliferative disorders and very large volumes of blood were required for the initiation of lines from leucocytes of normal individuals. The chief technical improvement has been the incorporation of an inoculum of X-irradiated cells from an established line containing EB virus or by promoting blastogenesis by the use of a non-toxic, non-haemagglutinating PHA. A success rate of about 34% in the establishment of new lines has been reported using the co-cultivation technique (Steel, 1972). The success rate was higher using leucocytes from placental blood samples. Typically the cultured cells are left undisturbed for some weeks with cautious supplementation with fresh medium. By about six weeks of culture a successful 'take' will be accompanied by definite acid production by the metabolising cells as indicated by a pH drop of the culture fluid. Soon after this stage the proliferating cells may be carefully passaged for the first time. Success at this critical stage soon leads to the establishment of a continuously growing line. It is still not easy to establish new lymphoid lines and not many laboratories will want to attempt it. However, the maintenance of established lines is a much easier task and in some respects less difficult than maintaining glass adherent lines e.g. Hela, fibroblasts.

In our experience EB2, EB4 and EB5 lines have grown well in Eagle's MEM supplemented with 10% human or calf serum and EB2 cells were even grown for some time in serum-free medium. Other lines are more fastidious but grow well in RPMI 1640 medium supplemented with 10% of a good quality foetal calf serum. Eagle's medium or McCoy's medium may also be used provided it is supplemented with at least 10% of a good foetal calf serum. The cells grow well in flat bottles at a depth of 1–2 cm of medium. Twice or three times a week an equal volume of complete medium is added and half the cell suspension transferred to a fresh bottle. The caps are tightly closed and provided they are air-tight no gassing is required. It is surprisingly easy to contaminate one line with another, particularly if the bottle tops become interchanged and it is preferable to keep a separate bottle of medium for each line to avoid this. The cell concentration may reach 1×10^6 per ml at 2–3 days after passage and the cell viability is usually more than 70% but varies from 50–90% with different lines. Routine tests for mycoplasma should be made.

All the lines available are broadly lymphoid in morphology and do not attach to glass surfaces but there are marked differences in the mode of growth of different lines (see ch. 5). EB2 and EB4 cells grow diffusely whereas

some of the lines from normal individuals or patients with infectious mononucleosis grow in tight clumps. They are largely diploid.

3.1.15. Quantitation of transformation

Even assuming that the culture conditions (medium, serum, stimulant, duration of culture, temperature, pH and O₂ tension) and the purity of the leucocyte preparation can be adequately standardised, one is still faced with the difficulty of how to compare objectively the activity of different cultures. Radioactive counts made on the pooled cells from a culture incubated with an isotopic precursor aim to measure the net activity of the viable cells in that culture. Autoradiographic or morphological estimates are based upon the relative numbers of active and inactive cells. The proportion of blast cells appearing in the cultures is affected by various factors other than the degree of stimulation. Part of the small lymphocyte population may die and disintegrate, as a result of the manipulation procedures or of the lethal effect of the antigen or stimulant preparation on the sensitised cells. Most stimulants are toxic above a certain concentration. Some stimulants, e.g. endotoxins, are by their nature cytotoxic and tuberculin PPD is normally used in cultures at near toxic concentration. Preservatives in stimulants also represent a source of toxicity. Since the progeny of activated lymphocytes are themselves likely to be activated many blast cells may arise from a single precursor and the numbers could perhaps be doubling each day. Small lymphocytes and blast cells may die. The same factors will affect the proportion of cells in DNA synthesis, as determined by autoradiography. Any realistic assessment of the proportion of the lymphocyte population activated requires a correction based on death and division of lymphocytes (Wilson and Thomson, 1968).

Counting of the radioactivity of the washed cell pellet after the addition of a labelled DNA precursor measures the total activity obtained from the initial population of cells and is now the most accepted means of quantitation. The results obtained have sometimes been expressed in the form of an index or ratio (dpm 'stimulated', dpm 'unstimulated') or as an increment ('stimulated' minus 'unstimulated'). The ratio allows one to assess the significance of the stimulation in relation to background whereas this cannot be guessed from the increment. On the other hand the ratio is unreal as a quantitative measure of the activity induced because there is evidence that different stimuli are additive, i.e. each stimulus applied to the cells increases stimu-

lation by a definite increment. For example, it is a common observation that if human cells are cultured in a medium containing foetal calf serum instead of human serum the activity of both 'stimulated' and 'unstimulated' cultures are increased by about the same amount, but the increment is substantially unchanged. Cultures prepared from thymus or bone marrow cells invariably have a much higher background activity than peripheral blood cultures from the same animal. Much of this activity may be contributed by cells not responsive to stimulant and the reduced stimulation index compared with that of peripheral blood cells may be quite misleading. Ideally the reported results should indicate the dpm 'stimulated' and the dpm 'unstimulated' on which the index of increment is based.

Even with non-specific stimulants like PHA it has been shown that there are individual variations in the optimum dose and in the kinetics of the response so that it is highly desirable in a comparative series to measure activation at at least two dose levels and over two or more time intervals (McIntyre and Cole, 1969; Richter and Naspritz, 1967).

3.2. Culture of lymphocytes from lymph nodes, spleen, thymus, tonsil, appendix or bone marrow

Lymphoid tissues may be cultured as fragments or the tissue may be completely disrupted and the resulting cell suspension cultured in much the same way as peripheral lymphocytes. Fragments of large tissues such as human spleen or thymus should be chopped from the bulk of tissue as soon as possible to avoid the effects of anoxia and placed in a bottle containing cold medium for transport to the laboratory.

3.2.1. Raft culture

A simple technique introduced by Trowell (1959) is useful in the culture of lymph nodes and lymphoid tissue fragments. The essential feature is the provision of a square metal grid made by bending over the two ends of a flat piece of perforated metal to make short legs. The material used is stainless steel expanded metal 1.5 mm mesh, 0.005 inches thick. The grid stands in a shallow dish made of hard glass or fused silica. On top of the grid a piece of lens paper (27×27 mm) is placed on which the tissue fragments are seated after culture medium has been added to a depth just sufficient to wet the paper. The dish is accommodated in a suitable air-tight

chamber fitted with entrance and exit tubes and 5% CO₂ in O₂ is passed through to gas the cultures. The gas chambers may be made of stainless steel, aluminium, perspex or glass.

Rats or mice may be anaesthetised with ether or preferably with a 50% CO₂-50% O₂ mixture. Lymph nodes, spleen or thymus are then aseptically removed. The tissues are collected in medium in a sterile Petri dish and trimmed of fat and connective tissue. Lymph nodes from rats or mice may be cultured whole on the rafts or may be cut in two. Fragments of spleen, thymus or nodes should not exceed approximately 2 mm in cross-section otherwise necrosis may occur at the centre through lack of O₂.

3.2.2. Filter-well technique

A Millipore filter well technique has been developed for the study of primary immune reactions in organ culture (Globerson and Auerbach, 1965, 1966). Fragments of spleen or thymus (0.3 mm thick and 0.5 to 0.7 mm in diameter) or bone marrow (gently removed from femurs and left as an intact gel) are placed in Millipore filter wells produced by gluing Millipore filter paper (25 µ thick, 0.45 µ porosity) to the underside of a plexiglass strip to yield a chamber 3 mm in diameter and 1.0 mm deep. The mounts are suspended over 1.0 ml of Eagle's medium (supplemented with serum, chick embryo extract and antibiotics) in a plastic dish.

3.2.3. Disaggregation of lymphoid tissues

The advantages of culturing intact lymphoid tissues are that the architecture of the tissue is preserved, the cells are not submitted to mechanical trauma and the normal physiological interactions of cells are not disrupted. The disadvantages are that quantitative comparisons between fragments from the same tissue cultured with different agents are difficult and the tissues have to be sectioned for morphological study. There is still a good deal of art in whole fragment culture. It is often preferable to risk the trauma of disrupting the tissue in order to obtain cell suspensions. The way in which this is done will depend upon the toughness of the tissue and the yield of cells required, higher cell yields requiring rougher dispersion techniques. The following are examples:

- a) Rabbit spleen is trimmed of fat and connective tissue and transferred, without fluid, to a small Petri dish or bottle. The tissue is chopped for some

minutes with sharp cuticle scissors and medium is then added to suspend the cells. After this the suspension is passed through a square of sterile nylon gauze (fine mesh) to remove small clumps of tissue. This is a gentle but inefficient method.

b) Mouse spleen is placed in a sterile glass homogeniser, a few drops of medium are added and the spleen is disintegrated with a single squeeze and twist of the pestle. Medium is then added and the cells are lightly dispersed in the homogeniser and filtered as before. This method was suggested by Dr. D. Dresser. The suspensions obtained from mouse spleen, which is admittedly a very soft tissue, are remarkably free of tissue debris. Other very soft tissues, e.g. rat thymus, can be efficiently disaggregated by patient teasing apart with two pairs of forceps supplemented, if necessary, by pressing the small pieces against the base of the Petri dish with the flat end of a syringe. Cell suspensions are very easily obtained from bone-marrow gel by gentle sucking and squirting of medium with a syringe.

c) Rabbit spleen is chopped lightly with scissors and the pieces transferred to a coarse mesh stainless steel gauze set in a polypropylene frame. The tissue is forced through the sieve by pressing with the barrel of a disposable 2 ml syringe, Eagle's medium containing 20% rabbit serum being squirted through the filter between pressings until all the tissue has passed through the metal gauze. It is then filtered through a nylon gauze. With this technique, which is suitable for tougher lymphoid tissues, there is always an appreciable amount of cell disruption. To remove cell debris the cells are spun lightly, the supernate discarded and the cells resuspended in medium containing 20% serum. The cells are sometimes not easily dissociated again but if the suspension is left at 37 °C for about half an hour and then given a single brief shake most of the clumps disperse without apparent adverse effects on the cells. Viable cells are counted by dye-exclusion in a haemocytometer.

d) The solid tissue is finely minced with sharp scissors into a Petri dish containing culture medium and transferred to sterile 73 μ nylon monofilament cloth (from Nitex, Toblar, Ernest and Trauber Inc., 71 Murray Street, New York) held in place on a stainless steel screen in a two-piece stainless steel thimble. The cells are teased through the gauze by rotary pressure applied by the plunger head from a disposable 1.0 ml syringe with the aid of some additional medium. The cell suspension is then filtered through 35 μ nylon cloth without pressure (Weyzen and Chin, 1966).

e) The use of enzymes (trypsin, collagenase, pronase) which are common-

ly used for dispersing solid tissues is undesirable in lymphocyte stimulation work because of the risk of modifying the surface characteristics of the cells.

3.2.4. Counting of cells in lymphoid cell aggregates

Cell suspensions prepared from disrupted lymphoid tissues frequently contain small clumps of cells, the presence of which may seriously affect the accuracy of simple nucleated cell counts. A similar problem arises with cells in cultures containing agglutinating agents such as PHA or leucocyte antibody. Several techniques have been devised for counting lymphocytes in tissue fragments and cell agglutinates. Trowell (1959) transferred the tissue from a raft culture to a dish containing 3 ml of 5% citric acid and left it for one hour for nuclear fixation and cytoplasmic dissolution to occur. The fixed tissue was then transferred to a 25 ml stoppered flask (with 3×3 ml washings of citric acid) containing 200 stainless steel balls (1/16th of an inch in diameter). The flask was shaken on a mechanical shaker at 2000 vibrations per minute (amplitude 5 mm) for 5 min. This completely dissociated the cells and left a suspension of undamaged fixed nuclei. The nuclei were stained by adding 1.0 ml of 0.1% gentian violet. Normal lymphocyte nuclei could be distinguished in the counting chamber from pycnotic nuclei and reticulum cell nuclei.

Several other methods depend upon the fact that dead cells are digested by trypsin, whereas viable cells are not. One such method, based on this principle, utilises the wide-spectrum proteolytic enzyme pronase in place of trypsin, to which it was demonstrated to be superior (Stewart and Ingram, 1967). Samples (0.5 ml) of a lymphocyte suspension containing 10^6 lymphocytes per ml and 12.5 µg PHA per ml were suspended in pronase at pH 7.3 (5 mg/ml, 0.5 ml) and incubated at 37 °C for 20 min. The entire 1 ml sample was added to 10 ml of a white cell counting solution. The counting solution was prepared by diluting formaldehyde (10 ml) and glacial acetic acid (5 ml) to one litre with 0.85% saline; cetyl trimethyl ammonium bromide was added to a final concentration of 5 mg/ml just prior to addition of the solution to the cell suspension. The pronase treatment completely eliminated dead cells (those in the trial experiments had been killed by exposure to 56 °C for 30 min) with little residual debris. The suspensions of nuclei could be 'sized' as well as counted on the Coulter counter. The nuclear volume changes detected appeared to reflect a true change in the morphology resulting from activation. A similar method omitting the use of proteolytic

enzymes has also been described (Schellekens and Eijsvoogel, 1968). To the cell suspension (approx. 3×10^6 lymphocytes in 0.4 ml of medium), glacial acetic acid was added (0.1 ml). After repeated shaking the quaternary ammonium salt Cetavlon (0.1 ml of 40% w/v) was added and the mixture again thoroughly shaken. Subsequently 0.1 ml of the suspension was transferred to 50 ml of dust-free (filtered through 0.22/ μ Millipore) buffered saline for Coulter counter measurements. Cell numbers may also be assessed indirectly by a DNA assay technique, such as the one described by Tedesco and Mellman (1967). Aliquots (0.5 to 1.0 ml) of a cell suspension containing 0.2 to 2.0×10^6 cells were centrifuged and the supernate completely removed (cell buttons could be stored at this stage for at least 25 days at -20°C without significant loss of DNA). The cell buttons were resuspended in 0.5 ml of 5% perchloric acid, incubated at 70°C for 15 min and cooled. Estimations of the desoxyribose in this hydrolysate by the diphenylamine technique were highly reproducible. The DNA per cell of lymphocytes was similar to that of cultured fibroblasts and cell numbers could be calculated from DNA levels.

3.3. The preparation and properties of 'pure' lymphocytes

Most of the work on lymphocyte transformation has been done on leucocytes obtained from the peripheral blood. The degree of contamination of blood lymphocyte preparations with other leucocytes depends upon the separation technique employed. The leucocytes from sedimented heparinised blood contain most of the granulocytes, monocytes and platelets of the original blood whereas all the platelets and some of the granulocytes are removed by defibrination of the blood. Some red cells are always present. It cannot be assumed, without proof, that the contaminating cells have no effect on the transformation process. They may modify or break down or simply absorb the stimulant; they may provide nutritive materials acting in much the same way as a feeder-layer. Conversely they may use up limiting nutrients and they may produce enzymes which affect the lymphocyte surface. This is particularly true of granulocytes. In mixed cell reactions contaminating cells may provide part of the antigenic stimulus or they may impede the cell to cell contact necessary for transformation. A further factor is that extracts of granulocytes are known to be active in the breakdown of thymidine thus possibly complicating the interpretation of assessments of activity based on the incorporation of $^{3}\text{H-TdR}$.

Although experiments with lymphocyte populations which are naturally relatively pure, such as thoracic duct lymphocytes, leave no doubt that activation, at least with PHA, still occurs, these responses may be submaximal. Of necessity most experiments are performed on lymphocytes derived from peripheral blood and the problem becomes one of separating lymphocytes as completely as possible from other blood cells. Several new techniques for separating leucocytes into their several components have recently been introduced and some of the older techniques subjected to rigorous scrutiny.

3.3.1. Density gradient techniques

The original albumin gradient technique (Vallee et al., 1947) and the more recently introduced Ficoll (polysucrose) gradient techniques were evolved on the principle that if cells were placed over or in a density gradient of a high molecular weight material the cells would distribute themselves in bands corresponding to their density. The great difficulty experienced in standardising these techniques revealed that factors other than density, notably pH and osmolarity of the solutions used, were involved. The three gradient techniques with which useful results have been obtained employ bovine albumin, polysucrose or polysucrose-metrizoate.

a) Bovine albumin. Up to 5×10^8 cells are dispersed in 13 ml tubes in a continuous linear gradient of bovine albumin prepared in a balanced salt solution of pH 5.1 and osmolarity 0.294. After centrifugation at 3800 g for 45 min at 4 °C fractions are collected by pumping in bromobenzene to displace fluid through an exit at the top of the tube (technique of Shortman, 1968 and Shortman and Szenberg, 1969). The bovine albumin used had been dialysed to remove salts and stored frozen. The gradient was prepared with a light albumin solution (14.5% w/w), and a heavy albumin solution (28% w/w). The cells were incorporated into the heavy albumin solution to avoid the interfacial packing and distortion which occurs when cells are placed in medium on the top of a gradient in the usual fashion. It was further shown that cell density is not an absolute figure but is affected by external conditions such as tonicity. Cell aggregation, another complication, was minimised by lowering the pH to 5.1. At this pH erythrocytes swelled and decreased markedly in density, an effect not observed with lymphocytes. At 4% hypertonicity thymus cells shrank and became more dense.

Good viable lymphocytes of high purity may, however, be obtained by

procedures much less elaborate than the one described, e.g. by preparing a discontinuous albumin gradient in phosphate-buffered saline pH 7.0 over the range 14–28% albumin, the cells being incorporated into the upper albumin layer. It is advisable to check the salt content of the albumin solution (e.g. by adding a known volume to a strong base cation exchange resin in the hydrogen form, filtering and titrating the acid liberated) and correcting for this salt by dissolving the albumin in the appropriate proportions of water and buffer solutions. The cell band in which the lymphocytes are found is substantially free of granulocytes and red cells (which are denser than lymphocytes) but contains monocytes. If the cells are obtained from heparinised instead of defibrinated blood, platelets will also contaminate the lymphocyte fraction.

b) Polysucrose. The chief advantage of using Ficoll (polysucrose of mol. wt 400,000) instead of albumin in an otherwise similar technique, is that Ficoll, being uncharged, does not bind ions and does not alter the osmolarity of the solution. The Ficoll used by Gorczynski et al. (1970, 1971) was deionised by passage of a 36% w/v solution through a mixed bed resin (100 g of resin per 500 g Ficoll) and the solution sterilised by filtration. Cell clumping was found by these authors to be an even greater problem with Ficoll than with albumin solutions. A combination of low pH (5.5) and the use of a dispersing agent was found to be necessary. A buffer of osmolarity 0.294 M, composed of 0.01 M sucrose, 0.003 M KH_2PO_4 , 0.1165 M NaCl, 0.015 M 2-naphthol-6, 8 disulphonic acid (dipotassium salt) was used. Gradients of Ficoll from 10 to 29% were prepared in the buffer, water being added as necessary to allow for the osmolarity of the Ficoll. Density profiles for mouse spleen cells showed nucleated cells distributed over the density range 1.05 to 1.096, with red cells forming a sharp peak in the density range 1.083 to 1.096. Damaged cells sedimented in the denser region of the gradient. The whole nucleated cell distribution curve was shifted slightly to a higher density in the Ficoll gradients prepared at pH 7.2 compared with pH 5.5. When slight osmotic gradients were introduced peaks were generated in what was previously a homogeneous distribution of cells suggesting that if osmolarity could be varied in a reproducible fashion it could be the basis of a separation technique. It is possible that successful gradient separations which have been difficult to reproduce have been based on fortunate combinations of the density and osmolarity variants.

c) Polysucrose-metrizoate. A balanced solution of the sodium, calcium and magnesium salts of metrizoate (3-acetamido-2:6:6 tri-iodo-5-N methyl

acetamidobenzoate) sold under the trade names of Triosil or Hypaque as an intravenous contrast medium, has been used in conjunction with Ficoll in a simple reproducible technique for preparing lymphocytes directly from whole blood (Boyum, 1968; Perper and Mickelson, 1968; Thorsby and Bratlie, 1970) and, without the addition of Ficoll, for the separation of eosinophils (Day, 1970). Triosil 34% is prepared by adding 24.2 ml of water to 20 ml of Triosil 75. Ficoll 9% is prepared by dissolving 9 g of solid Ficoll in distilled water to 100 ml. Ten parts of Triosil 34% is mixed with 24 parts of Ficoll 9% and the solution sterilised by filtration. For use, defibrinated blood (2 ml) is carefully layered over Ficoll-Triosil (2 ml) in a sterile capped tube and centrifuged at 400 g in a swing-out head for 30–40 min at room temperature. Red cells and granulocytes sink to the bottom of the tube leaving lymphocytes and monocytes in a white band just below the interface. The density of the Ficoll-Triosil mixture is approx. 1.076 and it is approx. isotonic being compounded of a highly hypertonic solution of metrizoate (34% is about 3.4 times isotonic) and a highly hypotonic solution of poly-sucrose (osmolarity scarcely greater than that of water). Although developed for whole blood the method is also suitable for the purification of leucocyte-rich supernates of blood after separation of most of the red cells with gelatin, dextran or methyl cellulose. The supernate from, say 50 ml of blood, is centrifuged and the cells taken up in 2 ml of hepes buffered medium and layered over Ficoll-Triosil as for blood. In this way lymphocyte preparations almost completely free of red cells and granulocytes are obtained with contaminating monocytes. It is the technique of choice for routine purposes. When applying the technique to separate lymphocytes from the blood of other species, differences in cell density may be encountered which affect the yield of lymphocytes. This may be corrected by altering the concentration of Ficoll while retaining the Triosil at 34%. Low recovery of rabbit blood lymphocytes obtained with 9% Ficoll–34% Triosil, for example, may be corrected by using 12% Ficoll–34% Triosil.

3.3.2. Separation of cells on the basis of size by sedimentation velocity

Cells of similar density may be separated on the basis of size by their different rates of sedimentation in the earth's gravitational field. Successful fractionation is dependent upon the cells being placed cleanly in a wafer-thin band on the top of a shallow stabilising gradient in a suitable vessel from which fractions can be readily run off after a suitable sedimentation period.

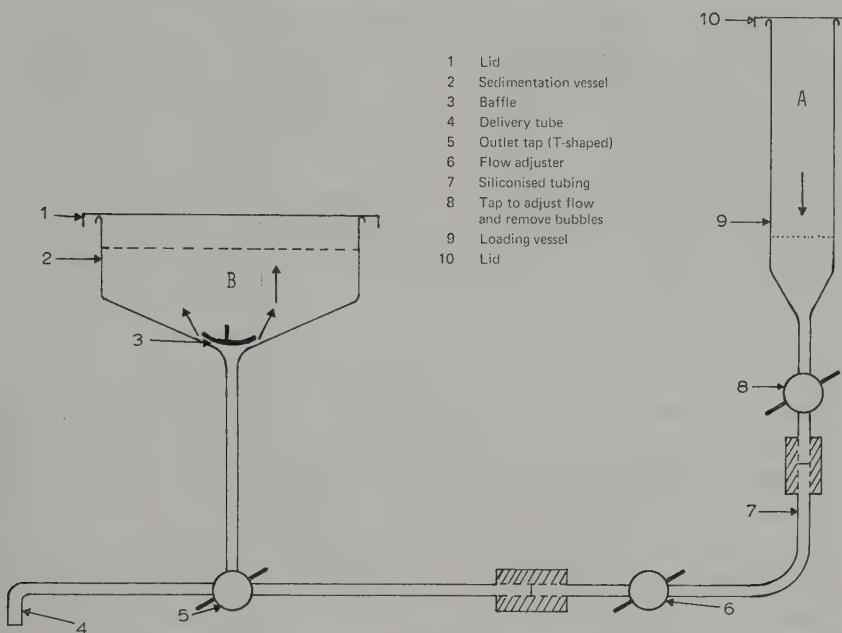


Fig. 3.1. A simple velocity sedimentation apparatus supplied by GK Scientific, Jacey Road, Shirley, Warwickshire. The sedimentation vessel (B) is 12 cm in diameter. A stock solution of 2% bovine serum albumin (BSA) is prepared by adding BSA (5 g) to RPMI 1640 medium (100 ml) buffered with Hepes (20 mM) and adding phosphate-buffered saline (140 ml) and foetal calf serum (10 ml). Dilutions of this solution are prepared by addition of PBS. The following solutions are then run consecutively into B via A: PBS (20 ml), cell suspension (10^8 cells in 20 ml of 0.2% BSA in PBS), 0.5% BSA (50 ml), 1% BSA (50 ml), 1.5% BSA (100 ml), 2% BSA (130 ml). Total loading time = 30 min. The apparatus is then left for 2½ hr and 10 ml fractions are run off from B via the outlet tap.

A suitable sedimentation chamber (based on that of Miller and Phillips, 1969) is shown in fig. 3.1. The flow rate and the shape of the baffle are important and markedly affect the cell band stability. Phosphate-buffered saline (20 ml) is first introduced slowly into the chamber, followed by the cell suspension in 0.2% bovine albumin and a discontinuous albumin gradient in phosphate buffered saline (0.2 to 2.0%) the filling taking 15 to 45 min. The cells are left undisturbed for say two hours (sedimentation velocity, $\text{mm/hr} = 0.25 r^2$ where r is the cell radius in microns) after which fractions (10 ml) are drawn off. Cell separation is approximately on the basis of size

and independent of shape. The number of cells which may be sedimented is severely limited by streaming to about $15 \times 10^6/\text{ml}$. The technique has already been used to separate haemopoietic stem cells from cells causing graft-versus-host disease (Phillips and Miller, 1970) and, in differentiating rosette-forming cells from myeloid stem cells (Edwards et al., 1970) and in defining the dimensions of the cells in mouse bone marrow and thymus which co-operate in the antibody response to sheep red cells as being those of small lymphocytes (Miller and Phillips, 1970).

3.3.3. Iron ingestion techniques

There are several variants of a technique which depends on removing phagocytic cells from blood by allowing them to ingest iron particles, and an automated technique (Technicon) utilising this principle is commercially available. Capacity for phagocytosis is associated with metabolic activity, hence it is important to use cells from recently collected blood and to avoid anticoagulants such as oxalate, EDTA or citrate which inhibit phagocytic activity. a) Carbonyl iron (SF grade 100 mg, particle diameter = 3 μ) is added to heparinised blood (10 ml) and the container slowly rotated at 37 °C for 30 min after which the phagocytic cells are removed with the aid of a strong magnet (Kuper et al., 1961). b) Carbonyl iron (100 mg, 3 μ diameter) and dextran (2.5 ml) are added to defibrinated blood (10 ml) and the cells removed from the supernate fluid after sedimentation for one hour at 37 °C. c) To 10 ml of sterile defibrinated blood at 37 °C add carbonyl iron powder (200 mg) and 3 ml of warm sterile 1% methyl cellulose in phosphate buffered saline. Rotate on a mixer at 37 °C for 30 min, decant to a warm sterile siliconed capped container to remove air bubbles and allow to sediment for 30 min at 37 °C. Red cells, granulocytes and monocytes settle down under the influence of gravity while many of the lymphocytes and some iron particles remain in suspension (Coulson and Chalmers, 1967). Most of the iron may be removed during subsequent centrifugation and resuspension of cells.

3.3.4. Surface adhesion techniques

Lymphocytes differ from most other nucleated cells in showing little tendency to adhere to glass, plastic or fibre surfaces. Phagocytic cells, on the

contrary, adhere rapidly to these surfaces and remain firmly attached during washing. This type of adhesion is an active process and requires a period of incubation at room temperature or (preferably) at 37 °C and the presence of fresh serum. It is also advisable to use freshly obtained cell suspensions, particularly with blood preparations, as neutrophils soon degenerate and lose the capacity to adhere. Methods of this type were originally designed to allow complete recovery of lymphocytes as a non-adherent population with complete removal of other leucocytes. None of the methods employed, however, give 100% recovery of lymphocytes and recovery of cells from glass and fibre columns is often very low. Moreover there is a preferential loss of B cells, which are 'stickier' than T cells so that the T:B cell ratio of the effluent cells is greater than that of the original population. This is particularly true of nylon fibre columns, which have even been used as a way of removing B lymphocytes from human blood or tonsil preparations. Columns of antigen or antiglobulin-coated glass, plastic or agarose beads have also been used to remove cells containing specific surface groupings. These techniques are beyond the scope of this review.

The simplest way of removing phagocytes is to incubate the leucocyte suspension in medium in a shallow layer in a Petri dish or a flat-sided bottle placed on a flat surface in an incubator at 37 °C for one hour or more. Non-adherent cells are resuspended by gentle rocking and the fluid poured off leaving surface-adherent cells behind with little loss of lymphocytes. The efficiency of this method increases as the serum concentration is increased during the incubation period but it is never possible to remove all the phagocytes. A number of techniques for separating leucocytes by passage through columns have developed from the original observations of Garvin (1961), who passed whole, heparinised blood through a column of siliconed glass beads to remove granulocytes and of Walker et al. (1961), who used cotton wool for a similar purpose.

3.3.5. Glass bead, glass fibre, cotton wool and nylon wool columns

Columns consisting of alternate layers of glass fibre (Pyrex fibre no. 3950, Corning glass) and glass beads (no. 100-15, Superbrite, Minnesota Mining Co.) have been used by Rabinowitz (1964) to remove phagocytes from blood leucocytes suspended in serum or plasma. For 5 ml of cell suspension columns 16 × 1 cm diameter were packed 85% full of alternate fibre and beads with a top layer of beads. A 37 °C water jacket was fitted. Cell

suspensions and wash solutions were introduced by injecting a needle through a rubber stopper fitted to the column inlet and a sleeve-type stopper sealed the outlet. Columns were left undisturbed at 37 °C for 30 min after introduction of leucocytes and then washed with fresh heparinised plasma, applied via a syringe, to give a flow rate of 15–20 drops per minute. Lymphocytes were obtained in about 50% yield, contaminated by erythrocytes but essentially free of phagocytes.

In applying glass bead columns for the fractionation of mouse spleen cells Shortman et al. (1971) found that several factors, in addition to the active adhesion so far considered, influenced cell retention and were responsible for losses of large numbers of lymphocytes. A 'size-filtration' effect was responsible for trapping of larger cells, but this effect was only significant when beads less than 100 μ in diameter were used. All columns tended to retain damaged cells under all conditions tested. A most important observation was that large numbers of lymphoid cells were trapped by 'physical adherence'. Unlike the 'active adhesion' of phagocytes this phenomenon was temperature independent and occurred at 4 °C as well as at 37 °C. Antibody-forming cells (and probably their precursors) were selectively retained on the column. The best recovery of lymphoid cells, with over 500-fold depletion of macrophages and approx. 50-fold depletion of active PMN leucocytes, was obtained with columns prepared and used in the following way: A column (14 cm high \times 1.8 cm diameter) fitted with a 37 °C water jacket and containing siliconed spherical glass beads (300–600 μ) was washed with 60% FCS-medium. Just before applying the cells 2 ml of 60% mouse serum in medium was run onto the top of the column to provide a density barrier for the cells. The debris-free mouse spleen cell suspensions (2 ml containing 2×10^7 to 2×10^8 cells in 50% mouse serum) was warmed to 37 °C and run onto the column, followed by an equal volume of 50% mouse serum-medium (with gentle stirring of the top of the bed at this stage to prevent blocking). The cells were washed through the column with one column-volume of 25% mouse serum-medium, allowing a residence time of 8–10 min of cells on the column. The method may be applied, with modification, to rat and mouse peritoneal exudate cells and to lymphoid cells from other tissues and species. When it was applied to bone marrow a column effluent consisting only of small to medium lymphocytes and erythroid cells was obtained. Glass bead columns are little used as a means of preparing small lymphocytes by 'size filtration' now that velocity sedimentation techniques are available. Yields of small lymphocytes from 'size fil-

tration' columns are low. Moreover although most of the cells exclude dye they are only about 50% as active as fresh cells in initiating graft-versus-host reactions (Shortman and Szenberg, 1969).

Simpler column methods have been used although they have not been as efficient. Chessin et al. (1966) passed the leucocyte-rich plasma from 500 ml of heparinised blood diluted with one volume of Eagle's medium, through a 100 ml syringe packed with 3 g of sterile glass wool filtering fibre (no. 3950 of Corning) at a temperature of 37 °C and a flow rate of 30 drops per minute. The cells were then washed through with an additional 100 ml of medium.

Columns of absorbent cotton wool have been widely used for removing blood phagocytes. Samples containing irregular distorted fibres have been shown to be superior, in terms of granulocyte retention, to those containing smooth regular fibres (Lamvik, 1966). Leucocyte-rich plasma (6 ml) is run into a sterile column (100 cm long × 13 cm in diameter) containing 400 mg of loosely packed cotton wool. The column is sealed, incubated at 37 °C for 30 min and then washed through with medium (6 ml). About 1% of the granulocytes and 50% of the lymphocytes pass through the column. In a simpler version of this technique 200 mg of coarse dry absorbent cotton wool is placed loosely in a 10 ml syringe. Leucocyte-rich serum (3 ml) is drawn up into the syringe, which is sealed and left at 37 °C for 30 min. The barrel is then pressed home to expel the fluid and non-adherent cells.

In all the methods described it is probable that more B than T lymphocytes are lost on the columns. Nylon fibre columns are also thought to remove large numbers of B cells as well as phagocytes. The following is a suitable procedure: One gram of nylon wool (previously washed for several days in water and dried) is packed into the barrel of a 10 ml glass or polypropylene syringe fitted with a short length of silicone rubber tubing. The syringe is sealed with aluminium foil and autoclaved. The rubber tubing is then closed with a small screw-clip and filled with hepes-buffered RPMI 1640 medium containing 10% heat-inactivated foetal calf serum warmed to 37 °C. Excess fluid is run off and 2 ml of the same medium containing 2×10^8 cells (prewarmed to 37 °C) is run into the nylon wool. The column is closed and incubated at 37 °C for 30–45 min. More medium is then slowly run through the column until about 10 ml of fluid has been collected.

3.3.6. Polystyrene bead columns

Columns containing polystyrene beads (styrene/divinyl benzene copolymers, 40–60 mesh; diameter 0.25–0.42 mm) have been used by Thomson et al. (1966). Columns (1.7 × 18 cm) maintained in buffered Tyrode's solution pH 7.4 were loaded by displacement of the buffer with leucocyte-rich serum (from defibrinated blood treated with gelatin). Ten ml of beads accommodated approximately 3.7 ml of fluid. After 30 min at 37 °C the column was unloaded by a single displacement with fresh buffered salt solution. The rate of flow was adjusted to complete this process in about 5 min. About 75% of the cells were recovered and the average proportion of lymphocytes was 81–99% of which less than 1% were pycnotic. Some degree of separation of lymphocytes from red cells occurred owing to a retarded passage of the red cells through the column. A red band remained at the base when most of the lymphocytes had been eluted. This method has been used for separating normal from leukaemic lymphocytes.

3.3.7. Removal of erythrocytes

Erythrocyte contamination of leucocyte preparations (usually 1 to 20 rbc per wbc) can be reduced by, (a) Methods depending upon the fact that erythrocytes are denser than lymphocytes. Red cells are almost entirely absent from leucocyte preparations centrifuged after layering over Ficoll-Triosil and this is probably the best way of removing them. (b) Methods depending on the susceptibility of erythrocytes to osmotic lysis (Fallon et al., 1962). The 'shock' solution may be pure water or 0.83% NH₄Cl solution (Agostoni and Ideo, 1965), red cells being permeable to ammonium ions. Centrifuge the cell suspension (10 ml) to deposit the cells. Remove the supernate (completely) to another tube. Flick the tube to disperse deposit. Add water (0.4 ml). Leave 30 sec, then quickly add back the supernate from the other tube. Alternatively instead of water add 0.83% NH₄Cl to the dispersed cell pellet (twice the volume of the cell button), leave 10 min and then add back the supernate. (c) Methods depending on the agglutination of red cells by antisera. The leucocytes from 20 ml of blood are taken up in 1 ml of medium and 0.1 ml of chicken anti-human erythrocyte antiserum added. After incubation at 37 °C for 15 min the cells are spun down, the supernate removed and replaced by 10 ml of fresh medium. Mix by gentle inversion and allow the red cell clumps to settle by sedimentation for $\frac{1}{2}$ –1 hr

at 37 °C (based on Sanderson, 1967). Erythrocytes from individuals of group A, B or AB may alternatively be removed by a similar treatment with sera containing the appropriate iso agglutinins or anti-H in the case of donors of group O.

3.3.8. Separation of T and B sub-populations

The many useful techniques now being developed for identifying and fractionating lymphocytes into sub-populations depend upon the presence of specific surface receptors or differences in density, charge or other physical characteristics (see ch. 2 for references and discussion).

Human T and B lymphocytes may be separated by forming E or EAC rosettes respectively following by sedimentation.

E rosettes: Defibrinated blood (20 ml) is treated with 3% gelatin in saline (7 ml). After one hour's sedimentation at 37 °C the leucocyte-rich supernate is removed, the leucocytes spun down, washed once in Hepes-buffered medium and taken up in 2 ml of this medium. This cell suspension is layered over Ficoll-Triosil (2 ml) and centrifuged for 30 min at 400 g. The lymphocytes are collected from the interfacial band and, after dilution with Hepes-buffered medium, are deposited by centrifugation and washed once in medium. The lymphocytes are then suspended in Hepes-buffered medium containing 20% inactivated foetal calf serum to a cell concentration of 4×10^6 per ml. A sheep red cell suspension is prepared from sterile sheep blood (collected in Alsever's solution and not more than one week old) by washing three times in PBS and suspending in PBS to 80×10^6 per ml. Equal volumes of each cell suspension are mixed in a flat-bottomed sterile bottle or vial and the cells deposited by centrifugation for 5 min at 200 g. The bottle is placed at 4 °C for 2 to 24 hr. The cells are then resuspended by gentle repeated inversions of the container and the number of rosettes (mononuclear cells to which at least 3 red cells are attached) are counted in a haemocytometer.

EAC rosettes: One ml of a 5% suspension of washed sheep red cells in PBS is mixed with an equal volume of haemolysin (rabbit anti-sheep red cell) diluted 1 in 2000 in PBS. After incubation at 37 °C for 30 min the cells are spun down, washed twice in PBS and resuspended in Hepes-buffered medium (1 ml). Fresh human serum (0.05 ml) is then added as a complement source and the suspension incubated at 37 °C for 30 min. The cells are then washed twice and resuspended in medium to 80×10^6 /ml. The EAC sus-

pension is mixed with an equal volume of the lymphocyte suspension (prepared as above), spun at 200 g for 5 min and left at room temperature for 30 min. The cells are resuspended by rapid and repeated inversion. Rosettes are counted. There is a possibility that under these conditions for the formation of EAC rosettes some EA and E rosettes are also formed. To avoid this some workers used IgM antibody rather than the crude haemolysin and human red cells instead of sheep, i.e. the EAC complex consists of human red cells (group O) coated with rabbit anti human red cell antibody of IgM class and exposed to fresh human serum (complement).

Separation of E or EAC rosettes from free lymphocytes would yield populations with the properties of T or B lymphocytes respectively. Some separation may be obtained simply by allowing the suspensions to settle for 30 min, removing the supernate, resuspending the cells in fresh medium and sedimenting for 30 min. After 3 or 4 such washes rosettes substantially free of non-rosetted cells are obtained but yields may be low. Superior methods are separation by density in an isopycnic gradient or by size in an isokinetic gradient (Cooper and Bain 1971; Pretlow and Luberoff, 1973). The Ficoll-Triosil and velocity sedimentation methods already described, also based on density or size, may also be used. Pure rosettes are difficult to obtain by these methods, however, because of break-up of rosettes during the separation and because of trapping of lymphocytes by the sedimenting rosettes.

3.3.9. Viability and responsiveness of 'purified' lymphocytes

Most authors have shown that the lymphocytes recovered from columns appeared morphologically intact and excluded vital dyes and that PHA induced some transformation. There are reasons for believing, however, that the lymphocytes may have been modified by some methods of purification. Thus Cooper and Rubin (1965) found that only a small proportion of their column-purified lymphocytes incorporated ^3H -cytidine into their RNA whereas most unprocessed lymphocytes are known to become labelled under the conditions used. Oppenheim et al. (1966) found that although column-purified lymphocytes still showed a marked response to PHA and leucocyte antibody they were consistently less responsive over a wide dose-range than matched lymphocytes which had not been processed. The response to streptolysin O, vaccinia vaccine, PPD and homologous lymphocytes was markedly diminished. The responses were not restored to normal

by using larger quantities of antigen or by prolonging the culture period. Most of the hyporesponsiveness of column-purified lymphocytes can be accounted for by trauma resulting not only from passage through the column itself but from the several washing stages to which the cells have usually been subjected. Some augmenting role of the other leucocytes cannot however be excluded. There are also indications that fractionation of the lymphoid cells present may occur, antibody synthesising and DNA-synthesising lymphoid cells being retained on glass bead columns along with granulocytes, macrophages and monocytes while small lymphocytes pass through the column (Plotz and Talal, 1967). Related studies have been made by Shortman et al. (1971).

The hypotonic shock treatment used to remove red cells has also been shown to affect the lymphocytes. Thomson et al. (1966) found that, in general, one third or more of the lymphocytes from 7 different donors suffered death as a result of the 30-sec exposure to water. The dead cells were distinguishable by a total homogenisation of chromatin and the absence of a recognisable nucleus indicating a bursting of the cell. The mortalities recorded after exposing column-separated lymphocytes for 30 sec to 0.05, 0.10, 0.15 and 0.2% saline were 32, 25, 22 and 13% respectively compared to 34% with water alone. The lymphocyte mortality from a single hypotonic shock progressively increased with increasing time of exposure to water. A shock time of 40–45 sec was required to kill half of a lymphocyte population. The lymphocyte population which survived one water treatment was resistant to a second shock. There was an average of about 5% death to the second shock and when a third shock was given only 1 or 2% of the lymphocytes died. Thus a certain proportion of the population of peripheral lymphocytes appears to be intrinsically more fragile than the rest. It is quite clear from these experiments that a change in the responsiveness of a lymphocyte population to activating agents cannot be predicted from a simple viability count.

3.3.10. Heat sensitivity of lymphocytes

Schrek (1966) tested the heat sensitivity of normal and leukaemic lymphocytes. Lymphocyte suspensions were heated in the range 42–50 °C and then incubated at 37 °C. The number of viable lymphocytes was assessed under the phase-contrast microscope on the 7th day. Exposure to 43 °C for 2 hr produced a 40–100% cytoidal effect on lymphocytes from 16 of 19 leukaemic

patients but produced less than 40% effect on the lymphocytes from all 18 normal persons tested. Almost all normal lymphocytes were killed by exposure to 50 °C for 3 min, 47 °C for 12 min or 45 °C for 50 min. Exposure to 43 °C for 3 hr produced only 50% cytoidal effect and at 42 °C an exposure of about 3 days was required to produce a 50% effect.

3.3.11. Preservation of lymphocytes

Lymphocytes separated from blood or lymphoid tissues have been successfully stored at low temperatures for long periods using techniques essentially similar to those used for storing other cells. Lymphocyte suspensions in medium to which dimethyl sulphoxide (DMSO) has been added are frozen in ampoules (or flat bags of a polyethylene-aluminium-foil-paper laminate if large volumes are to be frozen) at a slow controlled rate in a freezing apparatus and stored in a liquid nitrogen container. When required the ampoules are thawed quickly by gentle agitation in a warm water bath (37–40 °C). The ampoule is immediately opened and the contents diluted with cold (4 °C) medium. After centrifugation at low speed the supernate is discarded and the cells immediately resuspended in fresh medium. Delay at this stage or centrifuging at too high a speed causes clumping of lymphocytes.

It is well established that the efficiency of recovery of frozen cells, including lymphocytes, is critically dependent upon the rate of cooling. For most cells a cooling rate of 1 to 2 °C per minute is optimal. A cooling rate of 1 °C per minute is optimal for small lymphocytes but larger cells, e.g. normal or leukaemic blast cells, require a slower cooling rate (Knight et al., 1972). Lymphocytes pre-activated with a stimulant, e.g. con A or PPD, could be frozen one or two days after stimulation at a freezing rate and in a dimethyl sulphoxide concentration which allowed subsequent DNA synthesis of the stimulated cells while severely damaging the small lymphocytes present, thus producing an enrichment of mitogen-responding cells. Thus freezing can be used as a tool to select a portion of the lymphocyte population.

The following method (based on that of Bates and Sell, 1970) is suitable for routine preservation of lymphocytes: Lymphocytes suspended in Eagle's medium at 4 °C containing a final concentration of 7% dimethyl sulphoxide and a serum concentration of between 1 and 8%, are frozen in sealed ampoules at a rate of 1 to —25 to —30 °C followed by freezing at a rate of 3.5 to —125 °C. They are stored at —196 °C. For recovery the ampoules are

quickly agitated in a 37 °C bath. As soon as ice crystals have disappeared the ampoule is opened and the contents added to three volumes of medium at 4 °C. The cells are gently spun down, the supernate discarded and the cells resuspended in fresh culture medium. Under these conditions small lymphocytes are recovered but granulocytes, monocytes and red cells do not survive. Lymphocytes recovered from storage may be used for HL-A typing and are responsive to PHA, allogeneic lymphocytes and some antigens but show diminished responses in the MLR (Mangi and Mardiney, 1970). A high recovery rate requires a precisely controlled rate of freezing and care at the stage when supercooling is likely to occur (Knight et al. 1972). Cool the sample, after addition of DMSO, to 0.5 °C below the freezing point of the DMSO solution and then seed with an ice crystal before continuing to freeze. The cell concentration is normally about 5 or 10×10^6 per ml but may be very much higher without recovery rates being much affected.

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Section 3.1.

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CHAPTER 4

The activation of human and animal lymphocytes by common stimulants

4.1. *Choice of species and stimulant*

Many investigations, especially those concerned with the relation of an in vitro response to an immune state of the cell donor, or the relation of a mixed cell reaction to histocompatibility differences, are more easily performed with animal than human cells. While it is convenient with humans to culture the blood lymphocytes rather than spleen or lymph node cells, the reverse is often true of small animals and for this reason many of the results obtained with animal cells are not strictly comparable with those obtained with human cells. Larger animals, e.g. horse or pig, are a good source of blood lymphocytes for studies requiring very large numbers of cells. There are considerable variations in the survival of lymphocytes of different species, in the level of their response to various stimulants and the dose of stimulant required to achieve an optimum response. There are variations, too, in the numbers of lymphocytes in the blood of different species and in the differential blood picture (Schalm, 1967). Survival of cells of some species is poor in homologous serum (table 4.1) and better responses may be obtained in media supplemented with foetal calf serum, at the expense, usually, of an increased background stimulation.

Lymphocytes of most species are activated by the *Phaseolus vulgaris* mitogen (PHA), by other lectins e.g. pokeweed mitogen (PWM) and concanavalin A (con A) and by certain products of staphylococci, streptococci and other bacteria. These have all been called 'non-specific' stimulants because a high level response is obtained with the blood or tissue lymphocytes of most individuals and animals with no requirement for prior sensitisation to the substances concerned. Responses are also obtained with lymphocytes from cord blood and usually with cells from neonatal or

Table 4.1

Viability of cultured lymphocytes in 20% homologous serum in Eagle's medium.

Source of lymphocytes	Percent survival								
	Days of culture								
	1	2	3	4	5	6	7	14	21
Rabbit blood	61	43	26	22					
Rabbit thymus	43	30	12	7					
Rabbit appendix			26	23					
Rabbit spleen	69	47	38	28					
Guinea pig blood	37	33	24						
Rat blood	48	19	5						
Mouse blood	27	5	0						
Hamster blood	84	17	5						
Cat blood		60	43	35					
Human blood	85	73	66	62	58	55	50	15	8
Human thymus	76	66	54	39	30				
Pig blood	82	69	60	55	50	41	27		

embryonic thymus. It has long been known that only a proportion, albeit a high proportion, of the lymphocytes initially present are activated even with a potent stimulant under optimal conditions and that within the responding population there are extreme variations in the kinetics of the response. None of the mitogens are as yet well defined (see ch. 6) and with the impure preparations normally used there are considerable variations of response related to the batch of stimulant. This soon becomes evident if a dose-response curve is constructed for a mitogen. From fig. 4.1 it can be seen that both the optimal dose and the shape of the dose-response curve for con A vary with the particular product used. The same is also true of PHA, but a given commercial source (e.g. PHA from Burroughs Wellcome or PHA-M from Difco) tends to give a similar pattern of response on different occasions. There are indications that a single species of a mitogen in its purified form used at optimal concentration would stimulate a characteristic sub-population only. Even with the impure preparations of mitogens currently available there is clear evidence of some specificity of stimulation of sub-populations of lymphocytes. By this criterion common stimulants

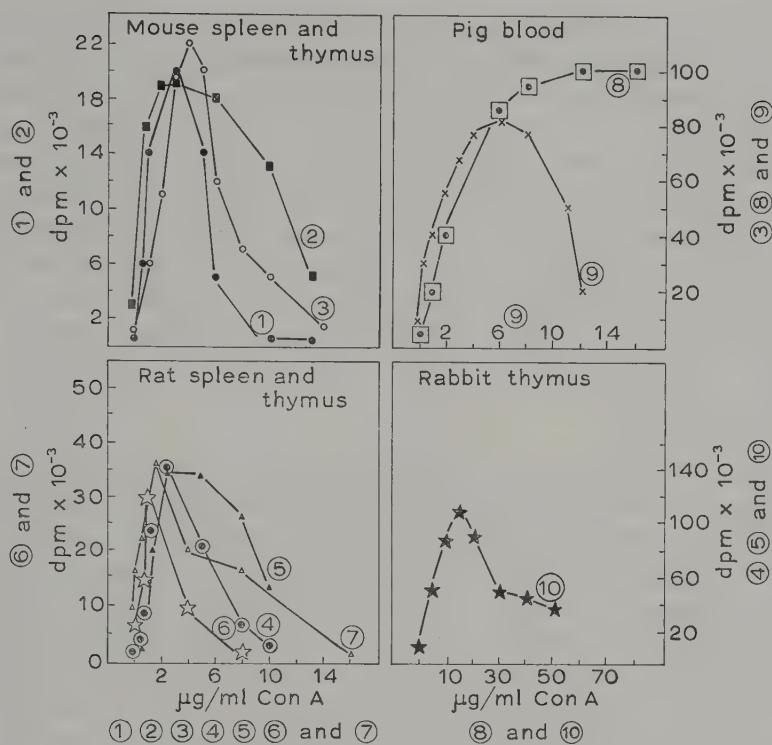


Fig. 4.1. Dose-response curves for con A using various con A products and lymphoid cells from different tissues and species. Con A sources = Miles-Yeda (MY), Calbiochem (CB), British Drug Houses (BDH) and a batch prepared by Dr. Pardoe (P). (1) ● = Mouse thymus cells, con A = P (2) ■ = mouse spleen cells, con A = P (3) ○ = Mouse spleen cells, con A = MY (4) ⊙ = rat thymus cells, con A = BDH (5) ▲ = rat spleen cells, con A = BDH (6) ★ = rat thymus cells, con A = MY (7) △ = rat thymus cells, con A = P (8) ▨ = pig blood cells, con A = CB (9) ✕ = pig blood cells, con A = CB (10) ★ = rabbit thymus cells, con A = CB. Ordinate = disintegrations per minute (dpm) representing ^3H -thymidine uptake by cells on the scale indicated. Abscissae = μg of con A per ml (final concentration) on the scale indicated.

cannot be said to be 'non-specific'. Indeed, it has been known for many years that some 'non-specific' stimulants, e.g. SF and anti-leucocyte serum can be shown to produce additive stimulatory effects, indicating that different cell receptors, and possibly different cell populations, are involved. Mitogens combining with a single surface determinant are likely to bring into a state of activation only that portion of the mitogen-responsive popu-

lation which bears these receptors. Possession of the receptors does not, however, guarantee responsiveness (most non-lymphoid cells also possess mitogen receptors) and maximal responsiveness appears to require subtleties of presentation. Excess of a mitogen frequently produces an inhibition of response which is more often a true inhibition rather than a cytotoxic effect. Some stimulants (e.g. endotoxins) are, however, lethal in any but low concentrations. Impure stimulants may also contain agents which combine with 'depressant' sites on the cell surface. Certain lectins from *Ricinus communis* will bind to human lymphocytes and very small amounts of these substances markedly inhibit lymphocyte activation (Kornfeld and Eider, 1974).

4.1.1. Primates

The great bulk of the work with primates has been done using PHA as the stimulant but PWM, con A, staphylococcal filtrate and streptolysin S have also been used. The reasons for the popularity of PHA are largely historical and this mitogen is not ideal in many respects, e.g. it is difficult to purify and is made up of a heterogeneous mixture of cell-binding agents, only some of which are lymphostimulatory. Commercial preparations, however, almost always show good activity and the optimal dose is not as critical as with many stimulants. Con A, preferred by many workers, especially for animal work, is easier to purify and is more active against cells of many animal lymphoid tissues, but often requires careful dose standardisation and attention to the presence of mitogen-binding proteins. There are individual differences in responsiveness to different types of PHA such as PHA-P and PHA-M which appear to reflect genuine differences in the properties of the individual's lymphocytes and cannot be explained by variations in the levels of serum inhibitors and mitogen-neutralising serum proteins (see § 3.1.15 for references). If care is taken to eliminate technical variables (e.g. those due to serum factors – see below) the response of an individual's lymphocytes to a particular PHA preparation is fairly constant over a period of up to twelve months when assessed morphologically or by ^{3}H -thymidine incorporation (see, for example, Hagen and Frølund, 1973), but, according to one report, the mitotic index is much less constant (Price and Timson, 1971).

There are marked individual variations in the response to a mitogen even when conditions have been carefully standardised, as recommended in ch. 3. A typical result is shown in fig. 4.2. It is obviously important in any com-

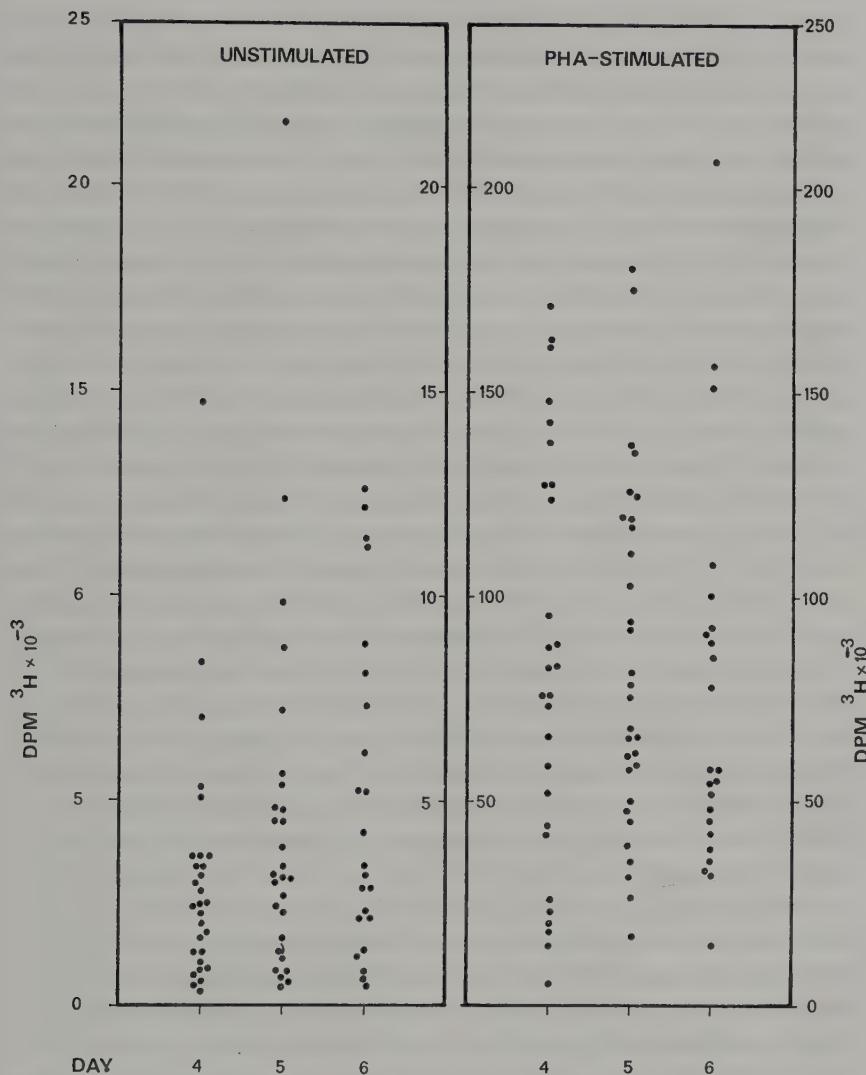


Fig. 4.2. Stimulation of blood lymphocytes of normal individuals with PHA. Each entry represents the mean ${}^3\text{H}$ -thymidine incorporation (expressed as disintegrations per minute, DPM) of 10^6 lymphocytes either unstimulated or stimulated with PHA. Culture conditions were standardised throughout the study. The maximal response with this batch of PHA regularly occurred on day 4. Note the considerable individual variations in the response to PHA. Based on data provided by Dr. G. Holmes.

parison with a 'disease group' that causes of variation in the 'control group' should, as far as possible, be understood. Day to day differences of the response of an individual to a stimulant are generally small but may be altered by a mild infection or other antigenic stimulus. Sex differences are small if one excludes pregnant women. Age is an important factor; low responses are obtained with cells of the very young and of the very old, in agreement with the more comprehensive studies in animals (Hagen and Frøland, 1973). Relatively low responses have been found for young people (under 26) compared to older adults (Pentycross, 1969). Decreased PHA responsiveness is, however, most marked in old age, particularly beyond 80 years of age (Hallgren et al., 1973). The progressive decline in PHA responsiveness in old age is related to a general decline in cellular immune reactivity. This is obviously a factor which must be taken into account in evaluating depressed lymphocyte transformation in chronic disease.

Since both PHA and con A are thought to stimulate the major part of the T sub-population (see ch. 11) responses of about equivalent intensity would be predicted when conditions were optimal for each stimulant. This, in fact, is what was found by Powell and Leon (1970). According to another report (Pienkowski et al., 1973) there are fewer blast cells in 72 hr con A cultures than the 60–80% found in PHA cultures but the number of cells in DNA synthesis was about the same (25%) in both cases. Despite the fact that PWM is thought to stimulate both T and B lymphocytes (see ch. 11) it has been shown that more peripheral lymphocytes are stimulated by PHA than by PWM when each is used at optimal concentration (Naspitz and Richter, 1968). This suggests that only sub-populations within the T and B populations are affected.

Generalisations about the responses of human lymphocytes to PHA and other stimulants are difficult when the mode of preparation so markedly affects the properties of the mitogen. One PHA preparation, to which human lymphocytes responded optimally at 0.02 mg/ml was toxic or inhibitory at higher doses and no response at all was obtained at 5.0 mg/ml (Rigas and Tisdale, 1969). A highly purified, very potent protein-free PHA of quite different properties was prepared by Goldberg et al. (1970). It lacked the red cell agglutinating activity of the usual glycoprotein PHA and differed from it in producing a dose-response curve which rose to a plateau in contrast to the usual bell-shaped dose-response curve. Some preparations of con A are also not toxic or inhibitory even when used at very high concentrations (see Pienkowski et al., 1973, and fig. 4.1). Others show only a

narrow optimum range. The shape of the dose-response curve does not appear to depend on the way the con A is added. If, instead of adding the con A 30 min before adding serum to the system, as recommended by Powell and Leon (1970), it is added in medium containing serum, the whole dose-response curve is simply shifted to a higher range. Con A is an agglutinin, but under the usual culture conditions agglutination is negligible in contrast to the marked erythro- and leuco-agglutination produced by PHA. There are receptors for most mitogens, however, on red cells and granulocytes and the presence of contaminating cells in lymphocyte cultures may affect the response, not simply by consuming mitogen, but by more effectively presenting it to the lymphocyte. The mitogenic activity of PWM is maximal at red cell : lymphocyte ratios of 0.5 to 20:1 whereas con A responses are inhibited at red cell : lymphocyte ratios of 5–10:1 (Yachnin, 1972). PHA responses are also affected by the presence of red cells. There are several reports that complete removal of red cells reduces PHA responses and conversely that addition of red cell membranes or pre-fixation of PHA on the red cell membranes improves the response (see § 12.1).

There are many serum factors which affect the response to mitogens and this has been particularly well demonstrated for PHA and con A. Some serum factors may compete with sites on the cell surfaces for stimulant and other factors in the serum may directly suppress the cellular response. Both PHA and con A react with α_2 macroglobulin, β -lipoprotein and IgM and to a lesser extent with α -glycoprotein, orosomucoid and IgA but not IgG (Morse, 1968). A human gamma globulin fraction has been shown to suppress the activation of human lymphocytes by stimulants (Cooperband et al., 1968) including that induced by two highly purified mitogens (Yachnin, 1972). Pregnancy serum or foetal serum or plasma contains higher concentrations of suppressive factors than normal adult serum or plasma (Ayoub and Kasakura, 1972; Hill et al., 1973). The level of stimulation produced by a given concentration of con A is markedly affected by the concentration of serum protein present and this is because serum contains several con A-combining proteins. Factors producing inhibition even at high con A concentrations are also present in unheated calf serum. They are removed by heating the serum at 56 °C, suggesting that they may be related to complement components (Forsdyke, 1973). A more general inhibitor in the α_2 -glycoprotein fraction of serum inhibits the responses of human lymphocytes to PHA, PWM and goat anti-leucocyte serum (Riggio et al., 1971). An inhibitory α_2 -glycoprotein was also found in extracts of

human placenta. The β -globulin fraction of serum has been reported to contain a lymphocyte growth factor which restored the impaired response to PHA observed when the growth medium was removed a few hours after setting up the cultures (Imrie and Mueller, 1968). The nature of one serum glycoprotein inhibitor is now known. When the glycoproteins were broken down to glycopeptides the most effective inhibitor of con A-induced human lymphocyte transformation was a glycopeptide derived from a human IgG₂, the carbohydrate component of which was a branched oligosaccharide with α -N-acetyl-neuraminic acid- β -galactose and β -N-acetyl-glucosamine in the peripheral positions linked to a core of three α -mannoses and then through two N-acetyl-glucosamines, one bearing α -fucose to the peptide. The presumption is that this structure, or something closely resembling it, is duplicated on the con A-binding site of the lymphocyte (Chase and Miller, 1973).

Lymphocytes from rhesus monkeys respond markedly to PHA and SF (Knight et al., 1965) and although studies have been fragmentary it is probable that lymphocytes of most other primates respond well to PHA and other common stimulants.

4.1.2. *Rabbits*

Rabbit blood lymphocytes survive and transform satisfactorily in vitro. It is unnecessary to resort to heart puncture to obtain blood. Provided reasonable precautions are taken (clean, sterile container, blood dripping freely into the container) blood may be obtained from the marginal ear vein without cultures prepared from it becoming contaminated. After addition of a half-volume of 3% gelatin in saline and sedimentation at 37 °C the leucocyte-rich serum contains $0.8\text{--}2.5 \times 10^6$ nucleated cells per ml, most of which are lymphocytes. This compares with an initial whole blood count of approx. $8 \times 10^6/\text{ml}$ leucocytes of which approx. 5×10^6 are lymphocytes, 2×10^6 heterophils, 0.4×10^6 monocytes, 0.4×10^6 basophils and 0.08×10^6 eosinophils. Both PHA and SF stimulate rabbit lymphocytes in medium containing 20% homologous serum but lower concentrations of both stimulants are required than are optimal for human leucocytes. High levels of 'spontaneous' transformation are often found if calf serum is used instead of homologous serum although some batches of foetal calf serum are satisfactory. Very good responses to con A are obtained in media containing 5% foetal calf serum.

Blast formation is maximal in the 24–48 hr period with PHA and between 48 and 72 hr when SF or con A is the stimulant. The morphological change

to a typical large blast cell with prominent nucleoli and basophilic cytoplasm occurs before DNA synthesis begins so that the time of maximal incorporation of ^3H -thymidine into DNA usually occurs later than the time at which the maximal number of blasts is found. The change in nuclear diameter during culture in relation to DNA synthesis is compared with that of human lymphocytes in figs. 4.3 and 4.4.

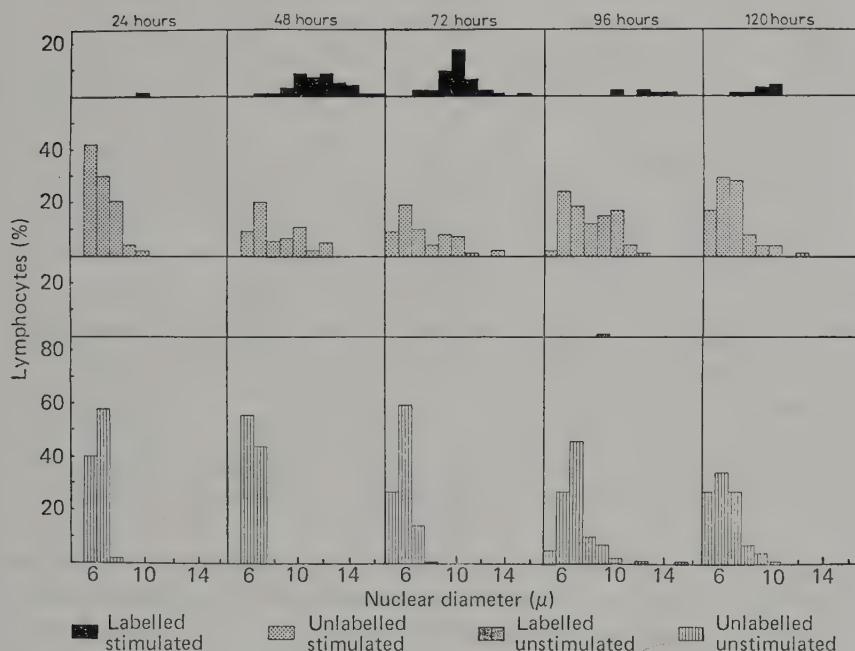


Fig. 4.3. Frequency distribution of cultured rabbit peripheral lymphocytes in relation to nuclear diameter. Stimulated cultures (which received SF) and control cultures (no stimulant) are classified as labelled or unlabelled on the basis of autoradiography under the standard conditions after incubation with ^3H -TdR for 2 hr. Nuclear size was estimated from two perpendicular measurements of the nuclear diameter. Reprinted from Knight et al. (1965).

4.1.3. Rats and mice

A considerable amount of attention has been given to methods of culture of lymphocytes of rats and mice. These are the species of choice for many types of experiment because inbred strains are available and large numbers

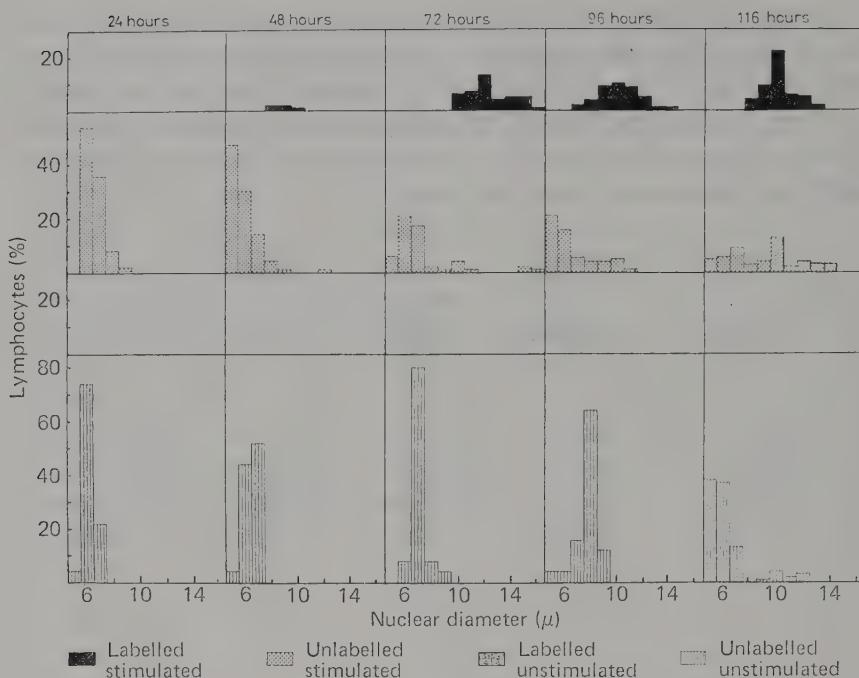


Fig. 4.4. Frequency distribution of human peripheral lymphocytes in relation to nuclear diameter. Culture conditions as for fig. 4.3. Reprinted from Knight et al. (1965).

of animals may be used. But rat and mouse lymphocytes are much less robust than human or rabbit lymphocytes and more fastidious in culture requirements. The number of lymphocytes obtainable from the blood of a mouse is small but may be increased by a factor of five or more by injecting the mouse i.p. three days prior to bleeding, with 0.1 ml of a 1 in 4 supernate of a *Bordetella pertussis* culture (Hayry and Defendi, 1970). The lymphocytosis produced is due to pre-existing lymphocytes, the migration of which from blood into lymphoid tissue is inhibited by the pertussis (Morse and Barron, 1970).

Mouse lymphocytes are particularly difficult to maintain *in vitro*. In conventional media supplemented with 5–20% homologous serum most cells are dead after 48 hr. Our own experience and that of most other authors is that RPMI 1640 supplemented with 5–10% of a good quality foetal calf serum is the best culture medium and this is recommended for general use. In other respects culture conditions may be those conventionally

used for human cells. A gas phase of 5% CO₂ 95% air is usual but Festenstein (1968) recommends 5% CO₂, 10% O₂, 85% N₂. It is important not to let the pH rise even for short periods as mouse cells are very sensitive to alkalinity. When lengthy manipulation of cell suspensions open to the atmosphere are to be performed, Hepes-buffered medium should be used. Of an initial $2 \times 10^6/\text{ml}$ mouse spleen cells cultured in RPMI 1640 medium supplemented with 5% foetal calf serum (heat inactivated just before use) approx. $1.4 \times 10^6/\text{ml}$ are viable after one day, $0.7 \times 10^6/\text{ml}$ after three days and $0.5 \times 10^6/\text{ml}$ after four days. The survival of rat lymphocytes is somewhat better, about 1.0 to $1.5 \times 10^6/\text{ml}$ being viable after three days (initial concentration $2 \times 10^6/\text{ml}$).

The serum present in the cultures is a major source of variation. According to Fowler et al. (1971) the serum concentration optimal for a PHA response may even vary with individual mice. Some workers think it is important to heat the foetal calf serum (FCS) at 56 °C for 30 min immediately before use; others like to remove low-titre antibodies to mouse cells said to be responsible for the 'background' stimulation by first absorbing with mouse spleen cells. Some prefer to use a medium containing a mixture of 5% rat serum and 5% FCS for culturing rat cells. Rat serum (10 or 20%) has been used successfully by Wilson (1967) with the stipulation that it should be freshly separated and not frozen. In Birmingham fresh rat serum has consistently been found to be inferior to FCS for culturing rat or mouse cells. Uncontrolled factors such as the diet of the rats used to supply the serum may be responsible for the discrepancy. Medium RPMI 1640 supplemented with 5% of a fresh selected human serum (heat inactivated) has been recommended for mouse cells with preferential addition of increments of fresh medium at 48 hr intervals (Adler et al., 1970). Mule serum (20% in RPMI 1630) has also been used for mouse spleen lymphocytes and was found to produce little 'background' stimulation (Mangi and Mardiney, 1970). Systematic studies on the culture of mouse blood lymphocytes suggest that a concentration of 3–8% FCS is optimal (Hayry and Defendi, 1970). Above 8% the 'background' thymidine incorporation in unstimulated cultures increased without improvement of the response to stimulant whereas, below 3% FCS cell survival rates sharply declined. Cell survival was poor in media containing rabbit, chicken, horse, lamb and most calf sera. Agammaglobulinaemic calf serum was no better than FCS. Mouse serum at a concentration greater than 5% was toxic and below 2% cell survival was poor. It also failed to enhance the properties of FCS.

There have recently been renewed attempts to culture mouse lymphocytes in a serum-free media. Sometimes low molecular weight factors (e.g. vitamins, non-essential amino acids) normally contributed by the serum have been added but good results have also been reported with conventional good quality media e.g. RPMI or Eagle's MEM. The advantages of a serum-free medium are the absence of background DNA synthesis or immunoglobulin synthesis resulting from the stimulatory action of the heterologous serum protein and the absence of suppressive and mitogen-consuming factors. Mouse spleen cells cultured in Eagle's MEM medium supplemented only with a Hepes buffer responded as well to con A as in the same medium supplemented with 10% foetal calf serum. The chief differences were a shift in the entire dose response curve with an optimum at 0.4 µg/ml con A in the absence compared with 4.0 µg/ml con A in the presence of serum and a reduction in cell survival, the serum-free values being about 90% of serum-containing on day 1 and 50% on day 5 (Coutinho et al., 1973). The mouse strain differences in con A responsiveness (see below) were unaffected. Activation of spleen cells by lipopolysaccharide (*E. coli* 0.55:B5) remained optimal at 100 µg/ml in both media. The kinetics of the responses (max. at day 3 for con A and day 2 for LPS) were also unaffected.

When cells do not grow well in common media it is customary, in tissue culture generally, to use 'feeder layers'. Surprisingly few attempts have been made to apply 'feeder layer' techniques to the culture of mouse lymphocytes although the presence of macrophages and other contaminating cells may well act as a 'feeder'. Some experiments with mouse thymocytes have shown that lymphocytes themselves may in some circumstances fulfill a growth-supporting function. Mouse thymocytes are normally very difficult to maintain and respond poorly if at all to PHA. When small numbers (e.g. 30×10^3) of human lymphocytes were added to 5×10^6 mouse thymocytes the response to PHA was markedly enhanced (Gery et al., 1971). The supernate from human lymphocyte cultures was also beneficial indicating that soluble agents of nutritional value were responsible. In contrast, the responses of mouse spleen cells were little improved by culture with human lymphocytes.

Rat and mouse lymphocytes respond to con A, PHA, PWM, lipopolysaccharide, PPD and other mitogens, the degree of response depending on many factors, e.g. age and strain of animal, whether or not pre-immunised and the proportions of T and B lymphocytes present in the tissue concerned. Spleen cells of C57/BL6 mice respond well to PHA-P, thymus and lymph

node cells less well (Adler et al., 1970). The optimum dilution of Difco PHA-M for stimulation of rat or mouse blood lymphocytes is about 1 in 150. A suitable concentration of Burroughs Wellcome PHA is approx. 10 µg/ml. These figures can only be an approximate guide because of the marked effect of serum concentration and other factors on the dose-response curve. With some brands of PHA the optimal dose may be as low as 1 µg/ml. Con A is a good stimulant for rat lymphocytes obtained from lymph nodes or thymus. Murine thymus and spleen cells also respond better to con A than to PHA (Stobo et al., 1972). Under conditions in which PHA induced 11–26% of the initial population of mouse spleen or lymph node cells to enter DNA synthesis over 1–4 days the corresponding figure for con A was 29–46%.

In exact studies of mitogen responses of mouse cells the age of the animals is an important factor to control. There is a decline in PHA-responsiveness in senescence corresponding with the decline in immunocompetence. There is very little response of spleen or thymus cells to PHA before the mice are 7 days of age, after which the response rises rapidly to a maximum at 40 days. In contrast, background DNA synthesis is high at birth and thereafter falls progressively (Adler et al., 1970). Changes in PHA responsiveness of mouse spleen cells appear to be small within the period of 3–14 months judging by investigations on AKR mice (Zatz et al., 1973). Thereafter mitogen responsiveness gradually declines. Age variations in responsiveness cannot be entirely divorced from strain differences (see below). Comparisons of the PHA-responsiveness of long-lived BC3F₁ mice (mean life-span 31 months) and medium-lived BALB/c mice (mean life-span 25 months) indicated that the earlier peak of maximum spleen cell activity and its earlier decay in BALB/c mice reflected mitogenic and life-span differences (Hori et al., 1973). In long-lived C57BL/6J F₁ hybrid mice a marked decline in responsiveness to PHA and PWM occurs during middle age and old age (Mathies et al., 1973).

There are marked 'strain' differences in the responses of mouse lymphocytes to PHA and con A (Hybertson and Bryan, 1967; Adler et al., 1970; Williams and Benacerraf, 1972). In one study lymphocytes from BALB/c mice responded most markedly to PHA followed by A/J and CBA strains and C57/BL6 least but the inferiority of C57/BL6 cells was not marked and was somewhat compensated for by the exceptionally low 'backgrounds' obtained with cells of this strain (Adler et al., 1970). Williams and Benacerraf (1972) also found that cells of BALB/cJ(H-2^d) mice responded appreciably better than those of C57BL/6J(H-2^b) mice with intermediate responses from

C3H/HeJ or AKR/J (both H-2^k) cells. The highest proportions of θ+ cells were found in the spleens of BALB/c mice but there was no direct relationship between the proportions of θ-bearing cells and responses to PHA and con A. It was noteworthy that good responders to PHA were good responders to con A and vice-versa. Since it is known that there are distinct receptors for these two mitogens this result indicates that mitogen responsiveness is controlled on a level other than that of antigen or mitogen recognition. A positive correlation of mitogen-responsiveness with the DNA synthetic activity of unstimulated cells suggests that the controlling factors may be metabolic in nature.

Similar comparisons have been made of the responses of lymphocytes of different rat strains to mitogens. Thymocytes of Lewis or Brown Norway (BN) rats responded identically to PHA over a wide dose range. A four-fold higher concentration of con A was required by BN compared with Lewis thymocytes to produce a maximal response (Williams et al., 1973). Lymph node cells behaved differently. Lewis lymph node cells were superior to those of BN rats in the level of response to con A or PHA at all mitogen concentrations but, as with thymocytes, BN cells required a higher con A concentration to respond maximally.

Many lectins have been tested for their effects on rat and mouse lymphocytes. Schumann et al. (1973) found that con A and three agglutinin preparations from *Phaseolus vulgaris* activated mouse T cells only and that PWM and *Robinia pseudoacacia* agglutinin stimulated both T and B cells of mice. Mitogenicity of *Ulex europaeus* agglutinin was confined to B cells of mice whereas another *Ulex* agglutinin, wheat germ agglutinins and soybean agglutinins were not mitogenic. Short-lived small lymphocytes of rats are more responsive to PWM than to PHA, responding to at least four times the level of long-lived lymphocytes (Stayner and Schwarz, 1969). In mice, spleen cells normally respond well to both PHA and PWM but the spleen cells of thymectomised, irradiated, bone marrow reconstituted mice respond to PWM only (Stockman et al., 1971) as would be predicted from the T and T+B reactivity respectively of these stimulants (see ch. 11). *E. coli* lipopolysaccharide (LPS) activates a proportion (part of the B population) of small lymphocytes in a normal mouse or rat spleen (see ch. 11). Cells in rat lymph nodes (presumably B cells), but not in the rat thymus, are also activated by LPS (Williams et al., 1973). The blast cells produced by LPS or PWM are not distinguishable under the light microscope from the blast cells produced with PHA but can be shown under the electron microscope

to have more endoplasmic reticulum in their cytoplasm (Shands et al., 1973).

4.1.4. Pigs, horses and sheep

For certain types of experiment requiring large numbers of blood lymphocytes it is convenient to work with blood of one of the larger animals. Of these the pig is probably the most useful. Pig blood contains large numbers of leucocytes (about 16×10^6 per ml, of which approx. half are lymphocytes), it is readily available from a local slaughterhouse, and may be collected in large quantities by cutting the aorta. It is our custom to defibrinate the blood by stirring large sticks in the collecting jar and, although bacterial contamination is an obvious hazard, in practice cultures from pig blood are rarely contaminated. After addition of a half-volume of gelatin and incubation, the supernate is siphoned off, the cells spun down and cultured in Eagle's medium containing 10 or 20% pig serum. Samples of pig serum vary a great deal in growth-supporting capacity and a preliminary trial is necessary to locate a good batch which may then be stored at -20°C in small aliquots. Pig blood lymphocytes usually respond well to PHA and con A (see fig. 4.1) but less consistently to SF. They may be obtained in greater than 99% purity by application of the Ficoll/Triosil technique (see ch. 3 § 1.4 and § 3.1).

Red cells are easily removed from horse blood by simple sedimentation and the lymphocytes obtained respond well to PHA. Cultures may be set up in horse or foetal calf serum. Very good responses to PHA-P ($1.25\text{--}2.5 \mu\text{g/ml}$) have been reported for horse blood lymphocytes cultured in 15–30% autologous serum. The response was proportional to cell concentration over the range $0.125\text{--}2.0 \times 10^6/\text{ml}$ with a maximum at 3–7 days (Lazary et al., 1973). If foetal calf serum is used, at least 1% is necessary for cell survival and maximum levels of RNA and DNA synthesis in PHA cultures occur with 3–20% foetal calf serum present (Walker and Lucas, 1969). As in other species the PHA dose requirement is dependent on the PHA/serum ratio. As in other species too the age of the animals is an important variable. Circulating lymphocytes from young foals respond much less well to PHA than those of adults (Lazary et al., 1973). Lymphocytes of all horses tested responded to bovine type tuberculin.

Sheep lymphocytes are difficult to separate from blood as the red cells do not form rouleaux. They have been obtained by direct application of the

Ficoll-Triosil technique to whole blood or by lysing the red cells of a whole blood sample by the NH₄Cl-Tris buffer osmotic shock technique. Sheep lymphocytes obtained directly from cannulated lymphatics survive well in media supplemented with foetal calf serum and respond well to PHA. The limiting factor is frequent contamination of the lymph during collection, with consequent fungal growth in cultures. The use of fresh sheep serum instead of foetal calf serum reduces the contamination rate (possibly because of antibodies in the sheep serum) and cell survival is just as good. Goat and ox blood lymphocytes also survive well and respond to PHA.

4.1.5. Guinea pigs, hamsters, cats, dogs, chickens, and other species

The guinea pig is the animal of choice for delayed hypersensitivity reactions and most in vitro experiments have been aimed at correlating a delayed reaction with specific response to antigen in vitro (Oppenheim et al., 1967). Guinea pig blood lymphocytes survive well in medium containing 20% foetal or precolostrum calf serum and respond to PHA-M and PHA-P (Phillips and Zweiman, 1970). Homologous sera occasionally support growth but there are great variations in the quality of individual sera and serum batches. 'Background' thymidine incorporation is only slightly higher in foetal calf than in homologous serum. The effect of culture conditions and cell concentration on growth rate and total growth have been fully explored by Eurenus and McIntyre (1970). Growth rate was best measured by ³H-TdR incorporation supplemented by a viability-corrected pronase-cetrimide cell count (see § 3.2.4). Pre-immunisation of the guinea pigs enhanced the response to PHA.

Hamster lymph node lymphocytes respond very well to con A and moderately well to PHA when cultured in RPMI 1640 + 10% foetal calf serum but bone marrow cells and thymus cells respond poorly (Singh and Tevethia, 1972). Good results have also been obtained with RPMI 1640 + 15% homologous serum. Under these conditions hamster lymphocytes respond to PHA, to xenogeneic but not allogeneic lymphocytes and, in the case of sensitised animals to PPD and haemocyanin (Fernald and Metzgar, 1971). Lymphocytes from Chinese hamsters respond well to PHA under similar conditions (Dejong and Anders, 1972). Hamsters appear to be unusual in that although their lymphocytes (lymph node, spleen or thymus) respond very well to con A or PWM using exactly the same dose of mitogen

as for rat, guinea pig or mouse this is not true for PHA. Maximal responses to PHA only occur with hamster lymphocytes when PHA concentrations 10–50 times those optimal for other rodents are used (Ron et al., 1973). Use of these high concentrations of PHA in cell cultures from other rodents was inhibitory. In contrast a plateau effect occurred at high PHA concentrations in hamster cultures. As in other species mitogen responses are age-dependent. Comparisons of responses of splenic lymphocytes of young, middle-aged and moderately old (80–94 weeks) MHA hamsters revealed a moderate age-related decline in responses to PHA and a lesser decline for PWM (Mathies et al., 1973).

The responses of chicken lymphocytes to stimulation are of much interest in relation to thymic or bursal derivation. Chicken lymphocytes survive poorly in culture and they are difficult to separate from blood because the larger nucleated chicken erythrocytes are not saucer-shaped and do not form rouleaux in the presence of dextran or gelatin. The following technique which is similar to that described by Greaves et al. (1968) is satisfactory: Heparinised blood (obtained usually from the wing of a chicken) is mixed with 3 volumes of Eagle's medium at 4 °C and centrifuged at 150 g for 6 min in a capped sterile container. The large erythrocytes, platelets and granulocytes are deposited at the bottom of the container leaving the lymphocytes, which are relatively small cells, in suspension. The cells are spun down, washed once in Eagle's medium containing 2% foetal calf serum, resuspended in RPMI 1640 medium containing 5% foetal calf serum to 2×10^6 lymphocytes per ml and cultured under the usual conditions. PHA responses are best measured over the 48–72 hr period. Thrombocytes inhibit the response to PHA and their removal by passage of the blood through cotton wool columns has been recommended but is not essential. Spleen cells respond almost as well as blood cells but cells obtained directly from the thymus, and especially from the bursa, are very fragile surviving for short periods only and responding poorly to PHA. Freshly separated chicken serum may replace the foetal calf serum (Alten et al., 1969) and sometimes serum-free medium has been used with good results. PHA responses up to ten times those in medium supplemented with autologous serum were obtained with chicken lymphocytes cultured in serum-free Eagle's MEM medium containing added non-essential amino acids, glutamine and insulin (Weber, 1970). The enhancement was obtained over a range of PHA concentrations and was probably not, therefore, due to removal of PHA-inhibitory serum factors. Similar good results have been obtained in

serum-free RPMI 1640 medium (Kirchner and Oppenheim, 1972). In serum-containing cultures best results were obtained at a cell concentration of 3×10^6 /ml and with 50 μl PHA-P or 70 μg con A per ml. In serum-free cultures optimal responses were obtained with 10×10^6 mononuclear cells/ml and mitogen concentrations of 0.5 μl PHA-P and 5 μg con A/ml.

Blood and spleen lymphocytes of bursectomised chickens respond normally to PHA but thymectomy drastically reduces responses indicating that, as in other species, PHA stimulates mainly T cells (Greaves et al., 1968). Similarly the responses of chicken spleen cells to con A are eliminated by a combination of thymectomy and irradiation whereas bursectomy and irradiation has little effect on con A responsiveness (Toivanen and Toivanen, 1973). Opinions are divided about the effect of *E. coli* lipopolysaccharide on chicken cells. Toivanen and Toivanen (1973) could not detect any activation of chicken lymphocytes whereas Weber (1973) detected proliferation of chromosomally identifiable bursa cells transferred to another host when spleen and blood cells were later tested with LPS.

Occasional experiments with many other species have been performed. Blood lymphocytes of cats and dogs survive well in vitro and respond well to PHA and SF. They have rarely been used experimentally. There may be special reasons (e.g. studies in comparative immunology) for choosing an unusual species such as the marsupials marmosa (Wolf et al., 1970) or kangaroo (Burton 1968a and b), the peripheral leucocytes of which respond well to PHA under conventional culture conditions. The opossum was chosen by Schneider and Rieke (1967) for a study of the asynchrony of DNA synthesis among individual chromosomes because its chromosomes are large in size and few in number and are well suited to autoradiographic analysis. Responsiveness of peripheral cells to PHA has been used to assess the effects of neonatal thymectomy of quokkas (Ashman et al., 1972). Responses of cells of neonatally thymectomised juvenile quokkas were less than half those of normal animals.

Unusual culture conditions may be required for the culture of fish and amphibian lymphocytes but no systematic studies have been performed to define them. Toad splenic lymphocytes appear to survive well in amphibian culture media supplemented with human, toad or foetal calf serum and have been shown to respond to PHA and allogeneic leucocytes (Volpe and Gebhardt, 1966; Goldshein and Cohen, 1972). Lymphocytes of the rainbow trout and other fish have been stimulated to cell division in Eagle's medium containing 10% foetal calf serum in an atmosphere of 100% O_2 at a tem-

perature of 19 °C (Heckman et al., 1971). It has been shown too that ammocoete larvae possess peripheral lymphoid cells which undergo activation when cultured with PHA, SF or allogeneic leucocytes (Cooper, 1969).

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The structure and ultrastructure of lymphocytes before and after activation

5.1. Resting and activated lymphocytes

The desirability of defining a cell by its functional potential and state of differentiation is unquestioned. While it is sometimes possible to measure the biological characteristics of lymphoid cell populations it is currently impossible to apply functional tests to each individual cell of the population. In practice mononuclear cells have to be classified by morphological criteria. Despite the distrust felt by many immunologists for any conclusions reached by purely morphological assessment, which is a reaction to the extravagant and misleading theories propounded by prominent histologists and haematologists of a previous generation, there is no doubt that a detailed knowledge of the structure of a cell can be very informative. Structure and function are interrelated and it is often possible to guess the metabolic activity and general properties of a cell from its morphology. Equally, one should be prepared to find considerable variation in the structure of cells in a population apparently performing a single function. It is salutary to note the varied size and form of cells of an established continuously growing cell line which, by biochemical criteria, is monoclonal. Lymphoid tissues, with the exception of the thymus and the bone marrow, contain few mitotically active cells although a proportion of the cells, even in blood, are clearly in an active phase. Blood cells have been most studied in humans, because of their importance in diagnostic haematology whereas in rodents thoracic duct or lymph node cells have received more attention. The changes which take place in tissue culture after exposure of lymphocytes to some of the known mitogens have been studied and a start has been made on morphological distinction of sub-populations.

5.1.1. Human blood lymphocytes

From their superficial appearance in Romanowsky-stained smears the mononuclear cells in human peripheral blood have been divided into seven different types by Torelli et al. (1963): a) small lymphocytes without distinct cytoplasm or with a very thin layer and nucleus containing large coarse chromatin masses, b) somewhat larger cells with easily visible cytoplasm and chromatin less pachychromatic than in small lymphocytes, c) large lymphocytes with abundant cytoplasm and eccentrically placed nucleus, d) large round cells with a central nucleus, a nucleolus and a very fine chromatin pattern and a narrow basophilic cytoplasm, e) cells of smaller size with scanty basophilic cytoplasm and a fine network of chromatin particles largely mixed with areas of coarse chromatin masses, f) cells of monocytic type usually with the characteristic features of the mature cell type but occasionally with narrower basophilic cytoplasm and a non-lobulated nucleus, g) cells of plasmacytic type, many showing features of immaturity, with a fine chromatin pattern. This haematological classification will include all the cell types recognised by immunologists from a functional viewpoint viz. resting T and B lymphocytes, activated T and B lymphocytes, K cells (possibly promonocytes), monocytes and plasma cells.

About 90% of the lymphocytes in peripheral blood or thoracic duct lymph are normally in a resting or inactive phase. Under the electron microscope most of these cells are seen to have a very sparse, finely granular cytoplasm with few organelles consisting of several large oval mitochondria, some scattered (not clustered) ribosomes, a poorly developed Golgi apparatus accompanied by one or two centrioles, a few pinocytotic vesicles and infrequent ergastoplasmic lamellae (Inman and Cooper, 1963; Zucker-Franklin, 1969). Of the two types of chromatin seen in nuclei, viz. heterochromatin (electron dense, coarse, closely packed) and euchromatin (much less dense, appearing as light staining areas, loosely packed) the nuclei of small lymphocytes contain heavy clumps of heterochromatin throughout the nucleoplasm and along the inner aspect of the nuclear membrane. Areas of euchromatin occupy the remainder of the nucleus and can be seen to extend towards nuclear pores in the double membrane (intervening space 200–400 Å) surrounding the nucleus (Zucker-Franklin, 1969). Nucleoli of resting cells are very small and are not readily seen. In general, the size of the nucleolus has been found to be proportional to the number of ribosomes in the cytoplasm.

Not all the small lymphocytes have the ultrastructural features of resting cells. There are always a few which appear more active. Some lymphocytes have a considerable amount of rough endoplasmic reticulum and ribosome clustering and there is a spectrum of cells extending to those with very little. Some lymphocytes are always found to contain fibrillar structures of 1–2 μ in length in the perinuclear region. Their significance is unknown. A few blood lymphocytes have the capacity of taking in particles (e.g. mycoplasmas or phage particles) which become attached to their plasma membranes but the majority do not. Lymphocytes of intermediate size are found with a nuclear chromatin pattern like that of small lymphocytes and a small nucleolus, but with some rough endoplasmic reticulum, some clustered ribosomes and an enlarged Golgi zone. Cells of this type account for about 2% of the lymphocytes in thoracic duct lymph but have been found to be present in much larger numbers in patients with raised serum immunoglobulin levels. Intermediate and large lymphocytes are also found in appreciably larger numbers in the blood of newborn infants and of adults during immune responses, e.g. to bacteria.

Little information is currently available on the comparative morphology of human T and B lymphocytes but this should soon be forthcoming. An examination of the cells forming sheep red cell (E) rosettes (T cells) and those forming EAC rosettes (B cells) by scanning electron microscopy has revealed that although there were multiple microvilli on the surface of both cell types they were fewer and shorter on T cells. B cells exhibited a complex villous surface. T cells appeared to be smaller and smoother (Sun-Lin et al., 1973). Thymocytes resembled peripheral T cells in being of smooth appearance (Polliack et al., 1973). Microvilli appeared to be the sole cell contact point between T cells and the sheep red cells whereas B cells made contact through both villous and non-villous regions. This mode of contact contrasted with that of monocytes, the microvilli of which appeared to bind red cells by a grabbing and pinching movement.

When observed in the living state at 37 °C by phase-contrast microscopy lymphocytes are recognisable, during the initial stages, as the round cells one sees in stained smears. But after a few hours in the incubator many of the cells show the characteristic amoeboid movements of lymphocytes. If a single cell is followed it will be seen to move randomly, often making contact with other cells or cell debris and there are periods of rest and periods of intense activity. The motile phase often begins by the protrusion of small pseudopodia. Lymphocytes when moving appear to have a definite

forward end and a definite tail. The tail (McFarland's 'uropod') trails behind the bulk of the cell keeping a remarkably constant orientation to the direction in which the cell moves. The forward moving end of the cell throws out large, blunt pseudopodia from both sides, sometimes together, forming a Y-shape and sometimes alternating, first one side and then the other (Marshall and Roberts, 1965).

5.1.2. Animal lymphocytes

Three types of mononuclear cell have been found in the thoracic duct lymph of rats (Marchesi and Gowans, 1964). The first and commonest was a small lymphocyte with a narrow rim of cytoplasm, a few prominent mitochondria and a few small cytoplasmic vesicles. The second contained more mitochondria than the small lymphocyte, numerous cytoplasmic vesicles and a prominent Golgi complex. The third type, in addition to cytoplasmic vesicles and mitochondria, contained a well-developed tubular endoplasmic reticulum. These cells, which would be classified as medium or large lymphocytes in stained smears, could well correspond to activated T and B lymphocytes respectively. Some of the larger cells were in DNA synthesis. No cells containing the electron-dense particles of rat monocytes were seen.

Guinea pig blood and thoracic duct lymphocytes appear to show the same general features. The number of mitochondria per cell increased after vaccination in normal but not thymectomised guinea pigs (Ernststrom and Larsson, 1967). Two types of blood lymphocytes have been identified in guinea pigs on the basis of the concentration and distribution of colloidal particles on the surface membrane of glutaraldehyde-fixed cells stained with thorotrast (Giacomelli et al., 1971). Lymphocytes in the thoracic duct of calves which have been depleted of lymphocytes by thoracic drainage have also been divided into two distinct populations according to the distribution of their ribosomes in the cytoplasm (Johnson et al., 1966). One class contained large numbers of membrane-free ribosomes dispersed more or less evenly throughout the cytoplasm whereas the other class showed prominent aggregation of ribosomes into groups of 5 to 8 sub-units (possibly T and B cells respectively).

5.1.3. Degenerating lymphocytes

Virtually all lymphocytes freshly separated from blood are viable. Some

dead cells are always present in dispersions from spleen, thymus or lymph nodes. After culture some degenerating cells are always found and this is an early event with cells of some tissues, e.g. mouse or chicken thymus. Most common stimulants, e.g. PHA, increase the death rate. It is not commonly realised that cells may die in many different ways and degenerate cells may appear in many different forms. In blood cultures granulocytes are the first cells to die. If whole blood is left at room temperature the lymphocytes survive for as long as twelve days, but by 24 hr most of the granulocytes have died (Aune et al., 1967). Loss of motility of granulocytes was followed by sudden death and degeneration of cytoplasm shown by diffuse staining. Nuclear pycnosis followed much later. Death of thymus lymphocytes by various noxious agents produced different lesions and appearances (Trowell, 1966). Cyanide or anoxia produced gross, watery swelling of all cytoplasmic components and nuclear breakdown. Swelling of cytoplasm and destruction of plasma and nuclear membranes were produced by —SH poisons. Cytoplasmic lysis was rapidly produced by tear gases or chloroform. Particles resembling ribosomes appeared in large numbers in the cytoplasm when death was induced by X-rays, cortisone, mitotic poisons or barbiturates. In lymphocyte cultures some dead cells disintegrate completely and are not found at time of harvest. Increased permeability to large molecules, including dye-protein complexes, is an early event and a useful indication of cell death, but dye-excluding cells which are obviously dead may be found at later times. Diffuse staining and loss of clarity of morphological features are a regular feature of cell death. Lymphocytes undergoing a different sort of degeneration are sometimes found in cultures e.g. of rabbit, dog or guinea pig lymphocytes, especially in the presence of agents like oxalate. A 'radial segmentation' of the nucleus occurs, but otherwise the cell morphology is normal (Norbery and Soderstrom, 1967).

5.1.4. Gross morphological changes induced by PHA and other stimulants

Lymphocyte transformation consists of a gradual change from a small or medium lymphocyte to a large blast cell so that sub-classifications of the intervening stages are inevitably arbitrary. Just as the starting population is heterogeneous so is the blast cell population arising from it and transformed cells which have travelled the same distance along the road to mitosis may well be morphologically dissimilar in other than broad features. The course

of transformation has been plotted by continuous phase-contrast observation of individual small lymphocytes from the time of activation right through to the mitotic stage and beyond (Marshall and Roberts, 1965). Lymphoblasts moved in the same way as small lymphocytes but were even more motile. In PHA cultures cell clumps were found in which cells were joined by their tails and stretched out radially, each making the movements characteristic of migrating lymphocytes. Following enlargement motility was temporarily lost before mitosis. The cell rounded up and became motionless and remained like this for about half an hour while the movements of the chromosomes occurred. The cell then pulled apart during a period of about 3 min forming two rounded up contiguous daughter cells. After a few minutes the daughter cells showed typical lymphocytic movements but often remained joined to each other by their tails for some hours. Neither the activated lymphocytes or their progeny were phagocytic but lymphoblasts are known to be stickier than small lymphocytes, as shown by their greater tendency to stick to glass (Marshall et al., 1969). The lymphocytes which are the first to transform in mixed lymphocyte cultures appear to be the most motile ones which make frequent contact with other cells and debris (McFarland and Schechter, 1970). It was not uncommon, in mixed cultures, to find two lymphocytes interacting by means of their uropods but no evidence could be found of direct cytoplasmic connections of the lymphocyte-lymphocyte or lymphocyte-macrophage type. Intercellular communication has been observed in PHA agglutinates of lymphocytes (Hulser and Peters, 1971) and labelled antigens have been reported to be passed directly to lymphocytes from macrophages, but, as far as activation is concerned, these events do not seem to have any importance. The formation of clones of 64 or more cells derived from a single small lymphocyte activated by antigens has been observed in micro-compartmentalised cultures (Marshall et al., 1969).

When Romanowsky-stained smears of PHA-cultures are examined at intervals, morphological changes may be detected by 24 hr of culture. Compared with the starting population of predominantly small and medium lymphocytes there is an increase in size of cells, the nuclear chromatin is less pachychromatic with more euchromatin and one or more nucleoli are easily seen. Less typical cells may have oval nuclei acentrically placed with not quite as high a nuclear : cytoplasm ratio. A few cells are already blast-like. By 48 hr a high proportion of the lymphocytes show some signs of activation. Many typical blast cells are present of 12–30 μ in diameter. They have a high nucleus to cytoplasm ratio with prominent nucleoli. Most of

the cytoplasm is basophilic with a crescent-shaped clear zone next to the nucleus. The blast cells have been classified into three types (Yoffey et al. 1965), a) with a very high N/C ratio and intense basophilic cytoplasm which contained a paler yellowish area; b) the most common type with a medium N/C ratio and a basophilic cytoplasm surrounding a round or ovoid leptochromatic nucleus containing several nucleoli or a single macronucleolus. The nucleus was commonly towards one pole with a perinuclear halo in the cytoplasm; c) cells larger than these two types with a medium N/C ratio and a relatively pale cytoplasm. By 72 hr larger blast cells are more common and mitoses are numerous at both 48 and 72 hr. Degenerating cells are also seen either as small lymphocytes with pycnotic nuclei or as diffusely stained larger cells. The surface of many blast cells may appear irregular and they may be vacuolated and fragile. The vacuoles seen in many blast cells in fixed preparations appear to contain lipid in the living cell. The sequence of events with other mitogens such as pokeweed or con A is very similar but the time schedule may be different. Morphology is more easily studied with mitogens which produce little agglutination.

5.1.5. Ultrastructure and cytochemistry of transformed cells

The chief features of PHA-transformed cells observed under the electron microscope are: a) a cytoplasm containing a highly developed Golgi apparatus and large numbers of ribosomes dispersed singly or in rosettes and increased numbers of large mitochondria; b) a nucleus in which most of the heterochromatin has been replaced by euchromatin and there are large and active-looking nucleoli (see table 5.1 and review by Douglas, 1972). The blast cells appearing in leucocyte cultures bear a distinct resemblance to the cells appearing in draining nodes in delayed hypersensitivity states, in regional nodes of animals bearing allografts and in lymphoid tissues during graft versus host reactions. The well-developed Golgi apparatus contains high concentrations of nucleoside diphosphatase and thiamine pyrophosphatase (Coulson, 1965). Within the Golgi region lysosomes are found (Inman, 1966). Fibrillar material is present in some areas of the cytoplasm, but little rough endoplasmic reticulum is found in PHA or MLR-generated blast cells although it is somewhat more abundant than in unstimulated cells. The presence of vesicles in the cytoplasm is indicative of increased micropinocytotic activity following stimulation. By a freeze-etching technique it has been shown that PHA causes a rapid increase in the

Table 5.1

The structure of PHA-transformed lymphocytes in relation to DNA synthesis*.

Structure	Small lymphocytes	Transformed (not in 'S')	Transformed (in 'S')
Nucleus			
chromatin	dense	more loosely packed	very loose
double membrane	regular	less regular more nuclear pores	very irregular large nuclear pores
nucleolus	not seen	often present	often present
Cytoplasm			
free ribosomes	trace to +	+ to ++	+++ to ++++
Golgi	poorly developed	moderately developed	very elaborate and extensive
micropinocytosis	+	++	+++
'rough' endoplasmic reticulum	nil	nil	nil

* Personal communication from Dr. D. R. Inman.

density of 70 Å globular particles in the cell membrane and in the number of nuclear pores (Scott and Marchesi, 1972).

The ultrastructure of cells produced with stimulants claimed to activate B cells is of great interest. The first indication that PWM differed from PHA in inducing morphological changes in lymphocytes came from the work of Douglas et al. (1967). Lymphoblasts produced from human peripheral lymphocytes with PWM contained more endoplasmic reticulum than those produced with PHA. A distinct intermediate-sized cell was described with an eccentric nucleus, clumped heterochromatin, a well developed Golgi apparatus, ribosomal aggregates and a well developed rough-surfaced endoplasmic reticulum. The cells were 10–20 µ in diameter under the electron microscope and 18–30 µ in smears. Others could detect little difference in the morphology of activated cells produced with PHA or PWM (Inman, 1966). More detailed histochemical studies have shown that similar morphological and metabolic changes are induced by PWM and PHA (Barker and Farnes, 1967). Glycogen storage, changes in lipid and hydrolytic and de-

hydrogenase activities were similar. This problem awaits the discovery of a mitogen which stimulates exclusively the B sub-population of human lymphocytes or the isolation of pure T and B sub-populations for stimulation. Mercuric salts induce very similar changes to PHA, the main difference being a more prominent aggregation of free ribosomes into polyribosome complexes (Schopf and Nagy, 1970). Increased levels of hydrolytic enzymes (acid phosphatase, β -glucuronidase) have been found in lymphocytes undergoing blastogenesis in response to PHA or PWM. The enzymes were localised in areas of cytoplasm and were not found in the lipid vacuoles. The lysosomes containing these enzymes have been found in greatest numbers in the Golgi region.

5.1.6. Lymphoblast structure in relation to the mitotic cycle

The ultrastructure of mitotic cells and cells in DNA synthesis (identified by autoradiography after a short pulse of ^3H -thymidine) in PHA cultures has been studied (Inman and Cooper, 1965). Cells in DNA synthesis tended to be larger than unlabelled blasts and the nuclear double membrane showed a far greater degree of irregularity giving a beady appearance. In some cases the outer membrane showed evaginations and in others it was fragmented, possibly indicating the first stages of break-down of the nuclear membrane prior to mitosis. Nuclear chromatin was loosely packed, especially in the central zone of the nucleus, and nuclear pores of exceptionally large diameter were present. Blast cells not in DNA synthesis had more densely packed chromatin, the nuclear membrane was more regular containing smaller pores and nucleoli were less frequent. Blast cells in DNA synthesis contained an average of twelve mitochondria per section, many of which were grossly swollen or elongated and contained cristae of bizarre form whereas comparable sections of unlabelled blast cells contained an average of seven mitochondria, more than half of which were bizarre in form. Labelled cells contained far higher concentrations of ribosomes, practically all of which were in the free form either single or arranged in rosettes of varying number. Rough endoplasmic reticulum was present in trace amounts in about half of the activated cells, whether labelled or not. Smooth endoplasmic reticulum, although present in trace amounts in half of the small lymphocytes examined, was more elaborate in activated cells and particularly the labelled ones. Micropinocytosis, multivesicular bodies and lipid containing bodies occurred with equal frequency in unlabelled and labelled cells.

The first change associated with mitosis is seen in the nuclear membrane at the end of prophase when it fragments to form vesicles which become disposed around the metaphase chromosomes to form an incomplete circle (Johnson and Roberts, 1964). During anaphase the chromosomes began to be covered by a membrane similar in structure to a nuclear membrane which appeared initially on the polar aspects of the chromosomes. During the later phases of cell division several layers of parallel membranes could be seen closely applied to each other in localised areas. In early metaphase the mitochondria appeared to pass through the disintegrating membrane into the nuclear area and in the later stages the mitochondria were often seen in close association with the membranes derived from or about to become the nuclear membrane.

5.1.7. Continuously growing lymphoid cells (LCL cells)

There are no known morphological differences which relate in any way to derivation from normal individuals or patients with leukaemia, Burkitt's lymphoma or infectious mononucleosis. Lymphoblasts from most established lines closely resemble, on superficial examination, the blasts produced from small lymphocytes by the action of mitogens. Although LCL are monoclonal they are never of uniform morphology and cells classifiable as stem cells, lymphoblasts, lymphocytes, plasmablasts and macrophages are all found, but no cells resembling granulocytic or erythroblastic cells or fibroblasts are present (Moore et al., 1968). Typically about 85% lymphoblasts, 10% medium and small lymphocytes and 5% multinuclear cells are found with some phagocytic cells and a few immature plasma cells. Many of the lines grow in a clumpy pattern. Sections of the clumps show necrotic cells at the centre with no evidence of differentiation or organisation. Fat-containing globules (0.3 to 1.0 μ) are a distinctive feature of many LCL, being commonly present in 70–90% of the cells. Cells containing ingested materials, including fragments of other cells, are found and almost all LCL cells have some phagocytic ability and will ingest ink particles and plastic spheres. Clusters and rosettes of ribosomes in chains with 6 to 12 pairs and as much as 120 to 140 nm in length are found. Fibrillar formations have been observed in the cytoplasm (Moore et al., 1968). The cells in some lines are highly motile though less so than normal lymphoblasts and they do not show the same amoeboid movements and hand-mirror motion. Locomotion of cells in a line derived from a leukaemic patient has been likened to that of

macrophages and monocytes rather than lymphocytes and in some Burkitt lines the cells show little motility (Clarkson, 1967). Most lymphoid cell lines commonly cultivated have the characteristics of B cells and this is reflected in their surface features, typically the presence of multiple microvilli. The less common lines with T cell characteristics have a smoother surface with a small proportion of the cells containing a relatively large number of surface digitations (Polliack et al., 1973).

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CHAPTER 6

Lymphocyte activating substances obtained from plants, bacteria and other sources

6.1. Plant mitogens

The term phytohaemagglutinin, or PHA, is now commonly used to describe material extracted from the red kidney bean, *Phaseolus vulgaris*, which has mitogenic as well as cell agglutinating properties. Many other substances with haemagglutinating properties are found in extracts of stems, roots and especially the seeds, of a variety of plants. The term phytohaemagglutinin may properly be applied, in its literal sense, to describe them all. To avoid confusion the letters PHA will be used in this book solely to describe the *Phaseolus vulgaris* mitogen.

By the early years of this century it was known that extracts of the seeds of many plants were able to agglutinate erythrocytes and leucocytes of many species. This property is now known to be due to proteins or glycoproteins called lectins, which bind to specific saccharide groups on the surface membrane of the cell. The structure and properties of lectins have been reviewed by Sharon and Lis (1972) and Lis and Sharon (1973). Almost all plant mitogens are also lectins and many were characterised as lectins well before their mitogenicity was discovered. Extracts of the seeds of many plants have now been tested to see if they possess mitogenic properties. These studies have shown that while the mitogenic properties of PHA are not unique, they are quite unusual. In three major studies, Barker (1969) found mitogenic activity in 16 of 166 species tested; Parker et al. (1969) found 9 of 90 species to have some activity, while Curtain and Simons (1972) found only 3 of 164 indigenous Australian species to be active. These figures probably overestimate the true frequency of mitogenic proteins, as the first two studies included a few extracts already known to be mitogenic, and the samples tested included a high proportion of species related to those known to con-

tain mitogens. The species studied by Barker (1969) included representatives of 22 plant families, but all the active species were members of the Leguminosae or the Phytolaccaceae. The two other studies produced occasional examples of mitogenic extracts of seeds from other families, but all the plant mitogens which have been studied in any detail come from these two families. Not all members of these families contain mitogens, and it is common to find close relatives of mitogen-containing species that are quite inactive.

Mitogenic material from three legumes (PHA from *Phaseolus vulgaris*, con A from *Canavalia ensiformis* and lentil mitogen from *Lens culinaris*) and one from *Phytolacca* (*americana*) have been purified and their chemical and biological properties studied in detail. These four mitogens are considered separately below. Three other legume mitogens, those from peas (Trowbridge 1973), *Wistaria floribunda* (Toyoshima et al., 1971) and *Bauhinia carronii* (Curtain and Simons, 1972) have also been purified and seem to have properties generally similar to other legume mitogens, while that from the wax bean, a variety of *Phaseolus vulgaris* (Takahashi et al., 1967), seems very similar, if not identical, to PHA (Kay and Oppenheim, 1971). A mitogen has also been purified from the mushroom *Agaricus campestris* (Young et al., 1971).

The precise relationship between erythroagglutination and mitogenicity has not been established, but not all lectins are mitogenic. Lectins from *Ricinus communis* indeed, inhibit DNA synthesis induced by other agents (see ch. 4). About one third of the plant species tested contain lectins, but probably less than one tenth contain mitogens. Many well characterised lectins can be shown to bind to the lymphocyte surface, but are not mitogenic. It has frequently been observed that treatment of erythrocytes with proteolytic or glycolytic enzymes enhances their agglutination by lectins and exposure of lymphocytes to proteases may enhance or reduce the activation induced by lectins (see § 6.3). Recently it has been shown that mouse spleen lymphocytes can be activated by the soybean lectin, which is not normally mitogenic, if they are first treated with neuraminidase (Novogrodsky and Katchalski, 1973). Plant mitogens are immunogenic and antisera have been readily produced, usually by immunisation of rabbits with the lectin emulsified in complete Freund's adjuvant.

6.1.1. PHA

PHA is the name given to the active principle in extracts from the red kidney

bean *Phaseolus vulgaris* which both agglutinates erythrocytes and activates lymphocytes. Most of the early studies on lymphocyte activation were carried out using either very impure preparations of PHA, such as those available commercially from Difco Laboratories (PHA-M) and from Wellcome Research Laboratories, or a partially purified, largely protein, fraction prepared by the method of Rigas and Osgood (1955) and available commercially from Difco Laboratories as PHA-P. PHA-P is still a very heterogeneous mixture of proteins, and at least 17 different bands can be distinguished by acrylamide gel electrophoresis (Allen et al., 1969).

When the only aim of an experiment is to obtain as many activated lymphocytes as possible, e.g. when metaphase preparations are required for chromosome analysis, the use of such unpurified preparations as stimulants is not only adequate but recommended, on the grounds both of economy and of consistency of results. In clinical surveys of PHA-responsiveness it is also preferable to use an impure commercial preparation. The choice of a purified preparation, the properties of which may be quite different from that of a purified product used in other laboratories, can only be a source of confusion (see § 4.1.1). However, for other types of experiment, especially for studies on the mechanism of action of PHA, or involving its injection into intact animals, it is necessary to use as pure a preparation as possible. Contaminants may interfere with the techniques used, or may themselves have irrelevant biological activities, and so a good deal of effort has been expended on the purification of the mitogenic principle.

The early studies were directed mainly towards determining whether the same component in PHA was responsible for both erythroagglutination and mitogenicity. It was quickly found that crude PHA contained fractions which agglutinated erythrocytes but which were not mitogenic, and that if the great majority of the erythroagglutinating activity was removed by repeated absorption with erythrocytes, at least some of the mitogenic activity was retained (Barkhan and Ballas, 1963; Kolodny and Hirschhorn, 1964; Norman et al., 1964). However, the mitogenic activity was found to be quite significantly reduced in those experiments in which its concentration was determined quantitatively. This problem was finally resolved when Weber (1969) and Allen et al. (1969) showed that cation-exchange chromatography on SE-Sephadex or CM-Sephadex led to the separation of two different mitogenic fractions. One of these fractions termed PHA-L by Allen et al. was essentially free of erythroagglutinating activity, while the other, PHA-H, was a potent erythroagglutinin.

The molecular weights of both these mitogens have been variously estimated at between 115,000 and 140,000. The PHA-H is slightly larger than the PHA-L, and is thought to be a mixture of three different but related proteins, which all possess both erythroagglutinating and mitogenic properties, but in different proportions (Yachnin and Svenson, 1972). Further work has shown that each of these proteins is a tetramer composed of subunits with molecular weights variously estimated at between 29,000 and 36,000 (Allan and Crumpton, 1971; Oh and Conard, 1972; Weber et al., 1972; Yachnin and Svenson, 1972). Two different types of subunits were found, one of which had all the mitogenic activity, while the other was responsible for erythroagglutination. PHA-L was found to be a pure tetramer of the mitogenic subunit, while PHA-H contained both types of subunit. The three bands within the PHA-H fraction found by Yachnin and Svenson (1972) correspond to the L_3H , L_2H_2 , and LH_3 subunit structures (Miller et al., 1973).

Even highly purified preparations of PHA contain some carbohydrate residues, mainly mannose and glucosamine (Weber, 1969; Allen et al., 1969). They also contain Ca^{2+} and rather smaller amounts of Mn^{2+} (Galbraith and Goldstein, 1970). The amino acid composition has been determined by several different groups, and shows some unusual features. There has been some variation from preparation to preparation, but the original report that the sulphur-containing amino acids, cysteine and methionine, were present only at very low levels, while those amino acids with carboxyl or hydroxyl groups in their side chains, especially aspartate, were unusually frequent (Rigas et al., 1966) has received numerous confirmations. The sequence of amino acid residues at the N-terminal ends of both the erythroagglutinating and mitogenic subunits has been determined by the Edman degradation method (Miller et al., 1973). The two subunits are different at six of the seven amino acids at the N terminal end, but their sequences from residue 8 to residue 24, the last amino acid determined, are identical. Serine was found to be the N terminal residue of PHA-L, and alanine was found in the equivalent position of the PHA-H subunit. In an earlier study Oh and Conard (1972) had reported that there were two separable non-erythroagglutinating mitogens in PHA, which both had histidine as their N terminal amino acid, but which had quite different tryptic and chymotryptic peptides.

PHA-L is now available commercially from Wellcome Research Laboratories and Medix Laboratories. Several procedures for the purification of PHA-L to homogeneity have now been published, including one in which

the final step is crystallisation from aqueous solution (Rasanen et al., 1973), but all the procedures are quite complex. Many lectins can be purified relatively simply by affinity chromatography, but as the precise binding specificity of PHA-L is not yet known, this method is not yet applicable. PHA-H binds very strongly to thyroglobulin, and has been purified successfully on columns of thyroglobulin covalently coupled to Sepharose (Matsumoto and Osawa, 1972).

The exact concentrations of PHA required for lymphocyte activation depend very much on the precise experimental conditions used, but for human lymphocytes in medium containing 10% serum the best PHA preparations are generally most active when present at 1–5 µg/ml. The specific activity of these preparations is about 10 times that of PHA-P, and about 100 times that of a crude PHA extract.

There is some doubt whether all PHA preparations prepared from different sources are identical. Crude extracts from different varieties of *Phaseolus vulgaris* were found to contain erythroagglutinins with different ranges of specificity (Jaffe et al., 1972). PHA from Wellcome Research Laboratories seems to bind more tightly, and to a larger number of receptors on the lymphocyte surface, than that obtained from Difco Laboratories (Kay, 1967; Skoog et al., 1974). Nevertheless, antibodies prepared to PHA-L from Wellcome Laboratories inhibit not only lymphocyte activation by PHA-H as efficiently as that by PHA-L, but they also completely neutralise all the mitogenic activity in crude extracts such as Difco PHA-M (Kay and Oppenheim, 1971), indicating that if different extracts contain different mitogenic factors they must be sufficiently similar to cross-react immunologically. Antisera raised against PHA did not cross-react with any of a number of other lectins tested, except that from the closely related wax bean, which is another variety of *Phaseolus vulgaris*.

In addition to their erythroagglutinating and mitogenic effects, crude PHA preparations have other properties. One of the first to be established was that PHA precipitated several different fractions of human serum, particularly α_2 -macroglobulins, β -lipoproteins and IgM immunoglobulins, although weaker reactions with a number of other components have been observed (Beckman, 1962; Morse, 1968). This precipitation of serum fractions is not essential for the mitogenic activity of PHA, and it seems likely that it is a property of PHA molecules containing two or more PHA-H subunits. Heating PHA to 85 °C for 5 min or absorbing it with erythrocytes were found by Holland and Holland (1965) to cause a parallel decrease in pre-

cipitation and agglutination of erythrocytes, without greatly affecting mitogenic activity, while Yachnin and Svenson (1972) showed that after CM-Sephadex chromatography serum precipitation was found only in the more cathodal PHA-H subfractions.

There have been occasional reports suggesting that different fractions within unpurified PHA preparations may stimulate DNA synthesis, RNA synthesis and protein synthesis (Rivera and Mueller, 1966; Cooperband et al., 1967; Harms-Ringdahl et al., 1973). However, all fractions which stimulate DNA synthesis also stimulate RNA and protein synthesis. It would be expected that low PHA concentrations, or higher concentrations if accompanied by inhibitory contaminants, might cause some stimulation of RNA and protein synthesis without being able to cause sufficient stimulation to initiate DNA synthesis, and it seems likely that explanations of this type could account for the results obtained. Suggestions that factors in PHA responsible for the secretion of interferon and lymphotoxin by lymphocytes may be different from the mitogenic components (Haber et al., 1972) may be more soundly based, but confirmation must await determination of the effects of highly purified PHA on the secretion of these agents.

6.1.2. Con A

Con A is the best characterised of the plant lectins. It may account for up to 3% of the protein of the jack bean (*Canavalia ensiformis*) and was first obtained in a crystalline state many years ago (Sumner, 1919). It was found to bind specifically to α -D-glucopyranosides and α -D-mannopyranosides, and to polysaccharides or glycoproteins containing such residues (Sumner and Howell, 1936a). The specificity of con A has been investigated in detail (see review by Sharon and Lis, 1972) and seems to be directed mainly towards the hydroxyl groups in the C-3, C-4 and C-6 positions of the pyranoside ring. Con A agglutinates erythrocytes, although not so powerfully as PHA, presumably by binding to saccharide residues on their surfaces. These properties of con A were known long before it was first reported to be mitogenic (Wecksler et al., 1968; Powell and Leon, 1970).

The purification of con A has been greatly aided by its ability to bind to cross-linked dextrans such as Sephadex. It can then be eluted with either 0.1 M glucose (Agrawal and Goldstein, 1967) or by lowering the pH to 2.0 (Olson and Liener, 1967). Unlike most lectins, it is a pure protein which does not contain any carbohydrate. At pH 7.0–7.5 it occurs mainly as a tetramer

of subunits with a molecular weight of 25,500, although below pH 6.0 it is found as a dimer.

The sequence of the 238 amino acid residues in the 25,500 molecular weight subunit, and its tertiary structure, as indicated by its electron density map at 2 Å resolution, have now been determined (Edelman et al., 1972). Like most plant lectins, it has a relatively high frequency of aspartate, serine and threonine residues, and a low level of sulphur-containing amino acids. There are two methionine residues, but no cysteine. The X-ray crystallographic data show that the main structural elements are two anti-parallel, pleated sheet arrangements, one of which is roughly perpendicular to the other. There is very little α -helix. The subunits are paired to form dimers, which are themselves paired to form tetramers which are roughly tetrahedral (fig. 6.1).

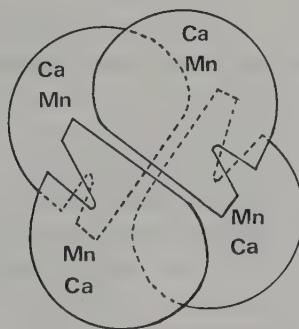


Fig. 6.1. Schematic representation of the tetrameric structure of con A showing the proposed binding sites of transition metals and calcium. Redrawn from Edelman et al., 1972.

The saccharide-binding activity of con A is dependent on it containing Ca^{2+} and a transition metal, normally Mn^{2+} (Sumner and Howell, 1936b; Yariv et al., 1968). The binding of the transition metal is itself essential for the binding of the Ca^{2+} . Each subunit contains one saccharide-binding site, one transition metal ion and one Ca^{2+} , but the two ions do not seem to be directly involved in the saccharide-binding site. The X-ray crystallographic studies show that the two ions are located close together, and that two aspartate residues are probably shared as ligands by both ions. They are, however, over 20 Å removed from the narrow but deep pocket which is thought to be the saccharide-binding site (Edelman et al., 1972). The role of

the metal ions is most probably to maintain the correct configuration of the rest of the molecule.

In most con A preparations a proportion of the subunits are cleaved to give N and C terminal fragments with molecular weights of about 13,000 and 11,000. The two fragments remain together and the biological properties of the subunit do not seem to be affected (Wang et al., 1971). The site of this apparently natural cleavage is between residues 119 and 120, which are on a loop away from the main part of the subunit that forms a connection between two parts of one of the pleated sheet structures (Edelman et al., 1972).

6.1.3. Lentil mitogen

Extracts of the lentil *Lens culinaris* (also called *Lens esculenta*) have long been known to contain a rather weak non-specific erythroagglutinin. More recently it has been shown that this lectin is also mitogenic (Toyoshima et al., 1970; Young et al., 1971). This mitogen has a receptor specificity similar to that of con A.

It can be purified by a number of different procedures. Like con A, it will attach to Sephadex, and can then be eluted with either 0.1 M glucose or by reducing the pH, but the most successful procedure is a combination of ammonium sulphate precipitation and chromatography on DEAE-cellulose (Entlicher et al., 1968; Howard et al., 1971). The preparation so obtained can be separated into two components by starch gel electrophoresis, acrylamide gel electrophoresis in sodium dodecyl sulphate or chromatography on CM-cellulose. Both components react with phosphomannans but not with dextrans, and both have similar erythroagglutinating and mitogenic properties (Young et al., 1971).

The two active components are very similar (Howard et al., 1971). Both have molecular weights of 49,000, and are composed of two identical subunits. They cannot be distinguished from one another by immunological methods. They have very similar amino acid compositions, with the usual high frequencies of aspartate, serine and threonine residues, and very low levels of cysteine and methionine. The N and C terminal amino acids are the same for both components, and their tryptic peptide maps are very similar. Fifteen peptides are common to both, while one component contains a single unique peptide, the other between one and three unique peptides. The lectins contain about 3% carbohydrate, mainly glucose and glucosamine.

Like con A, the lentil mitogen contains both Ca^{2+} and Mn^{2+} , and removal of the Mn^{2+} results in irreversible inactivation of the mitogen (Paulova et al., 1971). Equilibrium dialysis has been used to show that the mitogen binds simple saccharides such as mannose and methyl-D-glucopyranoside, but with a fifty-fold lower affinity than con A. The data suggested that there was one saccharide-binding site per subunit (Stein et al., 1971).

The mitogen can be extracted from lentil seeds, but not from other parts of mature lentil plants (Howard et al., 1972). On germination, activity is associated with both cotyledon and embryo, but not with the seed coat. Roots and stem formed at very early stages in development contain some activity, but all activity disappears after two to three weeks of growth, along with the loss of the cotyledon.

6.1.4. Pokeweed mitogen (PWM)

Extracts of several parts of the pokeweed (*Phytolacca americana*) have been found to be mitogenic (Farnes et al., 1964). This mitogen differs from those described above in that it has little erythroagglutinating activity, and was not known as a lectin prior to the recognition of its mitogenic properties. It is also not derived from a legume, and in the mouse it affects both B and T lymphocytes, while the mitogens from legumes described above seem to activate only the T lymphocytes (see § 11.1).

The mitogen has normally been extracted from the roots, although it can also be found in stem, leaves and berries. It has been purified to homogeneity by a complex procedure involving heat coagulation, trichloroacetic acid precipitation, chromatography on hydroxyapatite and preparative acrylamide gel electrophoresis (Reisfeld et al., 1967). It has a molecular weight of 32,000 and appears to be a single glycoprotein chain containing about 5% carbohydrate. Cysteine is the most frequent amino acid, accounting for 20% of the amino acid residues, with 66 residues per molecule. No free sulphhydryl groups have been detected, so the molecule must have 33 intrachain disulphide bridges, and thus rather a rigid structure. There are also high frequencies of glycine and of the acidic residues aspartate and glutamate, but no detectable methionine. The amino acid composition is quite different from that of the legume mitogens described previously, but the high cysteine and glycine content is reminiscent of the wheat germ lectin, a well characterised non-mitogenic lectin.

More recently, two distinct mitogens have been purified from the roots of

the shoriku, *Phytolacca esculenta* (Tokuyama, 1973). These both have amino acid compositions very similar to that of the pokeweed mitogen. One had a molecular weight of 32,000, almost identical to that of the pokeweed mitogen, while the other had a molecular weight of only 18,000, but indistinguishable biological activities. The activity of these mitogens is not affected by prolonged dialysis against distilled water, but low concentrations of Ca^{2+} or Mg^{2+} did increase the stability of the mitogen to incubation at 37 °C.

6.2. *Products of micro-organisms*

It is likely that many common bacteria produce lymphostimulatory substances. Since bacterial products from gut flora, or sites of infection, may enter the body and reach the lymphoid tissues, these substances may activate lymphocytes in vivo. They are therefore important not merely as laboratory reagents and it is surprising that, as yet, no systematic studies have been reported. A survey of 28 bacteria showed that unidentified products of 7 of them produced a marked activation of monkey lymphocytes (see § 9.1). Some of these substances were unstable and were lost by incubation at 37 °C for a few days. There has been a tendency to assume that lymphocyte activation produced by culture with a bacterial substance is always an immunospecific activation dependent on prior encounter of the donor, perhaps unknowingly, with the bacteria concerned. There are now several examples of bacterial products which appear to act as 'non-specific' mitogens in the sense that this term is applied to PHA and other plant mitogens. Even tuberculin PPD may act non-specifically when used at very high concentrations (see § 9.11).

6.2.1. *Staphylococcal mitogens*

All strains of *Staphylococcus aureus* appear to produce diffusible mitogenic material. If 199 medium (minus antibiotics) is inoculated with the organism, incubated for 5 days, and filtered to remove the cocci, the filtrate is mitogenic to human and rabbit lymphocytes when present at a final dilution of 1 in 10 to 1 in 100. Monkey and pig lymphocytes are also affected but rat and mouse lymphocytes are not. Mitogenic activity is to be found in aqueous extracts of acetone-ether dried organisms. Haemolysin preparations (α , β and δ) and

Staph. protein A products (Rodey et al., 1972) invariably contain appreciable amounts of lymphocyte-activating material. A careful analytical study has shown that there are two extracellular non-dialysable lymphocyte mitogens in a broth in which *Staph. aureus* strain DA352 has been grown (Kreger et al., 1972). The mitogens could be purified and obtained free of detectable amounts of other Staph. products by ethanol precipitation followed by isoelectric focussing. Most of the activity of filtrates could be recovered in an ammonium sulphate precipitate (90% saturation) or by precipitation with 2.5 volumes of chilled ethanol or acetone. The mitogens were quite distinct from the α , β or δ haemolysins, leucocidin, protein A, esterase, lipase, protease, DNase, coagulase, hyaluronidase, RNase, staphylokinase, lysozyme and acid or alkaline phosphatase.

6.2.2. Streptococcal mitogens

A lymphocyte mitogen found in commercial preparations of streptolysin S has been investigated by Taranta et al. (1969). Activity was regularly found in filtrates of β -haemolytic streptococci whereas filtrates from *Diplococcus pneumoniae*, *Strep. viridans* or *Staph. aureus* grown under the same conditions were inactive. However, it soon became evident that the mitogen was not the haemolysin since the non-haemolytic mutant C203U strain produced as much mitogen as the C203S haemolytic strain. Moreover the transforming activity was less heat-labile than the haemolytic activity and, unlike the haemolytic activity, was totally resistant to trypan blue, lecithin and chymotrypsin. Omission of an 'RNA core' from the suspension medium of C203S produced a filtrate with transforming but lacking haemolytic activity. A clear cut separation of the two activities was obtained by chromatographic fractionation of streptolysin S preparations.

Streptolysin O preparations contain material which activates the lymphocytes of some donors but not others. There is some evidence that it may be acting in an antigen-specific manner on lymphocytes of donors who have been pre-immunised. The active material is not the lysin and it is not lost with decay of lytic activity on storage. Streptokinase-streptodornase preparations contain a material of β -globulin-like characteristics and mol. wt. 21,500 which elicits delayed hypersensitivity reactions in man (Tomar et al., 1972). This material also has lymphocyte transforming activity, but it is not known if it is acting specifically or as a non-specific mitogen.

6.2.3. Endotoxins

Lipopolysaccharides with endotoxin activity produced by *E. coli* 0113 and 0111:B4 are mitogenic for mouse spleen cells with selectivity for B lymphocytes (see § 11.1.2 and § 4.1.2). They are also active against rat spleen cells but have little effect on human or rabbit blood lymphocytes. Endotoxins from *Salmonellae*, e.g. some smooth and rough strains of *Salmonella typhimurium*, are also mitogenic for mouse spleen cells (Peavy et al., 1973).

Lipopolysaccharides (LPS) from Gram-negative bacteria consist of an O-antigen polysaccharide, which is responsible for the serological specificity of the molecule and a 'core' polysaccharide, with groups common to many bacteria, which is linked through a 2-keto-3-deoxy octonate trisaccharide to a lipid component, termed lipid A. The lipid A part of the molecule consists of phosphorylated glucosamine disaccharide units with ester- and amide-linked fatty acids. It is present in LPS from both rough and smooth variants. It has been shown, both with the *Coli* and *Salmonella* LPS, that the lipid portion of the molecule is essential for mitogenic activity; the isolated polysaccharides are inactive (Andersson et al., 1973; Peavy et al., 1973; Rosenstreich et al., 1973). Mild acid hydrolysis of the LPS yielded a lipid fraction as active or more active than the intact molecule and a polysaccharide lacking mitogenic activity. Procedures which degraded or modified the lipid part of the molecule (e.g. mild alkaline hydrolysis which cleaves off the ester-linked fatty acids), while preserving the antigenic integrity of the polysaccharides, destroyed mitogenicity. Lipid preparations were shown to be fully active against highly purified mouse B lymphocytes (prepared by treating mouse spleen cells with anti-θ serum and complement, for example) whereas they had no effect on highly purified T lymphocytes. These experiments demonstrate conclusively that the lipid moiety of LPS are B cell mitogens, whereas the polysaccharide part of the molecule has no mitogenic activity. The basis of the activation reaction is not known, but it has been postulated that the lipid A interacts with the lipid bilayer of the plasma membrane in such a way as to produce a critical change in the conformation of protein or other membrane components (Andersson et al., 1973).

6.2.4. A mycoplasma mitogen

A massive blastogenesis of the lymphocytes of DA rats, grown for two days over syngeneic embryo monolayers, was shown to be due to the presence of *M. pulmonis* (the Negroni agent) in the culture (Ginsburg and Nicolet, 1973).

The agent failed to grow in cell-free medium, but grew luxuriantly in the presence of lymphocytes or other cells. Although the mycoplasma itself was heat and acid labile the lymphocyte-activating property was more stable. However, the active material apparently remained attached to the mycoplasma as culture supernates passed through a Millipore filter (0.22 µm) were inactive. Lymphocytes from SPF rats, as well as those kept under normal animal house conditions, were activated by the agent and therefore this reaction must be classified as one due to a 'non-specific' mitogen and not an antigen producing an immunospecific activation.

6.3. Other mitogens

Some degree of lymphocyte activation has been produced by a wide range of substances including periodate, zinc ions, mercury ions, proteolytic enzymes, extracts of walnuts and extracts of polymorphonuclear leucocytes. Low concentrations of cyclic GMP increase the uptake of ^3H -thymidine of human blood lymphocytes (see ch. 12). Mild activation may also be produced by physical agents such as heat, cold or ultrasonication. The preferential stimulation of B lymphocytes by dextran sulphate, polynucleotides and other polyanions will be discussed in ch. 11, § 1.2.

The formation of aldehyde groups on the surface of lymphocytes has been shown to give rise to blastogenic activation. Two different techniques have been used. In the first, mouse spleen cells have been incubated with neuraminidase to expose galactosyl residues on the cell surface from which the aldehyde groupings have been formed by a second incubation with galactose oxidase (Novogrodsky and Katchalski, 1973). The same authors have shown that human lymphocytes may be activated by incubation with periodate. The periodate was thought to act by oxidation of surface sialoglycoprotein with the formation of aldehyde groups which, it was postulated, interacted with amino groups on the cell surface, the resultant cross-linking causing a triggering of the cell. The periodate may also act indirectly, by modifying the surface of some cells (lymphocytes or macrophages) sufficiently for them to act as 'stimulator' cells in a mixed cell reaction (O'Brien et al., 1974). Mitomycin-treated human lymphocytes, exposed to periodate at 0 °C, washed and added to twice this number of autologous lymphocytes, produced a stimulation of the order of 8–10 times the background thymidine incorporation, with a maximum at 3 days.

Several zinc and mercury salts and at least one organic mercurial will

activate human lymphocytes (see Ruhl et al., 1971 and Caron et al. 1970 for references). Arsenic salts (trivalent or pentavalent) appear, by contrast, to be inactive. Optimal stimulation with Zn salts occurs at concentrations between 2.25 and 2.75×10^{-4} M. Few blasts are seen until after 3 days of culture, but by day 6 about 25% of the surviving cells (Zn compounds are somewhat toxic) may be blast cells. Maximal responses to HgCl_2 were also found on day 6 at an optimal concentration of 3.5×10^{-5} M.

Exposure of human lymphocytes to trypsin or chymotrypsin for a short period, followed by culture, produces about 2–14% blast cells by 16 hr. Papain is also effective, giving rise to about 2–7% blasts by 144 hr of culture (Mazzei et al., 1966). In contrast other workers have not detected trypsin-induced activation but have observed a marked reduction in PHA and PWM responsiveness following protease treatment (see Hough and Stevenson, 1973 for references). Exposure of human lymphocytes to 0.1 to 1.0 mg/ml crystalline trypsin in the presence of deoxyribonuclease for 90 min at 37 °C markedly reduced responsiveness to PHA and PWM over the first 48 hr of culture without affecting cell survival. By 66 hr of culture, levels of DNA synthesis in PHA and PWM-stimulated lymphocytes pretreated with 0.1 mg/ml trypsin approached that of the control (non-trypsinised) lymphocytes, possibly indicating resynthesis of mitogen receptors during this interval or simply recovery of cell function. The apparently conflicting findings are probably explained by differences in the purity and potency of the various proteolytic enzyme preparations used. Very mild proteolysis of lymphocytes may enhance responsiveness, but stripping off substantial amounts of surface material clearly reduces mitogen responsiveness until surface material is resynthesised. Some elegant studies by Steffen (1974), using a reversible aminopterin-blocking procedure to detect arrival of activated cells at G_1/S , have shown that, as the number of receptors on the lymphocyte surface is progressively reduced by pronase treatment, the optimal PHA concentration also alters. Mild treatment with pronase produced an increase in the numbers of cells arriving at G_1/S when the cells were stimulated with low concentrations of PHA, and a decrease at high PHA concentrations. More rigorous pronase treatment produced the opposite effect.

Morphological changes may be induced in human lymphocytes by exposing them to ultrasonication (20KC, 15 watts at horn tip) for 2–8 sec (Turk et al., 1969). By 4 days of culture up to 43% of the cells present were blast cells or 'altered' forms. Lysosomal activation also occurred but there was no demonstrable enhancement of RNA or DNA synthesis.

6.4. Effects on living animals of the injection of PHA and other mitogen preparations

It would be surprising if the injection of PHA into an animal produced a simple activation of blood lymphocytes of the type seen in vitro, although this appears to have been the expectation of some investigators. Both the coating of lymphocytes with mitogen and the activation resulting from it are events likely to lead to removal of the affected lymphocytes from the blood and their sequestering in spleen or elsewhere. The fact that PHA is an antigen, and may initiate a sequence of events in lymphoid tissue like any other antigen, is another complication of the in vivo situation. Furthermore, many other cells apart from lymphocytes have receptors for lectins and may become altered functionally and in surface structure by attachment of the lectin.

The i.v. injection of PHA-P into rabbits induces marked morphological changes in the spleen, less prominent changes in the liver and lymph nodes and a leucopenia followed by a relatively protracted leucocytosis (Blennerhassett et al., 1969). Hyperplasia of the white pulp was seen and later, areas of myelopoiesis and erythropoiesis appeared in the red pulp. These changes were not due to PHA acting simply as an antigen as similar effects were produced with PHA-M but not with a conventional protein antigen. A single i.p. injection of PHA-P or PHA-M into mice has also been found to enhance spleen cell activity, but to have little effect on bone marrow (Lozzia et al., 1969). An initial leucopenia was followed by a marked leucocytosis in the case of PHA-P, with no absolute rise in the number of lymphocytes. A neutrophilia and lymphocytosis followed the administration of PHA-M.

The injection of PWM i.p. into rats increased the spleen weight and increased the cellularity of the red pulp with many blast cells evident. Lymph nodes showed some disorganisation of the normal structure with an increased number of immature lymphocytes and plasma cells (Jorgensen et al., 1972). There were no changes in blood haemoglobin, leucocyte or lymphocyte counts or blood lymphocyte morphology. The spleen changes were similar to those described in mice treated with PHA. The thymus appeared to be unaffected.

Injection of con A-stimulated allogeneic mouse lymphocytes into lethally irradiated mice resulted in a survival rate of 60%, with production of a chimera, whereas mice injected with unstimulated allogeneic spleen cells all died of graft-versus-host disease (Ledney, 1972). Con A was superior to

PHA-P used in the same way. High concentrations of con A were used in these experiments (100 to 1000 µg/ml at a spleen cell concentration of $10-20 \times 10^6/\text{ml}$). A surprising and interesting finding was that 40% of lethally irradiated mice injected with con A-treated *syngeneic* spleen cells suffered wasting and diarrhoea. The experiments tended to show that con A modified the biological properties of both syngeneic and allogeneic lymphocytes.

Injection of mice with con A two days prior to challenge with sheep red cells causes a marked reduction in the number of plaque-forming cells produced in the spleen (Egan et al., 1974). The effect was less pronounced if the con A was injected at longer intervals before, or at the same time as, or after antigen. Much earlier work with PHA also showed that introduction of the lectin prior to sheep red cells reduced the haemolysin response whereas injection of PHA after the antigen did not reduce, and sometimes increased, the numbers of plaque forming cells in the spleen.

It will be seen from this brief outline of the literature that almost all the *in vivo* experiments have been attempts to enhance haemopoietic cell mitotic activity by injection of lectins or of lectin-treated lymphocytes. It will be apparent from areas of investigation discussed elsewhere in this book that lectins and bacterial mitogens might be used in other ways. Some types of tumour cells and normal tissue cells, for instance, are killed by activated lymphocytes only if PHA, PWM or con A is present at the killing stage when it appears to act not as a mitogen, but as a 'sticky' or membrane-modifying substance (see ch. 10). Cell killing in tissues or tumours infiltrated with lymphocytes might therefore be enhanced by local injection of PHA or other lectin. Promising results have been obtained by treating some leukaemic patients with 'immunotherapy' consisting of multiple injections of BCG. It is quite possible that a suitable 'non-specific' T cell stimulant would produce a more profound stimulation of T cell activity without appreciable antibody production. Plant lectins are unsatisfactory because they are agglutinins but the 'non-specific' staphylococcal and streptococcal mitogens, the purification of which has been discussed earlier in this chapter, are not and they deserve a trial in this type of experiment.

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Section 6.1.

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The activation of lymphocytes by foreign cells

When leucocytes from the blood of two unrelated individuals are mixed together and cultured for several days, the proportion of lymphoblasts in the mixed cultures is always significantly greater than in the corresponding unmixed cultures (Bain et al., 1964). This mixed lymphocyte reaction (MLR) is a general phenomenon: it occurs with homologous mixtures of lymphocytes from many and possibly all species and with lymphocytes from various lymphoid tissues (reviewed by Sorensen, 1972). The activation is associated with transplantation antigens on the surface of the lymphocytes and is normally a two-way reaction, both populations acting as 'stimulator' and 'responder' cells. Although apparently immunospecific in nature, being abolished specifically in one direction when one donor is rendered tolerant to antigens of the other, it is an inborn and not an acquired reactivity, no prior immunisation of one donor with cells of the other being necessary. Since the reaction is also observed in mixed cultures of rat thymocytes from young rats of two inbred strains it is clear that reactivity is not related to prior antigenic exposure even in utero. Despite early incorrect reports that a one-way stimulation could be obtained when one cell population was disrupted by freeze-thawing it is now agreed that stimulating capacity is retained only by intact cells or cells arrested by X-irradiation or treatment with drugs (Hardy and Ling, 1969). Only certain cells carrying antigens of the transplantation type appear to be capable of acting as 'stimulator' cells in the unimmunised animal. Human lymphocytes have no 'natural' reactivity to erythrocytes from an individual of a different ABO group despite the occurrence of 'natural' isoagglutinins in the serum. Some nucleated cells (e.g. fibroblasts) are known to be poor stimulators of allogeneic lymphocytes but cells of few tissues have as yet been tested for this capacity.

7.1. The mixed lymphocyte reaction with human cells

That lymphoblasts appear when leucocytes from two human donors are cultured together was first clearly shown by Bain et al. (1964) following the observation of Dr. Robert Schrek that blast cells were observed in cultures of leucocytes from a pool of donors. In smears from one pair 38 cells per 1000 were labelled (after addition of ^3H -thymidine to cultures on the fifth day) compared with 8 and 4 in the unmixed controls; in the second pair the corresponding figures were 8, 3 and 3, and in the third pair 22, 2 and 1. Leucocytes were shown to be responsible for the stimulation: when plasma, erythrocytes or platelets from one donor were added to the leucocytes of another there was no evidence of activation. No constant differences between the cell counts of mixtures and controls were found at the end of the culture period as might have been expected had a vigorous reaction of homograft rejection occurred. The mixed cell stimulation phenomenon is readily reproduced. Some increment of mixing is observed in all cultures set up from cells of unrelated human donors provided a sensitive assay system is used. In more than 600 mixed cultures from unrelated subjects in one series no negative combinations were found (Bach, 1970). The reaction is reduced or absent in mixtures of cells from closely related individuals and it was the observation that no reaction occurs in mixtures of leucocytes of monozygotic twins and that cells of fraternal twins and siblings react less vigorously than those of unrelated individuals which early suggested a possible use of the MLR as a histocompatibility test (Bain and Lowenstein, 1964).

The sequence of morphological changes in lymphocytes activated in mixed cultures is similar to that seen in cultures containing soluble stimulants except that fewer lymphoblasts are seen and the peak of activity is later, usually between day 5 and day 7 but sometimes later (Alling and Kahan, 1969). Each cell population has a dual role and the response observed is dependent on the responsiveness and the stimulating capacity of each population. Optimal proportions in this two-way reaction are usually not far removed from 1:1. If the stimulatory role of the allogeneic cell be regarded as that of a source of antigen presented on a cell surface it would be expected that the stimulation would be dose dependent just as it is in the case of soluble stimulants. It might be suspected too, that in mixtures where only minor antigenic differences are involved, relatively large numbers of cells would be needed to produce a significant degree of stimulation.

Lymphoblasts, because of their greater metabolic activity and probable associated increased rate of synthesis of surface antigens and their larger surface area, would, it is anticipated, be more effective than small lymphocytes in stimulating allogeneic lymphocytes. It has indeed been shown that both the numbers of 'stimulator' lymphocytes (Bach and Bach, 1970) and their state of activity (Lowe, 1971) affect the potency of the stimulus to an allogeneic population. In general, the response increases with increasing numbers of 'stimulator' lymphocytes and may not reach a maximum with weakly stimulating combinations until the ratio of 'stimulator': 'responder' is 2 or 3. In some strong combinations peak values occur at lower 'stimulator' concentrations and maximal stimulation across species (e.g. mouse lymphocytes stimulating rat cells) usually occurs at even lower concentrations with quite marked inhibition at high 'stimulator' cell concentrations.

The stimulation is usually measured by the enhanced level of DNA synthesis (as assessed by scintillation counting or autoradiography) or by conventional morphological criteria but occasionally other methods have been employed. One method which attracted attention because it measured a very early change in the mixed cell cultures was the increased uptake of acridine orange. However, recent observations suggest that what was being measured was a 'crowding' effect of the cells and not the dissociation of the nucleoprotein preparatory to nuclear activation as was originally thought (see Bolund et al., 1970).

The capacity of lymphocytes to stimulate and respond is present at birth. Activation occurs in mixtures of leucocytes from two cord bloods and may be as vigorous or more vigorous than that found in mixed cultures from blood of adults (Lamvik, 1966; Leikin et al., 1968). Lymphocytes from human thymus, tonsil and appendix as well as from blood are activated by allogeneic lymphocytes (Schwarz, 1967). Lymphocytes stored in liquid nitrogen have been shown to react in the MLR provided a good freezing-down technique is used (see § 3.3.11). Cultures have usually been set up from the impure leucocyte-rich serum or plasma following sedimentation of the red cells from defibrinated or heparinised blood. If enough cells are available purification of the lymphocytes over Ficoll-Triosil improves the responses and Ficoll-Triosil purification is essential when cultures are to be set up from preparations containing many dead cells and debris e.g. cell suspensions prepared from lymph nodes obtained post-mortem. Micro-cultures in plastic trays have been used (ch. 3) and so have whole blood cultures (Shons et al., 1973). Sometimes cultures have been set up in the absence of serum

and it has been shown that under these conditions mercaptoethanol and other reducing agents improve responses (Heber-Katz and Click, 1972).

7.1.1. Relation to genetic disparity and HL-A antigens

Three factors have been responsible for our considerable knowledge of the relationship of the MLR to histocompatibility differences: a) the relative precision and reproducibility of the HL-A typing technique which has evolved during the last few years. The test is based upon the reaction of homologous antibody and complement with lymphocytes in a specific cytotoxic test; b) the dissection of the two-way MLR into two one-way reactions (see next section); c) family studies, especially when HL-A typing has also been performed.

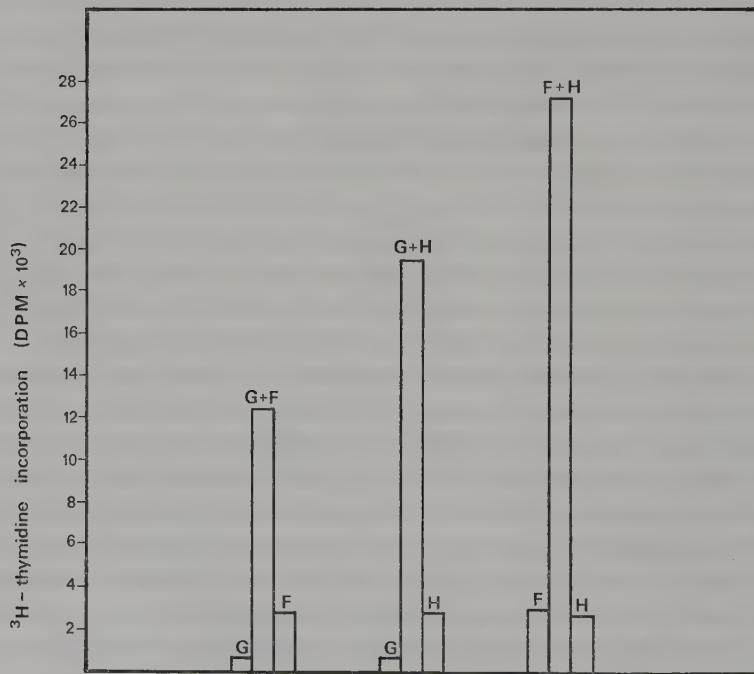


Fig. 7.1. Two-way MLRs with the lymphocytes of three unrelated individuals of identical or dissimilar HL-A type. G and F = identical HL-A type; H was the third donor.

It was established soon after the introduction of the MLR into histocompatibility testing that there invariably is less interstimulation in leucocyte mixtures from siblings and parent-child combinations than in mixtures from unrelated individuals (Bain and Lowenstein, 1964; Chalmers et al., 1966). More recent studies have had the advantage of being performed on leucocytes of HL-A typed individuals. According to current opinion HL-A antigens can be classified into two allelic series, the expression of which is governed by two genetic loci. Allelic antigens of the first (LA) series are 1,2,3,9,10,11,W28, and of the second (Four) series 5,7,8,12,13,W5,W10,W14, W15,W17,W18,W22 and W27, where W indicates a workshop designation of antigens under investigation (Allen, 1970). The most marked correlation of MLR reactivity with differences of HL-A type has been in studies within families (see review by Bach et al., 1972). Cells from about 28% of siblings fail to stimulate in a one-way reaction (Bach and Bach, 1971). Mixed cells from many related individuals of identical HL-A phenotype (sibling-sibling or parent-sibling) are unreactive in the MLR (Amos and Bach, 1968; Schellekens et al., 1970) suggesting that activation in lymphocyte mixtures is determined by differences at the HL-A locus or a very closely linked locus. Cases have been reported, however, of positive MLRs between siblings found to be identical at HL-A by genotyping and between mother and child of serologically identical HL-A phenotype, but these were rather rare, occurring only twice in 60 pairs in one study. Negative MLRs from randomly chosen unrelated individuals are very uncommon. Mixtures of cells from unrelated individuals of apparently similar HL-A phenotype have usually been found to show evidence of interstimulation but sometimes of lower intensity as judged by comparison with HL-A dissimilar mixtures set up at the same time (see fig. 7.1 for an example encountered in Birmingham).

The HL-A identity of the two donors has usually not been completely defined. By selecting individuals in whom the antigens of the two sub-loci could be considered to be serologically completely identical (assuming that no normal individual can possess more than two antigens of either allelic series) the dependence of MLR reactivity on the HL-A phenotype could be tested. Four such persons (selected from a group of 500 persons by Dr. Eijsvogel and his colleagues) were of phenotype 4a neg, 4b pos, 7a neg, 7b neg, HL-A7 pos, HL-A8 pos, HL-A1 pos, HL-A2 neg, HL-A3 pos, HL-A9 neg. A lack of stimulation in the MLR between cells of two of these individuals was established, comparable to that found in MLRs between HL-A identical siblings. The results remained negative when varying num-

bers of stimulating cells were used. In the other five possible combinations positive reactions were obtained. Each of the non-stimulating pair appeared to have an HL-A identical sibling. Mixtures of leucocytes of these four individuals cultured in pairs in all possible combinations showed no increment of stimulation. Cells of a fifth individual of the same phenotype were also found to give negative MLRs when mixed with cells of any of the other four individuals. Cells of three other individuals also of HL-A 1,3,7,8 phenotype produced, on the other hand, positive MLRs when mixed with cells of any other member of the trio or of any four individuals of the first group (Eijsvogel et al., 1971). These results could be explained by postulating the existence of HL-A antigens detectable by MLR but not serologically, or by non-HL-A antigens affecting the MLR. The second explanation, it was thought, would conflict with the marked coincidence of MLR negativity with HL-A compatibility observed in tests with siblings.

In further analysing the relationship of the MLR to histocompatibility it should be understood that there are two ways of approaching the problem, the first immunochemical, the second genetic. Are known transplantation antigens present on the various blood cells directly involved in the MLR? Blood group antigens can be completely disregarded. Mixtures from donors of different ABO groups show the same levels of transformation as those of similar ABO groups (Lamvik, 1966). Erythrocyte antigens and isoagglutinins do not appear to influence the MLR. On the other hand there is very good evidence of a direct or indirect connection with expression of HL-A antigens. Yet no good evidence has been forthcoming that isolated pure or impure HL-A antigens have any direct lymphostimulatory effect as was once claimed. Recent evidence points much more to a genetic association of MLR reactivity with expression of HL-A antigens without their being as yet any clue as to the form of phenotypic expression governing MLR reactivity. The whole problem has been greatly illuminated by some elegant family studies. One family of seven siblings (table 7.1) described by Dupont et al. (1971) contained two HL-A identical siblings (nos. 1 and 4) and one recombinant (no. 5) representing a crossing-over between the father's LA and Four determinants. It will be seen (table 7.1) that isolated Four antigen differences gave unequivocally positive responses. These results would suggest either that MLR reactivity is directly elicited by Four locus antigens or that another locus (MLC locus) controlling the MLR is closely linked on the chromosome to the Four locus. There are other considerations which suggest that the second possibility and not the first is correct. One of these is the fact that

Table 7.1

MLR between siblings of known HL-A type. Cells of siblings nos. (1) to (7) and an unrelated donor (U) were mixed in one-way MLR in all combinations. ^3H -TdR was present from 96–120 hr. Antigens 1 and 2 are of the LA locus, and antigens 7 and FJH of the Four locus. From data of Dupont et al. (1971).

		Stimulator cells							
no.	Responder cells HL-A type	(1) 2,FJH/3,AA	(2) 1,7/3,AA	(3) 2,FJH/1,8	(4) 2,FJH/3,AA	(5) 1,FJH/3,AA	(6) 1,7/1,8	(7) 1,7/3,AA	U 2,10/5,MK
(1)	2, FJH/3, AA	(96)	716	828	251	269	1359	599	1049
(2)	1, 7/3, AA	783	(183)	2303	912	790	1219	257	384
(3)	2, FJH/1, 8	317	603	(388)	1057	669	964	809	935
(4)	2, FJH, 3, AA	108	522	684	(241)	106	1079	430	933
(5)	1, FJH, 3, AA	140	808	1035	261	(264)	1516	941	1508
(6)	1, 7/1, 8	2074	1037	1228	2026	1506	(307)	1051	1412
(7)	1, 7/3, AA	879	130	1377	757	761	894	(100)	1072
(8)	2, 10/5, MK	1615	1278	1818	2377	1629	1128	1199	(362)

positive MLRs are nearly always obtained with mixtures of cells from unrelated individuals even though they type identically for Four antigens. Also, as already indicated, there are rare examples of siblings of HL-A identity whose cells stimulate in a MLR.

The HL-A antigens have been referred to as serologically-detectable (S_d) antigens and the hypothetical MLC locus (or MLR locus) antigens as lymphocyte-detectable (L_d) antigens (Bach et al., 1972). While this is a convenient way of describing two types of reaction it is as well to bear in mind that, although a MLC locus phenomenon exists, we are not certain that L_d antigens exist. Another uncertain area is the cytotoxic specificity of the blast cells generated in a MLR (see § 7.1.7 and § 10.2.1). There is some evidence in both mouse and human systems that although L_d differences determine the level of stimulation in the MLR it is the S_d antigens present on the stimulator cells which determine the specificity of the killing reaction (see Bach et al., 1972).

7.1.2. One-way reactions using various cells as stimulators

If the MLR is simply a special example of the activation of lymphocytes by exposing them to cells bearing foreign antigens it ought to be possible to produce a similar lymphocyte activation with other blood and tissue cells. Not all foreign antigens on cell surfaces are capable of activating the lymphocytes of non-immunised individuals as is shown by the negative results obtained with human red cells of another group even when they are present in high concentration (Hardy and Ling, 1969). Even nucleated alligator red cells, which must bear many foreign antigens, produce only slight stimulation of lymphocytes of unimmunised humans (Harris et al., 1970). It appears that only living cells bearing antigens of a certain type are capable of acting as 'stimulator' cells and, from what has been said in the previous section, it would appear likely that only MLC locus or related antigens fulfil this role, although, of course, many other cell surface antigens will stimulate the lymphocytes of immunised individuals.

When lymphocytes and other nucleated cells capable of DNA synthesis are to be tested as 'stimulator' cells measurement of lymphocyte activation is simplified if DNA synthesis of the 'stimulator' cells is inhibited by a method producing minimal modification of the cell. Two methods have been almost exclusively used. a) *X-irradiation*. Small lymphocytes suffer a delayed death as the result of X-irradiation without entering DNA synthesis. When

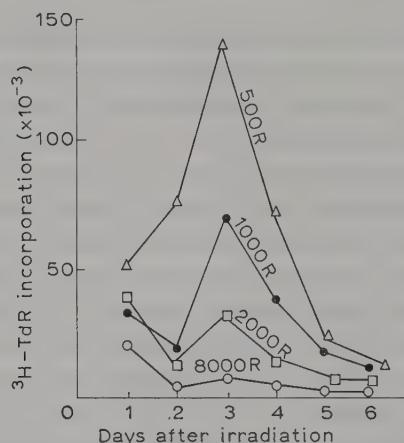


Fig. 7.2. Decay of DNA synthesis of EB4 cells after X-irradiation with 500 r, 1000 r, 2000 r or 8000 r. Initially 5×10^5 viable EB4 cells were present in 1 ml of medium.

X-irradiation is shortly followed by exposure to stimulation (e.g. with allogeneic cells; see ch. 10) more cells are able to survive at a given dose of X-irradiation and at low doses some will enter DNA synthesis. For a truly one-way reaction to occur it has been shown that a dose of the order of 4000 r is required (Kasakura and Lowenstein, 1968). Cell populations which are normally dividing need even higher doses of X-rays to arrest DNA synthesis. For efficient arrest of DNA synthesis within 48 hr, cells of Burkitt's lymphoma lines required a dose of 4000 to 8000 r (fig. 7.2) and cells of most lines require about the same dose. The great advantage of X-irradiation as a means of mitotic arrest is that no inhibitory substances likely to affect the responding population are introduced. The disadvantage is that the 'stimulator' population is dying off during the culture period. b) *Drugs which arrest DNA synthesis such as mitomycin* (Bach and Voynow, 1966). 'Stimulator' cells are incubated at a cell concentration of $10^7/\text{ml}$ for 20 min at 37°C with mitomycin C (25 $\mu\text{g}/\text{ml}$) and washed three times. Probably because of its simplicity, this method has been the preferred in most matching tests with human lymphocytes. There is a danger of carry-over of drug from 'stimulator' to 'responder' population but this has not seriously interfered with the interpretation of the results in the opinion of most workers. A full experiment should include a two-way reaction (untreated cells), two one-way reactions (one population treated with mitomycin or X-irradiation, one untreated population), and a background control (both populations treated). If normally dividing populations of cells, e.g. from cell lines, are to be used as 'stimulator' cells more intensive mitomycin treatment will be required and escape from mitotic arrest may occur over long periods of incubation.

Sex typing of chromosomes is a third method available. Suitable chromosome preparations from colchicine-arrested cultures of mixed male-female cultures are sexed and the extent to which the reaction has proceeded in the two directions assessed (Ceppellini et al., 1965; Oppenheim et al., 1965). This method is too time-consuming for routine use but is valuable as a way of tracing the course of a two-way reaction. It is, of course, based on counting the numbers of mitoses, which may not necessarily correlate exactly with the degree of activation as assessed by morphological change or thymidine incorporation. Nuclear chromatin markers on small lymphocytes and blast cells have also sometimes been exploited in a similar manner. One-way stimulation may be achieved without treatment of 'stimulator' cells if these cells are unlikely to enter DNA synthesis (e.g. freshly isolated macrophages

or thyroid epithelial cells). In one experiment of this type macrophages (obtained from monocytes by placing 10 ml of a blood leucocyte suspension in a 20 ml flat bottle and leaving it at 37 °C for one week) were separated from non-adherent cells (lymphocytes and red cells) by careful washing. When lymphocytes from a donor of the opposite sex were added to such a preparation and metaphase spreads prepared one week later for sex chromosome analysis it appeared that the reaction was not completely one-way (Marshall et al., 1966). About 10% of the dividing cells were of the same sex as the macrophage donor, the mitoses presumably originating from residual lymphocytes in the macrophage preparation. Since the macrophage preparation was not completely pure it is also not certain from these experiments that the stimulation was produced by the macrophages.

Direct sensitisation of lymphoid cells by allogeneic macrophages is supported by the experiments of Dyminski and Argyris (1971) but others have obtained negative results. There is even more uncertainty about a possible direct 'stimulator' role of other leucocytes in mixed leucocyte culture. This doubt persists because of the great difficulty in obtaining leucocytes of one class completely free of contaminating cells. Polymorphonuclear leucocytes have been reported to stimulate allogeneic lymphocytes but this is contrary to the experience of most of us who find that a normal MLR is enhanced by removal of granulocytes which appear not to stimulate and, in fact, to have an inhibitory effect (Mardiney et al., 1972; Ragle and Cowan, 1973). This is almost certainly true of the neutrophil but there appear to have been no investigations on isolated eosinophils or basophils. There is a single report of mitotic activation of lymphocytes by allogeneic blood platelets (Dausset and Rappaport, 1966) but some of us have not been able to confirm this. As far as blood cells are concerned it is only lymphocytes which have been shown unequivocally to have the capacity to act as 'stimulator' cells as well, of course, as being the only 'responder' cells.

A number of cell types freshly obtained from normal tissues or tumours have been tested as 'stimulator' cells using lymphocytes of normal individuals as 'responder' cells. Epidermal cells prepared from trypsin-dispersed human skin are definitely active in the human as they have been shown to be in the rat system (see § 7.1.5), the order of stimulation per cell being somewhat less than that of a lymphocyte. Trypsin-dispersed breast tumour cells are inactive (Ling et al., 1973). Cells from various tumours such as HeLa cells, KB cells, melanoma cells and osteogenic sarcoma cells are also inactive (Han, 1972). There have been few attempts to stimulate human lymphocytes

with animal tumour cells but in our experience the mouse L5178Y lymphoma is a poor 'stimulator', whereas Hersh (1971) found the mouse LP59 fibrosarcoma to be a good 'stimulator'.

There are at least two difficulties which limit a simple interpretation of these findings. Firstly, when cells from a solid tissue are to be tested it is obviously necessary to prepare a single cell suspension and this involves subjection of the tissue to enzymic disruption usually with trypsin. It is known that trypsin strips off protein and carbohydrate from cell surfaces and that in doing so it alters the properties of cells. Hence a negative result may be artefactual due to 'trypsin-stripping'. However, epidermal cells retain activity after trypsinisation and tumour cell dispersions inactive when freshly prepared do not acquire 'stimulator' potential when maintained in culture although it is known that cell receptors are resynthesised during 24 hr of culture.

A different objection may be raised when cells from continuously growing lines are used as 'stimulators'. They may be said to have lost the original antigenic surface characteristics of tissue cells. Although of course it is true that long term cultured cells do not retain many characteristics of the differentiated tissue cells they do retain surface antigens of certain types, notably species, blood group and transplantation antigens. By absorption tests HL-A antigens have been demonstrated on HeLa cells and on fibroblasts (Seigler and Metzgar, 1970) and by direct tests on cells of lymphoid cell lines (see later). Freshly isolated cells from most human tissues have also been shown to express HL-A antigens. They have been shown to be present on 'fibroblasts' grown from explants of human skin but these cells (as reported by Schellekens and Eijssvoogel, 1970 and confirmed in our laboratory) do not stimulate allogeneic lymphocytes. One suggestion to account for this failure to stimulate, despite the presence of surface histocompatibility antigens, and the similar failure of platelets, said to contain HL-A antigens in appreciable concentration, has been a difference in the distribution of antigens on the cell surface. The antigens on fibroblasts, it is suggested, may be evenly distributed over the cell surface, whereas on lymphocytes they may occur in high concentration in small areas, possibly on the uropod. Most of this speculation preceded the demonstration that HL-A antigens are not directly involved in the MLR. However, the same arguments may be applied to the hypothetical MLC locus antigens.

There is, without doubt, more to the role of the 'stimulator' cell than the possession of certain antigens. Leucocytes disrupted by freeze-thawing are

totally inactive as stimulants (see Hardy and Ling, 1969 and Schellekens and Eijsvoogel, 1970 for references) and the lack of activity is not due to the release of inhibiting enzymes. Similarly, cells from lymphoid cell lines when killed by freezing and thawing or even by careful freeze-drying, are quite inactive. Heat-killed lymphocytes which retain, as far as can be ascertained serologically, unmodified HL-A antigens on their surface, are inactive in the MLR (Schellekens and Eijsvoogel, 1970; Gordon and Maclean, 1965). Intact lymphocytes killed by ultra violet light are also ineffective (Lindahl-Kiessling and Safwenberg, 1971). Soluble HL-A preparations derived from human spleens by autolysis or controlled proteolysis have been reported to initiate blastogenesis in lymphocyte cultures but we have not been able to confirm this (Ling et al., 1973). A highly purified serologically active HL-A fraction kindly supplied by Dr. A. Sanderson was also inactive as a stimulant. The most spectacular responses yet reported in mixed cell reactions occur when human lymphocytes (from adult or cord blood) are cultured with X-irradiated cells from human lymphoid cell lines (LCL). Very high levels of stimulation are obtained when 2×10^6 blood lymphocytes are mixed with 2×10^5 X-irradiated EB2 cells or with cells from very many other cell lines derived from normal adult or placental blood or from patients with infectious mononucleosis or Burkitt's lymphoma. These cells are known to express HL-A antigens in greater amounts than normal lymphocytes and all the HL-A antigens found on the normal cells of the original donor are present (Mackintosh et al., 1973). It is, of course, possible that MLC locus antigens accompany the HL-A antigens and that these too are present in supra-normal amounts. The potency of these cells as 'stimulators' is influenced by the growth rate of the culture from which they are derived – the better the growth the better the stimulation. This may be accounted for by better presentation of antigens or by a higher rate of antigen synthesis and membrane turnover.

The two extreme views on the role of the 'stimulator' cell in mixed cell reactions have been (a) that it must be a lymphoid cell and that the MLR is a special type of reaction occurring only between two lymphoid cells; (b) that any nucleated cell bearing foreign antigens of the transplantation type would stimulate lymphocytes just as it must in the body at the first stage of a homograft reaction. The results obtained would not appear to make either alternative acceptable. It is generally agreed that some allogeneic cells, e.g. skin fibroblasts, HeLa cells which bear transplantation antigens do not stimulate lymphocytes. It is also agreed that T and B lymphocytes, LCL cells and

epidermal cells will stimulate. There are many other single reports or disputed reports suggesting that endothelial cells and various tumour cells will stimulate. In the mouse and rat systems (see § 7.1.5) it is well established that surface-adherent cells ('fibroblasts') from a whole embryo will stimulate lymphocytes. It is also known that specifically cytotoxic cells are sometimes generated in mixed cell reactions and that sometimes they are not (see § 7.1.7). Are all these examples of mitotic activation of lymphocytes by various foreign cells descriptive of a single type of reaction or do they conceal two or more distinct types of reaction? Before considering this question further the many direct observations of the events occurring during the course of the MLR must be considered.

7.1.3. *Nature of the cell-cell interaction in the MLR*

The MLR appears to arise from direct cell-cell interactions and activation does not occur when the two populations are separated by a Millipore membrane (Chapman and Dutton, 1965). Morphological observations comparing the behaviour of lymphocytes in mixed and unmixed cultures over a period of 6 days suggest that lymphocyte-lymphocyte and lymphocyte-macrophage interactions via a lymphocyte foot appendage (uropod) are a characteristic of the MLR (see ch. 5).

It is still uncertain whether the mixed lymphocyte activation is simply the result of a lymphocyte-lymphocyte interaction or whether the macrophage is essential as a 'helper' cell. The basis for the 'helper' theory is the marked diminution in intensity of the MLR when lymphocytes are 'purified' from other leucocytes by passage through glass bead columns or by one of the other standard techniques (Levis and Robbins, 1970). Activity has been restored by whole leucocyte preparations, macrophages or supernates of macrophage cultures. For example, the unresponsiveness to allogeneic lymphocytes observed in 19/21 cases when lymphocytes were purified almost completely from macrophages could be corrected by the addition of macrophages or supernates from macrophage cultures (Bach et al., 1971). Yet very good stimulation in MLRs may be obtained with mixtures of relatively pure lymphocytes from thymuses (Schwarz, 1966) and thoracic duct. In rabbit mixed cell reactions macrophages seem to have an inhibitory effect (Chapman and Dutton, 1965) and their presence makes no obvious difference to rat MLRs (Wilson, 1967). It is in the human MLR that reports of a positive role of macrophages have been most persistent. There appears to be good

evidence that the depressed MLR reactivity of purified lymphocytes is not simply due to cell damage during purification (Twomey et al., 1970). The supportive role of macrophages appears to be most marked at low cell densities (Levanthal and Oppenheim, 1969) and it does not have an immunological basis since macrophages from either immune or non-immune donors are effective (Seeger and Oppenheim, 1970). It may be important to note, in view of the many discrepant reports, that the macrophage effect is much more likely to be found in cultures set up in large flat containers (e.g. Petri dishes) rather than in conditions which promote cell crowding (e.g. round-bottom tubes) suggesting a mechanical or clustering role of macrophages, but it is possible that an active substance is released into the medium as reported by Bach et al. (1971). Some dynamic role of the 'stimulator' lymphocyte may also play a part since high levels of stimulation are only obtained when 'stimulator' as well as 'stimulated' populations of lymphocytes are viable and the level of stimulation appears to be related to the metabolic activity of the 'stimulator' lymphocyte as well as to the antigens on its surface. As stated already, lymphocytes must be living to retain 'stimulator' activity. It has been shown by various workers that 'stimulator' activity is destroyed by treating lymphocytes with ethanol, phenol, formalin, iodoacetate and other cell-damaging agents. Any substance affecting the cell surface may modify the MLR. Pre-incubation with sucrose inhibits the response (Rode and Gordon, 1969). Heparin has been reported to inhibit the MLR (Currie, 1967), but we have been unable to confirm this.

Antisera containing antibodies to antigens on the lymphocyte surface are potent inhibitors of the MLR. The sensitivity of the MLR to inhibition by anti-lymphocyte sera (ALS) is so marked that this is a possible way of assaying the immunosuppressive potential of antisera prepared in animals. It is more sensitive than a cytotoxic assay for heterologous ALS and is even capable of detecting homologous antibodies to human lymphocytes not detectable in the cytotoxic test (Grumet and Levanthal, 1970). Fab fragments of equine ALG, although not toxic to lymphocytes, markedly inhibit the MLR (Richie et al., 1972). Alloantisera to HL-A antigens on the surface of 'stimulator' lymphocytes completely inhibit the MLR (Ceppellini et al., 1971). Careful studies in the rat system have shown that only antibodies to surface antigens of the 'stimulator' cells block the MLR (Milton et al., 1973). It was not necessary for the antibody to be directed against the particular Ag-B antigens carried by the stimulating cell and absent from the responding cell. Antibody to other surface antigens on the 'stimulator' cells also inhib-

ited the MLR. Antibodies to surface antigens of the 'responder' cells did not block the reaction. Blocking experiments of this type evidently do not give any information, as was once thought, about the nature of the interacting sites on the 'responder' and 'stimulator' cells. It was once thought that the reported inhibition of the MLR by anti-light chain antibodies (or the Fab monomer fragments prepared from these) could be taken as evidence that the T cell receptor implicated in the MLR contained light chain determinants and was possibly acting as a cell-bound antibody (Greaves et al., 1971).

Mild treatment with proteolytic enzymes appears to enhance lymphocyte responsiveness in the MLR. After prolonged trypsinisation lymphocytes lose most of their capacity to respond. Treatment of either the 'stimulator' or 'responder' cells with neuraminidase increases the response significantly according to one report (Han and Pauly, 1973). Lundgren and Simmon (1971) also found that neuraminidase caused a marked increase in the stimulatory capacity of allogeneic lymphocytes but could detect no effect on the 'responder' cells. The general conclusion to be drawn from all these experiments is that the state of the surface of the 'stimulator' cell is of crucial importance in the MLR. Any chemical or serological modification of the cell surface depresses activity while removal of some cell surface components with enzymes enhances it. It also appears highly probable that interaction of membrane sites on 'stimulator' cells and 'responder' lymphocytes initiates the reaction although macrophages may have some indirect role. Whether the MLR is a unique reaction or whether non-lymphoid cells activate lymphocytes by an essentially similar mechanism is hard to decide. The MLR itself is not motivated by serologically detectable antigens, but there is no doubt that the immunogenicity of tissue cells is correlated with the concentrations of these antigens and that immunogenicity is closely related to lymphocyte activation *in vivo*. It may therefore be that the *in vitro* activation by, for example, epidermal cells, is simply a normal primary immunological reaction to serologically detectable antigen. With most antigens a primary response is not measurable, but an exceptionally immunogenic structure may produce a detectable activation during the usual 4–7 days of culture. If activation by non-lymphoid cells is really a different kind of reaction this ought to be reflected in the cell types involved and in their properties after activation. Further, pre-immunisation ought to enhance the reaction whereas it is known it does not in the case of the MLR. The requirements for the production of tolerance in the two reactions may also

be different. No definite information is currently available on any of these matters, but there are many hints and by the end of the chapter the reader may be able to formulate his own hypothesis.

7.1.4. Mixed lymphocyte reactions in various species

Mixed lymphocyte stimulation occurs in homologous mixtures of lymphocytes of many species, including monkey (Appelman and Balner, 1972), dog (Main et al., 1967; Kisken and Swenson, 1969; Rudolph et al., 1969), cat, rabbit (Chapman and Dutton, 1965; Daguillard and Richter, 1969; Harrison et al., 1971), pig (Bradley, 1973), chicken (Weber, 1970), rat and mouse (see below) and even in the ammocoete (Cooper, 1969). Hamster lymphocytes are stimulated by human and other xenogeneic lymphocytes but allogeneic mixtures of Golden Syrian and MHA strains do not stimulate each other (Fernald and Metzgar, 1971). Animal blood lymphocyte MLRs are slow reactions, as are human MLRs, with a maximum, if culture conditions are very good, at day 5–7. An earlier maximum is usually found when cells from spleen, thymus or lymph nodes are cultured.

Most investigations of the human MLR have been made on leucocytes obtained from blood whereas mixtures of animal lymphoid cells have frequently been of spleen, lymph node or thymus cells. There appear to be unexplained species differences in the intensity of the MLR in relation to tissue source. In rabbits the peripheral mixed cell reaction is of a low order in contrast to well defined mixed lymph node or mixed spleen cell reactions. The frequency of positive MLRs in mixtures of spleen cells of rabbits of an outbred but closed stock is almost 100% (Chapman and Dutton, 1965). There is little evidence of interstimulation in mixtures of rabbit thymocytes in contrast to the marked stimulation with mixtures of rat thymocytes. The responses of cells of very young animals are erratic. A mixed cell activation is not detectable in thymus–thymus cell mixtures from 8–15 day old rabbits and is slight in the corresponding spleen–spleen cell mixtures over a 24–48 hr period. The rabbit peripheral cell reaction is greatly enhanced if one population is first stimulated and X-irradiated before use in a one-way reaction (Lowe, 1971). Because of lack of knowledge about histocompatibility in rabbits the relation of MLR stimulation to antigenic disparity is undefined in this species but there appears to be some correlation of the MLR with skin graft survival (Harrison et al., 1971).

Very good MLRs are obtained with Ficoll–Triosil-purified pig blood lym-

phocytes and from extensive family studies it has been concluded that a single locus, with possibly 6 alleles, closely associated with but distinct from the locus controlling serologically detectable antigens, is responsible for stimulation in the MLR (Bradley, 1973). Good results are also obtained with dog lymphocytes (Kisken and Swenson, 1969) and in this species too MLR reactivity is controlled by a single locus (Templeton and Thomas, 1971). Great interest attaches to mixed bursal and mixed thymus cell reactions in the chicken.

Positive MLRs have been obtained with the lymphocytes from two closely related species, e.g. rat and mouse, but the stimulation in general decreases as the phylogenetic gap between species increases. Meaningful comparisons between species are difficult to make because of the better over-all performance of the lymphocytes of some species (e.g. human) and the poor performance of others (mouse, chicken). Human lymphocytes will, in fact, respond to mouse, dog, rat or rabbit lymphocytes and will stimulate mouse and dog lymphocytes (Widmer and Bach, 1972). Human-rat mixtures appear to be less active than human-human or rat-rat. It is possible that the inbuilt reactivity which is a characteristic feature of the MLR is evoked most readily by lymphocytes of the same species. Whereas rat MLRs are as good with mixed cells from germ-free rats as with cells from normal rat colonies the response of rat lymphocytes to human cells, which is normally small but measurable, disappears entirely when the rats are reared under germ-free conditions (Wilson and Fox, 1971). This suggests that at least some of the cross-species reactivity may be acquired as a result of exposure to related environmental antigens but there is no general agreement on this point. In the experiments of Nielsen (1972) the behaviour of lymphocytes of rats reared under germ-free conditions was not distinguishable from that of normal rat lymphocytes in the graft-versus-host and mixed lymphocyte reactions.

7.1.5. Rat and mouse mixed lymphocyte reactions

Because of the existence of inbred strains, the most significant work relating the MLR to histocompatibility differences has been done with rats and mice. It was early established that positive MLRs could regularly be obtained with lymphoid cell mixtures across the major species barrier. All 21 combinations of seven inbred strains of mice tested by Dutton (1965) gave positive mixed cell reactions but there was considerable variation in the magnitude of the responses of individual strain combinations from one experiment to another.

Evidence of interstimulation in mixtures of spleen cells of mice bearing different H-2 antigens was noted as early as 6 hr after mixing, and by 48 hr 1–4% of the total cell population in the mixtures was in DNA synthesis compared with very low numbers in control cultures. Many other workers subsequently confirmed that positive MLRs were obtained with strain combinations differing at the strong H-2 locus. It was also noticed that positive responses were obtained in mixtures from some strains compatible at the H-2 locus e.g. DBA/2 + BALB/C, AKR + C3H, DBA/2 + B10D2 and BALB/C + B10D2, and the presumption was made that this was due to differences at multiple non-H-2 loci (Dutton, 1965; Hayry and Defendi, 1970). In rats similar results were obtained, i.e. stimulation was regularly observed with cells from strains differing at the major Ag-B histocompatibility locus (Silvers et al., 1967).

If culture conditions are good enough, stimulation in mixed mouse lymph node and peripheral lymphocyte cultures continues to increase until about the 7th day of culture, but under culture conditions commonly used stimulation in mixed spleen cell cultures is of a much lower order with an ill-defined maximum on days 3–6 (Hayry and Defendi, 1970). Reproducible kinetics and a linear dose response curve may be obtained with mixed spleen cells under carefully standardised conditions (El-Arini and Osoba, 1973a). The maximal stimulation is always lower and earlier if culture conditions are not good. The optimal culture time for mixtures of rat thymocytes under good culture conditions appears to be about five days (Knight and Thorbecke, 1971).

In addition to the artificial methods of rendering the MLR unidirectional used in the human system (X-irradiation of one population or treatment with mitomycin) natural one-way reactions between parental and F₁ hybrid cells are readily available in rat and mouse studies. These avoid any risk of modification of the surface properties or viability of the 'stimulator' cells. Chromosomal analysis of parent-F₁ hybrid mixtures has confirmed that all of the mitotically activated cells are of parental type with no non-specific recruitment of the F₁ population (Wilson et al., 1968; Wilson and Nowell, 1971). 'Recruitment' of cells which have not been directly activated by the allogeneic cells (as might occur if a potent mitogenic lymphokine were liberated into the medium) does not appear to be a significant factor. There is an optimum number of 'stimulator' cells in one-way MLRs. In the rat system exposure of a constant number of 'responder' parental cells (2×10^6) to increasing numbers of F₁ lymphocytes produced maximal responses with approx. 0.5×10^6 F₁ cells (Wilson, 1967; Wilson et al., 1968). Further addi-

tion of F_1 cells did not increase the parental cell response. Experiments in which parental and F_1 hybrid cells have been compared as 'stimulator' cells show that it is the amount of antigen per 'stimulator' cell and not the total amount of antigen present in the system which determines the level of stimulation. A significantly higher stimulation by mitomycin-treated cells which were homozygous rather than heterozygous for a given foreign H-2 or H-1 allele has been demonstrated (Sorensen, 1972). This 'gene-dose' effect for one antigen suggests that the potency of a 'stimulator' cell depends upon the concentration of the appropriate antigens. It would be expected that a metabolically active lymphocyte expressing greater concentrations of allo-antigens would be a better 'stimulator' than the resting cell and that when a number of different foreign alleles are expressed on all the 'stimulator' cells these would summate to produce enhanced stimulation.

There has been general agreement that cells from strains of mice differing in H-2 or some non H-2 determinants stimulate each other in the MLR (see for example, Festenstein, 1966; Tridente et al., 1967; Tanaka et al., 1968; Adler et al., 1970 and Mangi and Mardiney, 1971). In general these studies indicated that strong stimulation in the MLR was associated with H-2 differences. In retrospect it seems to be clear, even from the early work, that allelic antigens of the H-2 locus could not be entirely responsible for MLR responses because good positive MLRs were obtained with certain strains of mice identical at the H-2 locus. The stimulation was at first attributed to the cumulative effect of antigens of multiple minor loci. In interpreting the very good stimulation obtained between the H-2 identical strains DBA/2 and BALB/c, Festenstein (1970) demonstrated that the reactivity segregated as a single genetic locus which he termed the M locus. Further information derived from studies of strain combinations in which the MLR did appear to correlate with H-2 differences.

In the mouse there are two major serologically defined sub-loci of H-2, the genetic location of which is at the extreme ends of the H-2 region and these are known as the K and D ends. Between these two ends there are other genetic loci, controlling quantitative differences in serum proteins (Ss-Slp) and the immune response (Ir) genes. The gene controlling the TL (thymus leukaemia) antigens is located outside the D end. Thus the order is: H-2K....Ir....Ss-Slp....H-2D....TL. As in the human MLR it was shown that the two sub-loci controlling the serologically determined histocompatibility antigens (H-2D and H-2K) were not equally related to MLR reactivity. Rychlikova et al. (1970), in studies with congenic mice, showed that

MLR stimulation was related much more closely to K-end than D-end differences. The M locus, which is now thought to possess the genes controlling the expression of the strongest lymphocyte activating determinants, is probably located in the Ir....Ss region (Festenstein, 1973; Meo et al., 1973). Other genes, controlling the expression of weaker lymphocyte activating determinants, may be spread over a large portion of the major histocompatibility complex between the K and D loci and perhaps outside them (Bach et al., 1973). Four allelic determinants (M-1–M-4) of the M locus have been detected and shown to be distributed among 12 inbred and combant strains of mice. Some M-locus alleles are weak or non-stimulatory (M-2) while others (e.g. M-1) are strongly stimulatory (Festenstein, 1973).

The MLR differs from normal antigen-specific activation reactions (see ch. 9) in that no prior sensitisation to antigens on the 'stimulator' cells is required. There is good evidence (e.g. from 'additive' stimulation experiments) that each allelic antigen coded for by the MLC locus contributes individually to the stimulation. How many cells respond to each allelic antigen? This is technically a difficult question to answer. If cultures are examined for activated cells at a very early stage only the early responders will be detected. On the other hand, by the time that all the cells capable of responding to the allogeneic cells have passed into an active blast stage, many of the early responders may be approaching their second or third division. Many other blasts and small lymphocytes (especially in mouse or chicken cell cultures) may have died. After taking account of all these factors (e.g. by sequential examination of mitotic figures from cultures exposed to pulses of ^3H -thymidine to detect first, second or third divisions by distribution of label on chromatids) a figure of the order of 2% was obtained for the number of rat lymphocytes responding across an Ag-B difference (Wilson et al., 1968). Since a similar proportion of the population would be separately responsive to lymphocytes bearing other histocompatibility isoantigens a considerable proportion of the population would be available only for this specialised reaction (on a one-cell, one-antigen theory) and therefore not able to take part in reactions to bacterial and other environmental antigens. It is possible, however, that the unipotentiality of the response to histocompatibility antigens of the species may not extend to antigens of other types and that a single cell may react to a bacterial antigen as well as to a cell bearing a histocompatibility antigen.

The high level of inborn reactivity in the MLR and the special involvement of species specific histocompatibility antigens single it out as a special

type of phenomenon. An immunological basis for the reaction is indicated by the fact that reactivity is specifically abolished by neonatally induced tolerance. Lymphocytes from rats injected intravenously at birth with bone marrow cells of another strain of rat are unresponsive in mixed cultures to lymphocytes of the tolerance-inducing strain while retaining reactivity to lymphocytes of rats of different Ag-B type (Schwarz, 1968; Wilson and Nowell, 1970). Not only were lymphocytes from tolerised rats unresponsive to the strain antigens concerned, but they were also incapable of being 'recruited' to the active state when present in cultures containing normal syngeneic lymphocytes (from an untreated rat) which had been stimulated with the allogeneic cells concerned.

Although reactivity in the MLR is an inborn capacity in rodents as well as in humans, the response of rat thymocytes to foreign lymphocytes has been shown to depend on the age of the animal. Thymus cells from newborn rats react less well than thymocytes of adult rats to an allogeneic cell stimulus. At birth, rat thymocytes do not react to xenogeneic (mouse) cells, but at seven days react equally well to rat and mouse lymphocytes (Knight and Thorbecke, 1972). Both the 'stimulator' and 'responder' cells in the rat thymus are part of the minor population of larger cells. Spleen cells of mice less than three weeks of age (before substantial T cell colonisation) are not stimulated by allogeneic cells (Adler et al., 1970). MLR reactivity declines in old age. Responses of C57B1 lymphocytes are maximal by 21 to 25 weeks of age and remain high at 90 to 94 weeks but are low by 150 weeks of age (Konen et al., 1973). Similar effects have been noted for CBA mice and for the shorter-lived A strain and NZB mice.

Rat and mouse lymphocytes are stimulated by some non-lymphoid cells. Blood lymphocytes from adult BN rats were shown by Main et al. (1971) to be markedly activated by allogeneic epidermal cells obtained by trypsinisation of skin of AC1 rats, the level of stimulation at five days of culture being of the order of ten times the response to the same number of AC1 leucocytes. The effectiveness of epidermal cells (from rats and mice including 'nude' mice) as stimulator cells for rat blood, lymph node and thymus cells has been confirmed (Lane, unpublished) but there is less agreement about the level of stimulation, results ranging from very low degrees of stimulation to the high levels reported by Main and his colleagues. The 'stimulator' capacity of mouse lymphomas is also very variable and the factors responsible have not been defined. Many rat tissue cells (thyroid, kidney, brain) produce little or no stimulation of allogeneic lymphocytes (Lane, unpublished). Lymphocytes

from rats and mice are stimulated by monolayers prepared from trypsin-dispersions of whole mouse embryos (Berke et al., 1969). The cells in this mixed population have not been identified but epidermal cells account for part of the stimulation (Lane, unpublished). One interesting facet of the embryo cell stimulation is that the potentially responsive population may be separated from the unreactive cells by allowing the lymphocytes to settle onto the embryo monolayer. Non-adhering lymphocytes showed a decreased ability to undergo sensitisation against embryo cells syngeneic to the ones used for adsorption, but were able to become sensitised against unrelated embryo cells of another H-2 phenotype (Altman et al., 1974; Wekerle et al., 1973). Whether H-2 antigens or M locus antigens are involved in these embryo monolayer reactions has not been determined.

7.1.6. Characterisation of the responding sub-population

There has been almost universal agreement that animals with depressed levels of T lymphocytes (usually brought about by neonatal thymectomy) show markedly diminished capacity to respond in a MLR (see Knight et al., 1973 for references). Children with a congenital absence of a thymus also lack MLR-responsive cells. Responder cells are present in diminished numbers in the blood even after adult thymectomy. It seems very probable that in the mouse only T cells may act as responder cells but both T and B lymphocytes possess 'stimulator' capacity (Harrison, 1973; Cheers and Sprent, 1973). Electrophoretically separated mouse spleen T cells respond to PHA and allogeneic lymphocytes but not to B cell stimulants (Andersson et al., 1973). However, PHA-responsive and MLR-responsive cells do not behave identically on density gradients prepared with albumin (Newlin, 1973). Thymus cells of some species e.g. rat, respond well in a MLR whereas those of other species (human, rabbit and some mice) respond poorly. It seems very likely that in most species responder cells are T cells but the certainty of this conclusion may well depend on the timing of the experiment both in regard to age of animal and time of examination of the MLR culture. Although T cell mitoses predominate in the early stages of a MLR culture, B cell mitoses are to be found at later times (Piguet and Vassalli, 1972).

The 'responder' T cells may be sub-divided into sub-populations which respond specifically only to allogeneic cells carrying a particular antigen on their surface. This follows from the many demonstrations of additive effects obtained in several species when a single 'responder' population is exposed

in one-way MLRs to several 'stimulator' populations. It has been shown that the increased activity of the 'responder' cells is positively correlated with the pool size when cells from two or more donors are used as stimulators (Han and Pauly, 1973). More direct confirmation of responsiveness within a population has been obtained from 'suicide' experiments. In these experiments the responding cells have been eliminated by one of several devices and the responsiveness of the surviving population to the original and new stimuli re-examined. Human lymphocytes activated by incubation with allogeneic lymphocytes for 24 hr and then exposed to ^3H -thymidine of very high specific activity (18 Ci/mmol, 5 $\mu\text{Ci}/\text{ml}$) for 18 hr lose the capacity to respond to the original stimulator cells but retain the capacity to respond to lymphocytes bearing different antigens (Salmon et al., 1971). When one of the lymphocyte donors had been preimmunised with keyhole limpet haemocyanin (KLH) their cells retained the capacity to respond to KLH after the hot thymidine treatment. The cells of both donors retained the capacity to respond to PHA. Similar results have been obtained by Zoschke and Bach (1971) using bromodeoxyuridine as the agent of lethal synthesis. The bromodeoxyuridine was added to one-way mixed cultures of human lymphocytes after DNA synthesis had begun. Those cells incorporating the light-sensitive DNA precursor were eliminated by exposing the cells to strong white light. The surviving lymphocytes were unable to respond to cells of the original donor but were responsive to other stimuli.

MLR reactivity appears to develop very early during embryonic life. Human foetal liver cells react to allogeneic cells as early as at 5 to 10 weeks of gestation (Meo et al., 1972; Carr et al., 1972). In older foetuses lymphocytes from blood, spleen and thymus were reactive whereas bone marrow cells were not. A significant reactivity was detected to all allogeneic lymphocyte 'stimulator' cells tested but reactivity against parental cells was about half that against cells of unrelated donors.

7.1.7. Relationship to homograft immunity, graft-versus-host reactions and lymphocyte transfer tests

At least three distinct loci are present within the major histocompatibility region of the chromosome in man viz. the Four locus, the MLC locus near to it and the LA locus. Much current work is concerned with the relationship of differences at these loci to graft survival. When MLRs were performed between nine HL-A identical unrelated donor/recipient pairs in one investi-

gation (Koch et al., 1971) two of the pairs did not stimulate but skin graft survival was no greater when grafts were exchanged between these than the other donors. Survival of grafts was, in fact, significantly longer (mean 11.4 days) than that of grafts between HL-A non-identical unrelated individuals (mean 10.0 days) but was much shorter than that between HL-A identical siblings (mean 18.9 days). This example serves to illustrate that histocompatibility differences are very incompletely defined by the three loci.

Rejection of a primary skin graft is considered to be principally a 'cell-mediated' process. It does not occur unless there is opportunity for the host lymphocytes to become sensitised to the graft antigens and this appears to be the primary immunological event. Sensitisation is accompanied by blast transformation and there is an obvious superficial resemblance to the morphological changes of the MLR. What is unclear is exactly how antigens are presented to the lymphocyte *in vivo*. Is it a whole live cell of the graft which activates the lymphocyte in a peripheral sensitisation reaction, or are the graft cells broken down and their antigens presented to lymphocytes after adherence to a macrophage or a lymphocyte in the draining node? The *in vitro* MLR is essentially a lymphocyte-lymphocyte interaction with a possible auxiliary role of the macrophage. The difficulty of obtaining a lymphocyte activation *in vitro* with cells other than lymphocytes as activator cells has already been discussed. This would suggest that the cellular events which make up the afferent arc of a homograft reaction may not be described by a simple direct lymphocyte activation such as is observed in the MLR. Homograft immunity is markedly enhanced by immunisation whereas the MLR is little affected. The MLR is easily demonstrable only when a major histocompatibility barrier is crossed whereas homograft sensitisation occurs even when only minor antigenic differences exist e.g. the MLR is negative in mixture of cells from HL-A identical siblings but skin grafts exchanged between these siblings are always rejected. The end result of homograft sensitisation is the destruction of the graft cells. It has never been shown that active destruction of lymphocytes occurs in a normal MLR, although cells from lymphoid cell lines and many other cell types are killed by activated lymphocytes produced by a mixed cell stimulation. There is really no good reason for regarding the MLR as an *in vitro* counterpart of a typical homograft reaction.

There is much closer resemblance of the MLR to the graft-versus-host reaction. Lymphocytes injected into a tolerant recipient whose cells bear antigens not present on the donor lymphocytes (e.g. from a parent to an F_1

hybrid) produce profound changes in the tissues of the recipient. The principle manifestations of the reaction are to be seen in the lymphoid tissues of the recipient, the most obvious being a lymphoid cell proliferation partly of host and partly of donor cell origin with subsequent tissue destruction. The initial stages are suggestive of an uncontrolled mixed lymphocyte interaction leading to a total disorganisation of the lymphoid tissue and unusual patterns of migration of lymphocytes, together with tissue destruction by the activated lymphocytes. The peculiar specificity of the reaction (intensity proportional to genetic disparity within the species but allogeneic reactions more marked than xenogeneic) resembles that of the MLR and both appear to be basically lymphocyte-lymphocyte interactions. Immunisation, as in the MLR, has little effect when a major strain difference is involved but enhances a reaction across a very weak histocompatibility barrier. Recent estimates of the number of cells responsive to a single major antigen on an allogeneic lymphocyte in the MLR (about 2% of the population) are very similar to the proportion of an injected lymphocyte population calculated to be responsive in a graft-versus-host reaction (Nisbet et al., 1969). As in the MLR, cells active in a GVH reaction in a particular strain combination may be specifically removed by plating the lymphocytes over embryo monolayers. For example, the 4–6% of lymphocytes of H-2^d genotype removed by interacting them with monolayers of H-2^k or H-2^b derivation must have included most of the cells capable of producing a GVH reaction as supernate cells had markedly depressed GVH activity only in the specific combinations concerned (Lonai et al., 1973).

Mouse spleen cells active in the MLR and GVH reactions separate out together in methods based on velocity sedimentation or density gradient techniques (El-Arini and Osoba, 1973b). However, careful comparative studies have shown that there is not always a close relationship between the graft-versus-host reaction and the MLR. For example, it has been reported that both rat and mouse thymus cells induce splenomegaly when injected into neonatal mice but not when injected into neonatal rats. Yet both species of thymocytes were equally responsive to allogeneic and xenogeneic cells in the MLR (Knight and Thorbecke, 1971). Also the strong MLRs of the BALB/c-DBA/2 combination are not paralleled by strong GVH reactions.

When lymphocytes from a normal adult guinea pig are injected intradermally into another normal guinea pig an inflammatory reaction of delayed onset occurs which reaches its peak about 2 days after the injection (Brent and Medawar, 1963). A similar reaction occurs in humans. The

evidence for the guinea pig reaction being a graft-versus-host reaction is two-fold (Brent and Medawar, 1964). Firstly, the immunological contribution of the hosts may be severely reduced or abolished by irradiating them with 500–1500 r whole body irradiation without the intensity of the reaction being diminished. Secondly, the reactions between two unrelated inbred strains of guinea pigs, Wright strain 13 and Heston and their F₁ hybrids were as the theory would require. Heston and strain 13 lymphocytes excited reactions in F₁ (Hes × 13) but F₁ lymphocytes from F₁ hybrids gave rise to smaller reactions in Hartley guinea pigs than lymphocytes of either parental strains.

Although these observations seem to point conclusively to a graft-versus-host mechanism a similar skin reaction in hamsters behaves more like a MLR at the skin site than a local graft-versus-host reaction (Ramseier and Streilen, 1965). Lymph node cells obtained from CB hamsters sensitised to MHA hamsters were injected in the skin of normal and X-irradiated MHA hamsters. The strongly positive reactions in normal hosts were markedly diminished after X-irradiation. Since the skin remained viable and antigenically intact after the irradiation the skin reaction did not appear to be explicable as simply a reaction between the activated lymphoid cells and isoantigens in the host's skin. Severe cutaneous reactions could be obtained when mixtures of CB and MHA lymph node cells were injected. Yet the response to either component of the mixture was insignificant. Similar reactions were obtained when mixtures of lymphocytes from unsensitised hamsters were injected. Positive reactions were also produced by homologous mixtures of lymph node cells from rats, mice and guinea pigs and from mixtures of human leucocytes whereas there was never a significant reaction to cell suspensions originating from a single donor. Among the mouse strain combinations tested the greater was the antigenic disparity between the strains the greater the severity of the cutaneous reaction provoked. Lymphocytes from specifically sensitised donors were no better than those from normal donors. Antibodies played no part in the reaction. Phylogenetically unrelated lymphocytes are inactive in this test but mixtures of lymphocytes from closely related species (e.g. goat and ox) do produce a dermal reaction in the skin of sheep (Lafferty et al., 1972). It is clear that a good case could be made for the proposition that the lymphocyte transfer test and the MLR are closely related.

Other *in vivo* reactions also appear to be a direct consequence of a MLR *in vivo*. For example, Elkins (1971) showed that parental lymphocytes intro-

duced under the capsule of a parental kidney which had been previously grafted onto an F_1 hybrid actually produced kidney damage by a local cytotoxic reaction. The parental lymphocytes had evidently been activated by encountering F_1 hybrid lymphocytes in the parental kidney. Parental lymphocytes did not, however, damage the kidney of an irradiated F_1 hybrid. Damage to renal parenchyma occurred only when conditions were chosen to produce a local mixed lymphocyte activation (Lindquist and Guttman, 1971).

Lymphoblasts generated in the MLR have long been known to possess cytotoxic properties when mixed with other cells. Lymphoblasts from human MLRs will lyse Chang cells, Burkitt lymphoma cells and mouse lymphoma cells whereas they will not lyse human or sheep red cells or human fibroblasts. Early attempts to detect some HL-A based specificity for the killing reaction were unsuccessful. In other mixed cell reactions specificity has been readily shown. This is so for the mouse-embryo-sensitised lymphocytes and for mouse lymphocytes sensitised to certain lymphomas (see Ling et al., 1974 for references). In the normal MLR itself there has never been any evidence for one population of lymphocytes killing the other. Viability tests show little difference between survival of lymphocytes in mixed and unmixed cultures over long periods. If lymphocytes of one of the donors are pre-treated with PHA (followed usually by a 3-day incubation to convert them to blast cells, although this is unnecessary) it can be shown that they are susceptible to killing by activated cells generated in the MLR. There is considerable autochthonous killing in most experiments but conditions can be arranged such that there is some degree of specificity of killing with preferential killing of cells bearing the HL-A antigens of the 'stimulator' cells (see ch. 10 for further details). It is claimed that although serologically detectable antigens do not directly affect the stimulation stage of the MLR they do determine the specificity of killing at the effector stage. In mice, too, it has been reported that although mitotic activation may occur in MLRs between certain strains not differing in H-2 antigens the generation of cytotoxic cells during the MLR is dependent upon the presence of cells bearing foreign H-2 antigens. However, it would be wrong to overinterpret this evidence. It has been shown that target cells which have been stripped of the supposedly target H-2 antigens by 'capping' are nevertheless still sensitive to specific killing by sensitised T lymphocytes from immunised animals (Edidin and Henney, 1973).

Although both the responsive cells in the MLR and the cells which give

rise to cytotoxic lymphoblasts are known to be T lymphocytes they are not necessarily identical populations. There are T cells in thymus, spleen, lymph nodes and blood which are responsive to allogeneic lymphocytes but they differ in behaviour. In the thymus only a minority population (the larger cells) is responsive or capable of acting as 'stimulator' in the MLR and under most conditions the activated cells do not develop cytotoxic properties. On other evidence the T cells may be divided into two functionally distinct sub-populations, T_1 and T_2 (Cohen and Howe, 1973). The T_1 sub-population is thought to be present in high concentrations in spleen and thymus, to be largely non-recirculating, rapidly depleted by thymectomy, and to express the θ antigen in large amount. The T_2 sub-population is thought to be present in high concentration in lymph nodes and peripheral blood. T_2 cells are thought to recirculate, to be slowly depleted by adult thymectomy, and to express little θ antigen. In the MLR it is thought that the T_2 sub-population contains the cells which proliferate and the T_1 sub-population the effector cells.

From the point of view of tissue typing it is less important to know which antigens determine the course of the MLR than to know which are of predictive value in graft survival. Both HL-A and M-locus antigens are related to histocompatibility but our knowledge of both typing systems does not as yet provide more than very incomplete information about the likely fate of a tissue graft. There has recently been some feeling that antigens of the Four locus and the genetically related MLC locus are of the most importance. There appears to be a better correlation of graft survival with the MLR than was once thought (Cochrun et al., 1973). These developments are reviewed from time to time in the transplantation journals. The most informative experimental animal has been the pig. In a series of 30 renal allografts, siblings were bilaterally nephrectomised and given no immunosuppressants (Bradley, 1974). Pigs which were typed as identical in the MLR and by serological tests did not reject the grafts. MLR non-identical, serologically compatible siblings showed protracted rejection (71 to more than 303 days) and MLR identical, serologically non-identical siblings also showed protracted rejection (68 to greater than 90 days).

7.1.8. Stimulation by autochthonous cells

A perfectly physiological cell-cell interaction of MLR type occurs between

the thymocytes of mice 2–3 days old (responder cells) and spleen cells ('stimulator', possibly B cells) of more mature mice of the same strain (Howe, 1973). This syngeneic MLR can be regarded as a final stage in the differentiation of T lymphocytes prior to encountering foreign antigens. Other syngeneic activations are difficult to interpret (e.g. syngeneic rat epidermal cells and lymphocytes), but may partly be due to 'feeder layer' effects. When lymphocytes are activated by any agent and then X-irradiated and added to syngeneic resting lymphocytes a low degree of activation frequently occurs. It is sometimes referred to as 'contagious activation' and may mimic 'recruitment' in antigen-stimulated cultures.

A remarkable syngeneic activation takes place when rat or mouse lymphocytes are grown over syngeneic embryo monolayers in the absence of serum or any other foreign proteins. The activated cells acquire cytotoxicity to the syngeneic cells. Moreover, when injected into syngeneic animals they evoke a considerable reaction (? recruitment) in the draining lymph node (Cohen and Treves, 1974).

A biological role could be ascribed to the MLR if it could be shown that normal lymphocytes were activated by contact with abnormal cells in the body and that this led to the destruction of the cells concerned. This could occur not only by the complex processes involved in acquired immunity e.g. to tumour antigens, but by a simpler process involving only T cells activated in a local MLR. Such an event would be most likely to be initiated by cells bearing abnormal antigens of the type coded for by the MLC locus. Clearly such antigens would be more likely to be present on abnormal lymphoid cells than on cells of other tissues.

There are several examples of the existence of such a mechanism. It has been shown that cells from an LCL will activate in mixed culture the normal lymphocytes of the original donor and that these activated cells will destroy the autochthonous LCL cells. If abnormal cells of LCL type were formed in the body (e.g. during infectious mononucleosis) they would almost certainly be eliminated by this sort of reaction (see Ling et al., 1974 for references). Since the abnormal cells may be activated B lymphocytes which have been 'transformed' by EB virus it is possible that the many other viruses which grow in T or B lymphocytes may also produce abnormal cells capable of evoking an autochthonous MLR.

Because of the effectiveness of this surveillance mechanism it is unlikely that lymphoid cells bearing abnormal MLC locus antigens could ever survive in an individual with normal T cell reactivity. This would suggest that sur-

viving abnormal lymphocytes (e.g. leukaemic lymphocytes) do not possess abnormal MLC locus antigens or that for some reason the host has developed tolerance to them. It has been shown that abnormal lymphoid cells may sometimes stimulate the normal lymphocytes of an identical twin or HL-A identical sibling (Han and Wang, 1972; Mavligit et al., 1973) which suggests that 'blind spots' in MLR reactivity may occur. It is also possible that leukaemic and other abnormal lymphocytes may sometimes lack the normal lymphostimulatory property of B and T lymphocytes.

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Activation by antibodies

Because they believed the mitogenicity of PHA to be due to its leuco-agglutinating property Grasbeck et al. (1964) predicted that leucocyte antibodies should also be mitogenic. Several antisera prepared by immunising rabbits with human leucocytes were indeed shown to activate human lymphocytes in culture. The degree of stimulation achieved far exceeded the mild effect produced by normal rabbit serum (Elrod and Schrek, 1965).

8.1. Heterologous leucocyte antisera

Mitogenic antisera are readily produced by immunising rabbits with whole human leucocytes. A course of five i.v. injections at one week intervals with 10^9 thrice-washed leucocytes from sedimented heparinised blood has been found to be satisfactory (table 8.1). Sera from rabbits which were immunised twice weekly for five weeks with the leucocytes from 5 ml of blood were active at a dilution of 1 in 100 in culture (table 8.2). An active antiserum has also been prepared simply by injecting a rabbit on three occasions with large numbers of tonsillar lymphocytes. Antisera prepared against HeLa cells or human red cells had little or no mitogenic activity (table 8.2). Activity found in antisera using platelets or whole serum as immunogens could well have been due to contamination of these preparations with leucocytes or leucocyte fragments. Antisera prepared with disrupted leucocytes are in general inferior to those prepared with whole leucocytes but an antiserum which stimulated incorporation of $^{3}\text{H-TdR}$ into human lymphocytes has been prepared by immunising rabbits with a membrane glycoprotein preparation from papain-solubilised human lymphocyte membranes. An antiserum prepared using a similar membrane preparation from HeLa cells was not stimulatory although it was cytotoxic to both

Table 8.1

The letters A-F refer to the cell source and the numbers preceding them to the number of cultures in which they were used. Positive = at least 5% mitoses and 10% blasts. Negative = not more than 0.5% mitoses and 0.5% blasts. Culture period = 3-4 days with addition of colcemid 24 hr before the end of the incubation. (After Grasbeck et al., 1964.)

Serum from	Leucocyte	Haemag-	Leucoag-	Response			
	donor for immu- nisation	glutinin titre	glutinin titre	Positive	Inter- mediate	Negative	Toxic
Seven variously immunised rabbits	—	—	—			ADE	A
Five rabbits	ADG	1:2 ⁸	1:2 ⁹			D	E
immunised	ADG	1:2 ⁹	1:2 ¹⁷	1E			7E
with	A	1:2 ¹⁰	1:2 ⁹	5A 3B	3C	2D	E
leucocytes	A	1:2 ¹⁴	1:2 ⁹	3C 1D			1D
	A	1:2 ⁹	1:2 ⁷	4A 3B	2C	1D	

lymphoid and HeLa cells (Einstein et al., 1971). The blastogenic activity of the antiserum to lymphoid membranes was absorbed out completely with Raji lymphoid cell line cells.

8.1.1. Characterisation of the active component of the antiserum

Active antisera always contain haemagglutinins and leucoagglutinins. Absorption with packed erythrocytes was shown by Grasbeck et al. to cause a moderate decrease in both agglutinin titres (some leucocytes may be present in the packed erythrocytes) whereas the mitogenic potential was unaffected. Absorption with leucocytes, on the other hand, reduced the titre of the mitogenic factor and was accompanied by total, or almost total, disappearance of leucoagglutinating power while the haemagglutinin titre was unaffected.

Lymphocytes respond quickly to leucocyte antiserum, the time of maximal activity being comparable to that with PHA, whereas lymphocytes treated with tuberculin or homologous leucocytes respond more slowly. Activation probably begins as soon as antibody becomes fixed to the cells. The combination of antibody with its antigen is thought to occur very rapidly so that

Table 8.2

Characterisation of rabbit antisera. (From Holt et al., 1966.)

Antiserum	Immunogen	Reciprocal titre against			% blasts at	
		Red cells (Group O)	Red cells coated with IgG	Leuco- cytes	1/100	antiserum concn. 1/6
A1	Spleen cells (frozen)	800	640	200	2	—
5243	Leucocytes (pooled, frozen)	1600	20480	100	0.5	2.0
A29	HeLa	12000	4	20	0.5	1.0
B1	Serum dust (extruded platelets and erythrocytes)	400	160			0.8
S1	Washed platelets (ultrasonicated)	100	4			0.5
L1	Whole platelets (haemophiliac)	100	640			5.0
P11	Whole platelets	1600	640			2.0
A	Erythrocyte membranes	1600	160			1.0
Bu	Washed blood cells	3200	640		0.5	1.0
5317	Proteus					3
A13	none	4	4	4	—	1.0
A17	none	4	4	4		0.8
501	Y leucocytes	6400	160	200	22-41	—
502	S leucocytes	12000	2560	50	4-8	—
503	P. R. leucocytes	32	40	100	10-15	
504	J. leucocytes	32	5	100	10-18	
510	M. D. leucocytes	512	5	200	8-16	
5603	C. H. leucocytes	256	5	200	8-14	
511	IgM	4	5		0.5	1.0
Poly- valent	Whole serum	256	640		—	1.0
A62	IgM	128	2560		0.5	1.0
A66	IgM	64	1280			
6783	IgG	512	71800		0.5	0.5
A14	IgG	512	81920		0.5	1.0

evidence of activation ought to be obtained after exposure of lymphocytes to the antiserum for only a brief period. Definite activation can certainly be demonstrated in cells cultured after exposure to antiserum for only 10 min but it is difficult to obtain a high level of activity of washed cells after a pulse-stimulus of less than one hour's duration.

8.1.2. Effect of complement on the reaction

Cells cultured in the presence of antiserum incorporate increasing amounts of thymidine as the concentration of antiserum increases providing that complement is excluded. Fig. 8.1 depicts the DNA synthesis of human lymphocytes sensitised with antibody and cultured in the presence (dotted line) or absence (solid line) of complement. Complement was removed

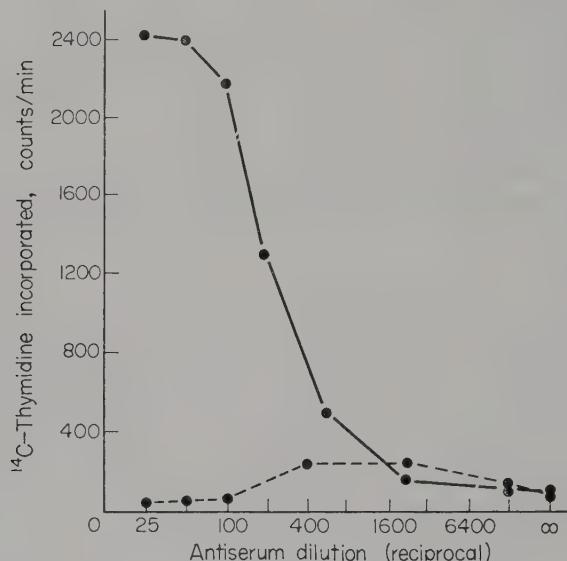


Fig. 8.1. The DNA synthesis of leucocytes cultured with no. 501 leucocyte antiserum in the presence and absence of complement. Washed human leucocytes (3×10^6) were suspended in Eagle's medium (2 ml) containing the diluted antiserum. After 30 min autologous raw or inactivated serum-gelatin (1 ml) was added to the cells which were then incubated at 37°C for 4 days. Broken line: cultures containing raw serum-gelatin. Unbroken line: cultures containing inactivated serum-gelatin. Reprinted from Holt et al., 1966.

(from both the antiserum and the human serum used to suspend the cells) by heating at 56 °C for 30 min. Washed human leucocytes (3×10^6) were suspended in Eagle's medium (2 ml) containing the diluted antiserum. After 30 min autologous raw or inactivated serum-gelatin (1 ml) was added to the cells which were then incubated for 4 days. ^{14}C -TdR (0.12 μCi) was added on the day before harvesting the cells. In the presence of complement increased thymidine incorporation was detectable at low levels of antiserum but the effect was eliminated by cell destruction at high antiserum dosage. The relative cytotoxic and stimulatory effect of a given antiserum in the presence of complement was markedly dependent upon the technique employed. When the antiserum was added to the cells for 30 min before fresh autologous serum was added marked destruction of cells occurred and a very low overall rate of DNA synthesis was observed (fig. 8.1). However, addition of the same antiserum to cells not separated from their own fresh plasma induced strong activation even at high antiserum dilutions although toxic effects still supervened at high antiserum levels. Fig. 8.2 shows that the DNA synthesis of the cells of two donors (S and P) cultured for 4 days with leucocyte antiserum. The appropriate amount of antiserum 501 or 502 was added directly to the leucocytes which were suspended in fresh autologous serum-gelatin and finally medium was added.

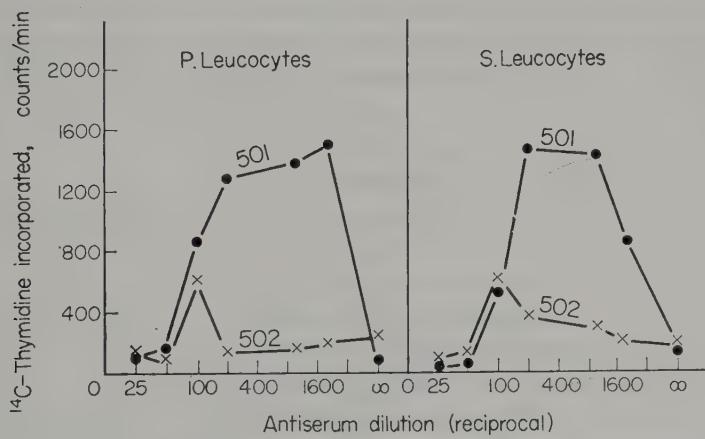


Fig. 8.2. The DNA synthesis of leucocytes of two donors (S. and P.) cultured with leucocyte antiserum. The appropriate amount of antiserum 501 and 502 was added directly to the leucocytes suspended in fresh autologous serum-gelatin. Culture period: 4 days. Reprinted from Holt et al., 1966.

Lymphocytes which have been coated with antibody are lysed immediately upon the addition of complement, whereas the cells appear to be able to resist low concentrations of antibody in the presence of complement added at the same time, presumably because a high surface concentration of antibody is never achieved. Whichever technique is employed the pattern of activation is always characteristic for a given antiserum.

Sensitivity to complement-induced lysis is retained for some time under the conditions of culture employed. To cultures containing 3×10^6 leucocytes and 0.5 ml of inactivated (decomplemented) serum and various concentrations of antiserum, a source of complement (0.5 ml of raw human serum-gelatin) was added at intervals between 15 min and 20 hr. The cultures were then continued for a total of four days and the incorporation of ^{14}C -TdR measured for the final 16 hr of this period. There was a steady loss of sensitivity to complement over this period but some cell damage occurred even when complement was added as long as 20 hr after the antiserum. Slightly different results were obtained when the cells were separated by centrifugation before addition of complement. Table 8.3 shows the results of an experiment in which cells were exposed to antiserum at a concentration of 1 in 30 under complement-free conditions. At the time indicated the cells were spun down and suspended in 0.1 ml of raw or inactivated autologous serum followed by 0.9 ml of Eagle's medium. The cells were further cultured until 72 hr, ^3H -TdR being present for the final 24 hr of culture. A substantial proportion of the antibody must have become cell-fixed during the one hour's incubation period. By 16 hr a good part of the lethality of the complement had been lost.

Table 8.3

Period of exposure to antiserum	^3H -TdR uptake 48–72 hr after culture in	
	Raw serum	Inact. serum
1 hr	487	9,200
16 hr	8,300	14,800
40 hr	10,600	15,300
72 hr	—	20,800

It is interesting to compare these findings with those of Oda and Puck (1961) using HeLa cells. An experiment which showed that HeLa cells, exposed to antiserum in the absence of complement and incubated, progressively lose their susceptibility to complement lysis was performed as follows: 100 cells were added to each Petri dish together with 1.5% of heated antiserum in inactivated growth medium. The plates were incubated at 37 °C for one hour to permit the cells to attach to the glass. The medium was then removed, the cells washed, new complement-free growth medium without antiserum was added and the plates reincubated. At various time intervals (up to 5 hr and a few samples beyond this time) complement was added and the incubation was continued for nine days. The number of colonies developing was counted and expressed as a % of the original cell inoculum. Survival was less than 10% when complement was added at 1 hr or less. Between 2 and 2½ hr 50% of the cells survived rising to approximately 70% at 5 hr. The reaction responsible for loss of sensitisation at 37 °C had an induction period of approximately 45 min before significant loss of sensitisation occurred.

8.1.3. Activity of immunoglobulin fractions and fragments

Confirmation of the antibody nature of the active substance has been obtained by locating the active substance in an immunoglobulin fraction. An active leucocyte antiserum (2 ml) was dialysed against phosphate buffer pH 7.5, 0.01 M containing 0.0175 M NaCl. The dialysed serum was applied to a 10 × 1 cm column of DEAE-Cellulose (Whatman C₂ grade). A 'break-through' fraction (I) corresponding to slow IgG globulins was washed through the column with the starting buffer. A second fraction (II) was eluted with the same buffer. It contained 'fast' IgG globulins. A third fraction (III) containing IgA was eluted with phosphate buffer pH 7.5, 0.01 M containing 0.3 M NaCl (IgM fraction). The fractions containing the protein in question were combined, concentrated to 2 ml at 4 °C by ultrafiltration, and filtered in a Hemmings filter to sterilise. Significant activity was found in both IgG fractions but not in the IgA or IgM fractions (table 8.4) although these contained appreciable concentrations of haemagglutinin.

Other antisera have shown mitogenic activity in the IgM fraction and there is no reason why leucocyte antibodies of all immunoglobulin classes should not be active. Antigen-binding fragments of leucocyte antibodies also have some activity. Divalent pepsin fragments, F(ab')₂ have been shown

Table 8.4
 Stimulatory activity of fractions of leucocyte antisera.
 P = phosphate buffer.

Fraction	Ig	¹⁴ C counts per min		R.B.C. titre
		1 in 50	1 in 20	
DEAE I (‘breakthrough’)	IgG (slow)	388	3260	1/400
DEAE II (0.01 M P pH 7.5 0.0175 M NaCl)	IgG (fast)	140	365	0
DEAE III (0.01 M P pH 7.5 0.08 M NaCl)	IgA	104	88	1/24
DEAE IV (0.01 M P pH 7.5 0.3 M NaCl)	IgM	67	109	1/200
Controls	—	20–100	—	—

to possess agglutinating capacity and to be able to stimulate ³H-uridine and ³H-thymidine uptake (Woodruff et al., 1967) under conditions in which the univalent fragments (Fab') were inactive. Another study also showed that divalent but not univalent fragments of a 7S fraction of an ALS were active and that the univalent fragments attached to the cell and could exert a ‘blocking’ effect (Fanger et al., 1970).

8.1.4. Specificity of heterologous leucocyte antisera

Heterologous leucocyte antisera are known to possess little or no capacity to discriminate between the leucocytes of different human donors as judged by leucoagglutination or cytolytic tests. In tests with five leucocyte antisera against lymphocytes of several donors it was likewise found that the relative stimulating capacity of the antisera was the same for all donors (Holt et al., 1966).

Antisera prepared by immunising rats and guinea pigs with whole rabbit lymphocytes were not good mitogens when tested against rabbit peripheral lymphocytes in culture although lymphoagglutinating and lymphotoxic

antibodies were detectable (Knight and Ling, 1967). Antisera prepared by immunising rabbits with an unfractionated homogenate of rat lymph node cells or with a cytoplasmic fraction were good stimulators of rat lymphocyte DNA synthesis (Warnatz et al., 1972). An antiserum to a microsomal fraction was less lymphostimulatory in spite of its high level of cytotoxic antibody. Antisera to fractions of PHA-stimulated lymph node cells were less stimulatory than antisera to unstimulated cells. An ALS prepared in rabbits against guinea pig lymphocytes has been shown to stimulate guinea pig lymph node cells (Foerster et al., 1969). It was shown that cell-bound immunoglobulin was not involved in the stimulation.

8.1.5. Effect of rheumatoid factor on sensitised lymphocytes

Rheumatoid factor is a macroglobulin found in the serum of some individuals, especially of patients with rheumatoid arthritis, which has the property of combining with altered gamma globulin of several species including man and rabbit. In the presence of rheumatoid factor the attachment of rabbit IgG antibody to the leucocyte surface will be followed immediately by the attachment of rheumatoid factor to the fixed antibody. Areas of the lymphocyte surface to which rabbit antibody is attached will now be enveloped by a much larger mass of protein which might augment or inhibit the activation. In practice some inhibition of ^3H -TdR incorporation into lymphocyte DNA occurred at low antiserum concentrations and some slight enhancement at intermediate antiserum concentrations in cultures containing rheumatoid factor euglobulin, compared with otherwise identical cultures from which rheumatoid factor was omitted. Although rheumatoid factor did modify the effect of antibody-induced lymphocyte activation the results would not suggest that lymphocyte activation by antiserum is simply the result of depositing a large amount of protein on the cell surface.

8.1.6. General properties of antibodies to cell antigens

The toxic effects of antibody and complement on mammalian cells have been reviewed by Green and Goldberg (1960) and Bitensky (1963). The combined actions of antibody and complement are lethal to most cell types studied. Blebs soon appear on the surface of affected cells which then become uniformly swollen by the entry of water. The swelling affects the mitochondria, the endoplasmic reticulum and the perinuclear space.

Studies with ascites tumour cells have shown that there is a rapid equilibration of small molecules between the cell interior and the medium. K^+ is lost from the cells and Na^+ passes rapidly into the cells. The cells quickly lose phosphates, amino acids and ribonucleotides. The way in which macromolecules such as protein and RNA appear in the medium is less certain. The fact that succinate metabolism was unaffected after amino acid turnover and glycolysis of the cell had been abolished suggested that large organelles such as mitochondria were retained within the cell and that immune cytolysis resulted in a leakage of intracellular macromolecules through a stretched but morphologically intact cell membrane. Other workers take the view that some rupture of the cell membrane must occur.

Complexing of antibody with soluble antigen in the growth medium does not appear to damage cells. Only antibody which is directed against cell surface sites may produce cytotoxic or activating or growth inhibitory effects. Lysis of nucleated cells by the action of antibody and complement occurs by a reaction similar to that which causes lysis of red cells. Thus holes have been detected by electron microscopy in the membranes of Krebs ascites tumour cells which have been lysed by rabbit antibody and complement which are similar to the holes produced in sheep erythrocytes with Forssman antibody and complement (Humphrey and Dourmashkin, 1965). Nucleated cells, however, appear to have some measure of resistance to lytic antibody. If complement is limiting, or for some other reason the cell resists lysis, other effects on cell metabolism may be observed e.g. lysosomal activation. These effects are consequential on cell surface changes and cannot be reproduced by adding antibody directly to isolated organelles.

8.1.7. In vivo effects of lymphocyte antibodies

The effects of injecting anti-lymphocyte serum (ALS) or isolated immunoglobulin fractions containing lymphocyte antibodies, into mice or other animals, including humans, have received a good deal of attention chiefly in connection with the prolongation of the life of grafts (see reviews by James, 1967 and Lance et al., 1973). The mode of action of ALS is still uncertain. The immunosuppressive properties of antisera do not correlate with their lympholytic titre in vitro in the presence of complement. This is not surprising since it is known that mononuclear cell killing of antibody-primed cells occurs at an antibody titre well beyond that at which complement-induced lysis ceases to be detectable. Moreover, many antibodies which

are non-lytic in vitro in the presence of complement (e.g. blood group iso-agglutinins) are very effective in priming cells for destruction in vivo. What is more surprising is that a persistent lymphopenia is not regularly produced by the administration of ALS to mice. This may be explained by replacement of long-lived lymphocytes which are initially eliminated by the ALS by a shorter-lived population. It is hard to believe that lymphocyte destruction does not play an important part in the immunosuppressive role of ALS. However, other explanations of its effects have been proposed e.g. competitive tolerance and competitive activation. 'Blindfolding' of lymphocytes, another hypothesis, is unlikely to be important since antibody does not remain on cell surfaces for more than about 24 hr, yet the effects of ALS are evident many weeks after administration. 'Sterile' or 'competitive' activation has been considered a possible explanation because of the known mitogenic effects of ALS in vitro, it being assumed, on doubtful evidence, that the progeny of ALS-activated lymphocytes would be unable to react to antigens to which the original population was responsive.

Antisera prepared against tissue cells of the species concerned (e.g. mouse skin) cross react with lymphocytes in cytotoxic effects and have been used as immunosuppressants. Antisera to cultured human lymphoid line cells (LCL) have also been shown to possess cytotoxic and immunosuppressive properties. A comparison of antisera to LCL and pooled fresh human lymphocytes showed that cross-reactivity was found; antibodies directed against one or other of the cell types were also present (Perper et al., 1970). Lymphocyte-specific, LCL-specific and common antigens were detected on each cell type. Absorption with liver reduced the cross specificity of each antiserum.

8.1.8. 'Blocking' properties of lymphocyte antibodies

Lymphocyte antibodies are very potent 'blocking' agents for preventing the responses of lymphocytes to many stimuli in culture. Antibodies which attach to the cell surface, without activating a lymphocyte, can often be detected by this means. Rabbit antisera to human lymphocytes markedly inhibit the MLR and similar inhibition of the rat MLR occurs when a heterologous ALS is added. Alloantisera to HL-A and other antigens are also suppressive in the MLR (see ch. 7) and it has been suggested that the enhancing property of an alloantiserum parallels its MLR-inhibiting titre (Gordon et al., 1971). It is obviously necessary that the cells 'blocked' by the antiserum should possess the antigens concerned but it cannot be

concluded from this type of experiment that the sites 'blocked' are those concerned in the stimulation. Antibody to any surface site appears to exert a blocking effect. An alloantiserum examined by Revillard (1973) contained a 7S antibody which inhibited antigen-induced proliferation as well as the MLR. It differed from a similar inhibition produced by heterologous antibody in that the inhibition could be reversed by replacing the culture medium after one or two days of culture. In the mouse MLR, too, alloantisera to surface antigens are inhibitory. An AKR anti-C3H θ serum completely abrogated a spleen-blood cell MLR (Tyan and Ness, 1972). Four allo-immune plasmas investigated by Buckley et al. (1972) blocked the responses of human lymphocytes to PHA-P, PWM, Candida antigen and mitomycin-treated allogeneic leucocytes. The plasma contained no antibodies to PHA-P, PWM or Candida. The 'blocking' and 'cytotoxic' agent survived heating of the plasma at 56 °C for 30 min and was present in greatest concentration in the IgG fraction. The inhibitory effects appeared to be due to a direct inhibition of the functioning of the responder cell. Cytotoxicity was associated with ability to fix complement (IgG₁ and IgG₄).

8.1.9. Homologous leucocyte antisera

Although human isoantisera to HL-A and other leucocyte antigens attach to the surface of lymphocytes, as evidenced by the 'blocking' and cytotoxic properties of these antisera, there are no reports that they are mitogenic. In the mouse, antisera to defined surface antigens on lymphocytes, such as the θ antigen, have not been shown to produce activation. In the rabbit, which appears to be the only species systematically studied, alloantisera with mitogenic properties have been produced with difficulty (Knight and Ling, 1967). Of the serum samples taken from nine recipient rabbits after a course of five injections (total of 150×10^6 lymphocytes) from an unrelated donor rabbit only one showed definite blastogenic activity in cultures of donor lymphocytes (table 8.5). Activity was detectable at a concentration of 1/100 and 1/10 in cultures containing decomplemented serum. After seven injections two other recipient rabbits produced active antisera. The most potent antiserum produced about 30% blast cells by the time of harvesting. When tested against the lymphocytes of eight other rabbits antiserum 4 was active in two cases, weakly active in three others and negative in two. There was a partial correlation with cytotoxic activity. Antisera to surface antigens evidently do not always produce activation.

Table 8.5

DNA synthesis of donor lymphocytes incubated with recipient serum. Each figure represents the mean counts per minute in triplicate cultures of donor cells in the presence of recipient serum before and after injection of leucocytes in each of the 9 combinations. The antiserum dilution in the cultures was 1/100. NZW - New Zealand White; NZR - New Zealand Red; DC - Dutch Cross.

Pair	Donor	Recipient	Counts per minute with serum after injection							
			0	3	4	5	6	7	8	10
1	NZW	NZW	132	127	74	133				
2	DC	NZW	50	98	34	88				
3	NZW	NZW	131	105	60	50				
4	DC	NZW	45	50		532	116	15,200		24,000
5	DC	NZW	157	81		81	120	60	129	119
6	DC	NZW	50	111		14	57	43	0	59
7	NZW	NZR	126	100		99	43	240*		
8	NZW	NZR	44	126		38	49	13	67	91
9	DC	NZR	50	70		46	182	800	38	3,000

* Donor animal 7 died at this point and further immunisations were not possible. (After Knight and Ling, 1967.)

8.2. Antisera to determinants on immunoglobulins and other serum proteins

Immunoglobulin determinants are readily demonstrable on the surface of B lymphocytes of man, mouse and other species and the combination of antisera with these determinants might well be expected to activate these cells. There is, however, no agreed body of opinion that antisera to immunoglobulin light or heavy chains, prepared across species, produce lymphocyte activation and there is doubt whether some of the positive results reported could be accounted for by contaminating antibodies to other cell surface antigens. One of us (N.R.L.) has consistently obtained negative results with rabbit and sheep antisera to human IgG, IgM, IgA and IgE and to light chains. Others have obtained responses with anti-gamma sera (Adinolfi et al., 1967) and monkey antisera to human immunoglobulin (Oppenheim et al., 1969). Definite but weak lymphocyte stimulation by an anti-F(ab')₂ anti-serum which had been carefully checked for contaminating antibodies to

non-immunoglobulin cell membrane antigens has been reported by Frøland and Natvig (1973). All investigations so far have been performed on impure blood cell preparations containing only a small proportion of B cells and a more decisive answer to this question will be obtained when pure B lymphocyte populations are tested.

The survival of lymphocytes in media containing antisera to human immunoglobulins is comparable to that of lymphocytes cultured in normal rabbit serum, indicating that if antibodies do attach to the lymphocyte surface they are not cytotoxic even over a long period. According to one report, however, antisera to whole IgG or to the Fc piece lyse lymphocytes,

Table 8.6
Relationship of lymphocyte transformation by antiallotype serum to allotypes of rabbit IgG. (After Sell and Gell, 1965.)

Serum	Allotypes of donor cells							
	1, 3, 4, 5	1, 3, 5	2, 4	2, 3, 4, 6	3, 4	1, 6	1, 3, 6	2, 3, 6
Percent blast transformation								
Autologous	1	1	1	1	1	1	1	1
Anti-1	14	17	—	1	1	16	3	1
Anti-2	1	—	9	5	—	—	—	2
Anti-3	1	1	—	1	1	—	1	1
Anti-3 + 5	—	—	—	14	9	—	2	13
Anti-4	26	1	47	28	29	—	—	—
Anti-5(+ 6)	8	27	1	3	—	2	—	7
Anti-6	1	—	—	9	1	21	10	20
14C-TdR uptake (disintegrations/10 min)								
Autologous	608	613	404	182	344	283	618	478
Anti-1	3090	4930	—	294	435	2990	7320	680
Anti-2	308	—	573	633	—	—	—	885
Anti-3	457	347	—	200	578	—	1775	515
Anti-3 + 5	—	—	—	2983	1390	—	2540	7320
Anti-4	7360	344	19950	3021	1708	—	—	—
Anti-5(+ 6)	1260	12070	138	452	—	1287	—	2980
Anti-6	246	—	—	868	309	8400	8070	7100

but only if they are pre-incubated at 37 °C before addition of antiserum (Welsh et al., 1971).

Guinea pig B lymphocytes have been reported to undergo proliferation in response to anti-immunoglobulin (Elfenbein et al., 1973) and so have chicken lymphocytes (Ivanyi, 1970). Rabbit lymphocytes appear to be exceptionally responsive to anti-immunoglobulin antisera. Specific antisera directed against any of the six well-characterised determinants of rabbit IgG (As 1,2,3,4,5,6) were shown by Sell and Gell (1965) to be capable of inducing blast transformation and DNA synthesis (table 8.6) when added to lymphocytes obtained from rabbits of the appropriate IgG allotype. Allotype antisera were prepared by long-term immunisation of rabbits with *Proteus vulgaris* coated with antibody from a suitable pre-immunised donor (Dubbiski et al., 1959). Several experiments were performed which demonstrated convincingly that the active transforming antibody was indeed directed against the allotypic determinant. Firstly, serum from an As 1,4 rabbit immunised with *Proteus vulgaris* and As 1,4 gamma globulin in an identical manner to that used for the As 1,3,5,6 animals and containing no demonstrable anti-As antibody was not effective in inducing blast transformation in vitro. Secondly, the blastogenic activity was lost after mixing the As4 antiserum with serum containing specific As4 allotype. Thirdly, loss of blastogenic activity by absorption with heat-killed target lymphocytes for one hour was accompanied by loss of allotype antibody as determined by indirect haemagglutination using sheep erythrocytes coated with antibody. Fourthly, the active antisera did not agglutinate the target lymphocytes as might be expected if a leucocyte antigen were involved, and the antisera were not cytotoxic in the presence of complement except at high concentrations. The active antibodies were shown to be present in the 7S IgG fraction on DEAE chromatography. Mixtures of antisera directed against two different allotypic determinants induced a level of transformation greater than the sum of the activities of the two antisera tested separately.

Two loci control the expression of the allotypic determinants. The a locus allotypes are located in the variable region of the H chains and are found in all classes of rabbit immunoglobulin. The b locus allotype is on the kappa light chain of rabbits, which is the predominant type of light chain in this species. Antisera prepared in sheep to rabbit IgA or IgM or IgG subunits (Sell, 1967a and b) have also been shown to be blastogenic. Sheep antisera to rabbit light chains retain substantial specific mitogenic activity for lymphocytes of rabbits of one allotype after absorption with normal

serum from rabbits of another allotype (Lowe, 1972). Transformation appears only to require the antigen combining portion of the molecule, as has been shown to be the case with anti-leucocyte sera. However, although in vitro transformation can be obtained with $F(ab')^2$ fragments of rabbit or sheep antibodies to allotypic determinants, activity of another type, viz. allotypic suppression in the newborn animal, is possible only with intact rabbit antibody. Confirmation of the presence of the allotypic determinants on the surface of the lymphocytes has been obtained by demonstration of the formation of rosettes in a mixed agglutination technique (Ferrarini et al., 1973). Surface immunoglobulin is considered, on evidence from other species, to be the hallmark of lymphocytes of the B class. On this basis a high proportion of the lymphocytes in rabbit blood and tissues (spleen, appendix, sacculus rotundus) must be considered to be B lymphocytes. The very poor response of thymocytes to anti-allotype sera under conditions in which a good response to other stimulants e.g. SF, is obtained also implicates a B rather than a T lymphocyte as the responder cell. However, a high proportion of lymphocytes from rabbit blood, spleen and other tissues also respond well to PHA and con A, which are usually considered to be T cell stimulants. It is possible that distinct populations respond to the two types of stimulant or it may be found that rabbit lymphocytes have different characteristics to those of other species. The spatial arrangement of immunoglobulin molecules on the surface of rabbit lymphocytes may be more favourable to triggering the cell after attachment of antibody than that of other species. Rabbit lymphoblasts produced by activation with anti-allotype serum resemble PHA blasts in containing abundant free ribosomes with virtually no endoplasmic reticulum (Marcuson and Roitt, 1969).

Serum proteins other than immunoglobulins have not been shown to evoke antibodies which are lymphostimulatory. An exception to this is α -macroglobulin. Sheep, goat or guinea pig antisera to rabbit α -macroglobulin are mitogenic to a maximum of 10% of rabbit peripheral lymphocytes (Sell, 1970).

It is clear that mitogenic antisera to serum globulins exert their effect by combining with sites on the cell surface and appear to act in the same manner as leucocyte antibodies. Antibodies to surface antigens do not always produce lymphocyte activation and triggering may be related to the type, density and distribution of receptors on the surface. Mitogenic antisera are not all cytolytic in the presence of complement. Moreover, none of the antibodies which attach to lymphocytes seem to prime them for destruction

by the cytotoxic mononuclear cells (K cells) known to be present in the cultures of blood or spleen cells, in spite of the IgG nature of many of the antibodies.

8.3. Significance of antibody-induced lymphocyte activation

The fact that lymphocytes are activated by anti-leucocyte sera provides confirmation of the hypothesis that there are surface 'trigger' sites on lymphocytes, but there is no reason to believe that agglutination or sub-lethal damage play any part in the activation. Even with cross-species antisera it is clear that there are great variations in the activating capacity of different antisera which are not correlated with agglutinin or lytic titre, suggesting that antibodies to some surface determinants are activatory and to some others are inactive or inhibitory. In rabbits only a proportion of homologous leucocyte antisera have activating capacity and in humans antisera containing lytic HL-A antibodies have not been shown to produce activation. These non-mitogenic antisera to HL-A and other surface antigens will inhibit MLRs and must therefore produce a durable change of the cell surface. There are no indications that antibodies to particular sites on the cell surface are specially inhibitory. There appears to be less variation of effect according to spatial disposition of inhibitory antibody than is the case with activating antibody.

The fate of surface-attached antibody will depend largely on the nature of the site to which it becomes attached. If it attaches to a 'mobile' antigen which is free to move in the plane of the membrane it will be rapidly removed by 'capping' and endocytosis. Other antibodies may be shed off into the medium with fragments of antigen attached to them. Antibodies to antigens which are neither 'mobile' nor undergoing rapid resynthesis may be retained for long periods on the cell surface. Lymphocytes coated with heterologous antibody gradually lose their susceptibility to complement-induced lysis over a 24 hr incubation period. If it is assumed that mitogenic antibodies are lost at the same rate as lytic antibodies there is ample opportunity for an activation event, similar to that produced with lectins, to occur.

Experiments with anti-immunoglobulin sera do not support the popular belief that surface immunoglobulin sites on lymphocytes are antigen-recognition 'trigger' sites. Indeed the rapid removal of the antibody-immunoglobulin complex by capping and endocytosis would make activation very improbable. Anti-immunoglobulin reagents cannot therefore be

considered as possible B-cell stimulants. In the mouse and in the human they do not activate B lymphocytes and it is improbable that they do so in other species. The apparent exception to this rule is the rabbit. It is well established that anti-allotype or anti-light chain antisera will activate rabbit blood and tissue lymphocytes. Although rabbit lymphocytes have not been fractionated into sub-populations it seems likely, if only because of the large numbers of peripheral cells responding to both anti-allotype and con A, that at least some of the cells bearing allotype determinants are T cells.

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Immunospecific activation of lymphocytes of pre-immunised donors

9.1. *Antigen-binding and antigen-reactive cells in normal and immunised animals*

It has been convenient to divide lymphocyte stimulating substances into specific and non-specific categories according to whether or not lymphocyte reactivity is restricted to individuals previously exposed to the material concerned. The dividing lines between inborn and acquired, non-specific and specific reactivity are by no means clearcut, as will become apparent later. As well as responding to plant mitogens, lymphocytes from unimmunised animals are frequently responsive to products of bacteria. For example, human and rabbit lymphocytes, even of the newborn, are activated by staphylococcal products. Two 'non-specific' protein mitogens of staphylococci have been described (see ch. 6). Mitogens active against the lymphocytes of almost all individuals are also produced by streptococci (ch. 6). Substances which are mitogenic to a substantial percentage of the lymphocytes of non-immunised monkeys are produced by *S. typhi*, *E. coli*, *N. gonorrhoea*, *Strep. pneumonia*, *Proteus vulgaris*, *C. pseudotuberculosis* and *C. pyogenes* (Mandelstam and Fauconnier, 1969). One must also include allogeneic lymphocytes, but not free transplantation antigens or cell fragments, as 'natural' activators (see ch. 7). The lymphocyte response to most antigens which have not previously been encountered is, however, slight and often not detectable by techniques commonly used. A clonal selection theory would predict that only about 1 in 10^5 cells would be responsive to a primary antigenic stimulus and this would certainly be beyond the limit of detection. Many antigens used are impure and responses observed may be the result of summation effects.

For the purposes of this review an *antigen-binding cell* is simply a cell

which binds antigen. An *antigen-reactive cell* is a cell activated to a blast state by antigen. It is generally believed that antigen-reactive cells bear specific receptors on their surface for the antigen concerned (i.e. that antigen-reactive cells are antigen-binding cells). There is some evidence that this is so from antigen-suicide experiments, but the problem is a very difficult one to investigate. The converse is not true (i.e. not all lymphocytes which bind an antigen are activated by it). Not only is there frequent artefactual binding of 'sticky' antigens and cell surface antigens (the non-immune sheep cell rosetting of T cells is an example) but the process of differentiation from a B cell to a plasma cell leads eventually to loss of antigen-reactivity while antigen-binding antibody is synthesised in increasing amount. It is envisaged that the important binding sites are 'trigger' sites, such that an exposure of a lymphocyte population to an antigen 'triggers' those lymphocytes bearing specific and appropriate receptors, there being a preferential stimulation of cells in the population which bind antigen with high affinity (Davie and Paul, 1973). The resultant mitotic activity brings about a clonal expansion thereby increasing the ease of detection of cells reactive to this antigen. A soluble antigen is not necessarily 'presented' to the lymphocyte in its soluble form. Indeed it may be more commonly and effectively 'presented' on the surface of another cell such as a macrophage, a monocyte or another lymphocyte. A responding lymphocyte would, however, still need to possess the appropriate specific receptors for the antigen on its surface. There are probably many different types of surface receptor and some may have little importance in activating the cell. B lymphocytes of all species contain surface immunoglobulin and in the mouse there are about 10^5 immunoglobulin molecules on the B cell surface. These receptors appear to be labile in that an antigenic surface may induce movement and 'capping' of the surface immunoglobulin molecules. Receptors are constantly being resynthesised by cells and the absence of cells bearing a particular specific receptor at one time does not mean that such cells may not later appear in the population.

The antigen-binding and antigen-reactive lymphocytes of lymphoid tissues were the subject of investigation many years before the existence of lymphocyte sub-populations was demonstrated, but it is now known that there are differences in the antigen-binding characteristics of B and T cells. For cells of unprimed animals there is a marked falling off in the numbers of antigen-binding cells of both types as the antigen concentration is decreased below saturation level. This remains the case for T cells after priming whereas the avidity of B cells for antigen is markedly increased (Roelants and Rydén,

1974). The same authors provide evidence for the presence on T cells of immunoglobulin antigen-receptors which have been actively synthesised by the cells. One view is that the receptors on T cells are immunoglobulin in nature with the Fc part of the molecule more deeply buried in the membrane than is the case for B cells. However, there is, as yet, no agreed body of opinion about the nature of the T-cell receptor for antigen.

The frequency of binding of cells for an antigen is greater than would be predicted on a clonal selection theory. Using a 'sandwich' technique (unlabelled haemocyanin KLM antigen and radio-iodine-labelled antibody) about 0.1 to 0.2% of mouse spleen cells have been shown to bind antigen (Unanue et al., 1973). Lower values in early studies were probably due to using sub-optimal concentrations of labelled antigen. In most animal studies antigen-binding capacity has been found to be lacking in thymocytes but some antigens are bound by human foetal thymocytes (Dwyer and Mackay, 1972) and by cells from chicken embryonic bursa and thymus (Dwyer and Warner, 1971). A few cells (about 0.02%) in the thymus of young mice will bind KLM antigen but these may not be typical thymocytes because the mouse thymus contains a few cells with surface immunoglobulin, many of which are not destroyed by antisera to the θ allelic antigen (Unanue et al., 1973).

It has long been known that the number of antigen-binding cells increases after immunisation. For example, when an animal is immunised with sheep red cells, there is an increase in the number of lymphocytes capable of rosetting with sheep cells. Using a bacterial cytoadherence technique a similar increase in binding cells can be demonstrated following immunisation with bacteria. The same phenomenon occurs with soluble antigens as can be shown with radio- or fluorescein-labelled antigen. The proportion of human blood lymphocytes binding radio-iodine-labelled flagellin rose from 0.5 to 4% by 14 days after immunisation (Dwyer and Mackay, 1970). Not more than 0.04% of cells in the lymph nodes of normal guinea pigs bind ^{125}I -DNP-guinea pig albumin, but 2–4 weeks after immunisation with the antigen in complete Freund's adjuvant the proportion rises dramatically to about 5% (Davie et al., 1971). The number of rabbit blood lymphocytes binding fluorescein-labelled PPD or thyroglobulin has also been shown to increase after immunisation (Hjort et al., 1968).

It has generally been found that antigen-binding is markedly inhibited by anti-immunoglobulin sera. There have been several reports, for example, that antisera to mouse immunoglobulin H and L chains inhibit the formation

of sheep red cell rosettes by mouse spleen cells tested at various times after immunisation (see Wilson, 1971). Some antigens are taken up rapidly at 4 °C in sufficient amount to produce near maximal responses of lymphocytes (Caron and Poutala, 1969). This would suggest that uptake is not a metabolically-dependent process, which is consistent with it's being simply the union of antigen with an antibody-like receptor. Sodium azide is sometimes added to ensure that only a simple binding reaction of this type occurs. In our experience, however, there is great variation in the ease with which antigens are taken up by lymphocyte preparations. Some antigens e.g. PPD (see later) are taken up by irradiated leucocytes in amount sufficient to induce a high level stimulation of autologous lymphocytes but this is not the case with other antigens e.g. bovine albumin or ovalbumin. Most of the antigen is taken up by monocytes and macrophages rather than by lymphocytes in experiments of this type. Receptors for antigen are removed from the lymphocyte by trypsinisation but they are reformed after incubation (Mackler et al., 1972). This is consistent with their being immunoglobulin in nature but non-immunoglobulin receptors, e.g. for mitogens, are also removed by protease and resynthesised.

Because of technical difficulties it has not been easy to prove that the antigen-binding cells include the antigen-reactive cells. It has, however, been shown that specific loss of immunocompetence follows the deletion of antigen-binding cells by incubation with very 'hot' antigen (e.g. Unanue, 1971). Also, by a combination of autoradiography for the detection of labelled antigen and sedimentation velocity for the fractionation of lymphoid cells of immunised animals, it has been shown that antigen-reactivity in vitro or in vivo is related to the number of antigen-binding cells in the various fractions (Diener et al., 1973). It is still uncertain to what extent antigen-reactivity is inborn and to what extent it is acquired and at what time. Antigen-reactive cells (e.g. to haemocyanin and some bacterial antigens) have been shown to be present in appreciable numbers in the blood of the newborn (Leikin et al., 1970). It is possible that clonal expansion may occur before birth as some antigens may be encountered by transplacental passage (Gill, 1973). The correlation reported between the responses of lymphocytes of mothers and offspring to various antigens would be compatible with this interpretation (Field and Caspary, 1971).

Two major questions arise concerning the activated cells found in antigen-stimulated lymphocyte cultures: a) What proportion of the activated cells are T cells and what proportion are B cells? b) How many of the activated

cells have arisen from lymphocytes bearing specific receptors for the antigen concerned?

Both T and B lymphocytes are known to be antigen-reactive *in vivo* (see later) and both T and B blast cells should therefore be found in cultures, the proportions depending on the degree of thymus-dependence of the antigen concerned. Most of the proliferative response of immune lymphoid cells to antigen is known to be contributed by T cells, but this result may be influenced by culture conditions. Just as antigen-binding cells of B type are more readily detected than those of T type, so the reverse is true of antigen-induced activation. Under conventional culture conditions B lymphocytes and their progeny survive less well than T cells and the final stages of differentiation to plasma cells are not seen. An even greater problem is that of possible 'recruitment' to the active state of cells not bearing the appropriate antigen receptors. This could occur either by the liberation of a mitogenic lymphokine into the medium during stimulation or by the exposure of lymphocyte-stimulating determinants on lymphocytes as a result of activation. One way in which 'recruitment' can be detected is by the use of cell markers (e.g. chromosome markers) in mixtures of histocompatible lymphocytes. Chromosome markers to identify syngeneic lymphocytes in mixed cell reactions have indicated that lymphocytes from tolerant rats are not 'recruited' when syngeneic normal lymphocytes are activated. However, the opposite result has been obtained in an antigen-stimulation system. When equal numbers of PPD-reactive lymphocytes from male Fischer rats and of PPD-unreactive lymphocytes of female Fischer rats were cultured together in the presence of PPD it was found that of 18 out of 730 metaphases clearly identifiable in the cultures 8 were of female karyotype (Larralde, 1970). Direct observation of antigen-stimulated lymphocytes placed in compartmentalised cultures has shown, nevertheless, that clonal proliferation does occur and that it proceeds through several generations (Marshall, 1969). 'Suicide' experiments using 'hot' thymidine or 'hot' antigen or bromodeoxyuridine + light to delete lymphocytes responding to a particular antigen have shown that specific deletion of cells responsive to one antigen may be achieved with retention of reactivity to other antigens and non-specific mitogens. While these experiments do not exclude the process of 'recruitment' they do suggest that the cells bearing specific receptors initiate the reaction and that 'recruitment' must be a late event. The 'suicide' experiments do not prove that lymphocytes react to only one antigen. Indeed this is improbable. Whether the receptors for two antigens are present on different or on the same cells some additive effects might

be expected if the population were exposed to both antigens at their optimal concentrations at the same time. With some stimulants and some antigens additive effects can clearly be shown, especially if they are used at sub-optimal concentrations, but inhibitory effects are just as frequently obtained presumably indicating that attachment to two sites on the same cell may produce inhibition in the same way that an excess of a mitogen like PHA or con A can also be inhibitory.

9.1.1. Responses of human lymphocytes to tuberculin PPD and other antigens

In the eleven years following the first report that blood lymphocytes from tuberculin positive, but not from tuberculin-negative individuals are mitotically activated by tuberculin in culture (Pearmain et al., 1963), the tuberculin system has become a classical reference point for antigen-specific activation of human lymphocytes and comparisons of leucocyte culture responses with tuberculin skin tests have frequently been made (e.g. Gump et al., 1967). In general the correlation between the two tests has been good. Miller and Jones (1973), for example, found that, in 25 of 34 subjects tested, the size of the skin test response to any strength of tuberculin could be predicted with one standard deviation from the degree of lymphocyte transformation. Loss of skin reactivity in old age has been shown to be paralleled by a loss of lymphocyte response to tuberculin. Recent exposure to antigen induces sensitivity. As early as the 13th day post-BCG vaccination, and before the skin test becomes positive, a lymphocyte response may be detected (Sarkany and Hales, 1968). However, it does not follow from these results that all the reaction measured is always attributable to acquired immunity. A high degree of reactivity to PPD may occur which cannot readily be accounted for by immunisation. A positive response to PPD is sometimes found during the first three months of life in the absence of exposure to tubercle antigens (Matsaniotis and Tsenghi, 1968).

The technical aspects of the test have received much attention. There is general agreement that unpurified lymphocytes work better than purified and some evidence of a specific requirement for macrophages (Schechter and McFarland, 1970). Some authors have used a crude preparation of old tuberculin (at a concentration of 1/2000) but usually the purified protein derivative (PPD) of human tuberculin has been used, frequently as an acetone and ether-dried powder brought into solution with very dilute NaOH or as a freeze-dried preparation. A dose of 250 OT units per ml of culture

containing 2×10^6 lymphocytes per ml has been recommended as the optimal dose for distinguishing between tuberculin positive and tuberculin negative donors (Coulson and Chalmers, 1967). Hinz and Chickosky (1972) have recommended a leucocyte concentration of 10^6 per ml with some non-lymphocyte leucocytes present, a PPD concentration of 1 $\mu\text{g}/\text{ml}$ and a culture period of 5 days. Alternative procedures include adsorbing the PPD on bentonite when it has been found to stimulate more effectively at low PPD concentrations and to induce faster responses (Nilsson and Moller, 1972). It has been clearly shown that the PPD is very effective if 'presented' on a macrophage (see § 9.1.3) and PPD on the surface of X-irradiated autologous lymphocytes will also induce activation (Kasakura, 1969). Intact tuberculin bacilli are more effective than soluble PPD (Zabriski and Falk, 1970). The amount of tuberculin PPD added to lymphocyte cultures in different laboratories has varied between 1 and 20 $\mu\text{g}/\text{ml}$. The amount of PPD present markedly affects the response and lymphocytes of tuberculin positive and negative individuals show different sensitivities (Nilsson, 1972) as judged by the level of DNA synthesis at 3–6 days, in cultures containing 0.1 to 100 $\mu\text{g}/\text{ml}$ PPD. Lymphocytes from skin-test negative individuals did not react at the lower PPD concentrations but were responsive at the highest concentrations. Lymphocytes from skin-test positive individuals responded at the lowest concentration of PPD and showed increased reactivity as the concentration was increased to 10 $\mu\text{g}/\text{ml}$.

Many searching investigations of the specificity of the PPD response have been made. Lymphocyte responses to Seibert's standard mammalian tuberculin PPD-S, PPD-B from a Battey strain, PPD-Y from the Bostrom Strain of *M. Kansii*, PPD-scotochromogen from the Watson strain and PPD-phlei have been compared with the reactions of the same individuals to an intra-dermal skin test with 0.1 μg of the same antigen (McFarland and Heilman, 1966). The lymphocyte response usually corresponded with the result of the skin test. Lymphocytes from patients with active tuberculosis were responsive to PPD provided care was taken to remove PPD-neutralising antibodies in the serum. A good correlation between the lymphocyte and cutaneous sensitivity was obtained in a survey of 208 persons using 2, 10 and 50 units of tuberculin in the Mantoux test (Lewi, 1970). No lymphoblasts were found in cultures from individuals showing no skin reaction to 50 units and up to 30% lymphoblasts in culture from subjects sensitive to 2 units. It is the protein and not the carbohydrate components of tuberculins which induce lymphocyte transformation (McFarland and Heilman, 1966; Janicki et al.,

1972). The carbohydrate fractions of tuberculin are capable of inducing intense skin reactions and affecting macrophage migration but will not activate lymphocytes (Chaparas et al., 1971).

It is probable that most of the responding cells are T cells although, as yet, this has not been demonstrated. Judging by experiments in mice PPD can also act as a non-specific stimulant of B cells if used in very high concentrations and at comparable high levels it has been shown that some lymphocytes in the blood of tuberculin-negative individuals will respond (Sultzner and Nilsson, 1972; Nilsson et al., 1973).

Of the many other antigens which have been used to stimulate human lymphocytes, smallpox vaccine (one vial of vaccine per 8 ml of culture) and tetanus toxoid (a final dilution of 1:250) are among the most common (Caron, 1967). A poor response to smallpox vaccine was obtained with lymphocytes of individuals immunised 20, 30 or 50 years previously (1-3% blasts at 5-6 days), a better response when the interval was 13-14 years, and a quicker and better response (6.0-9.8%) after 6 years or less. There was no correlation with the level of neutralising antibody (Gurvich and Svet-Moldavskaya, 1968). Products of various Gram-negative organisms will stimulate human lymphocytes. Typhoid-paratyphoid vaccine, once used as a 'specific' stimulant, has been shown to stimulate lymphocytes of unvaccinated as well as vaccinated donors (Heilman, 1970). The mitogenic activity of Brucella extracts was shown to reside in a protein fraction; the lipopolysaccharide fraction possessed only weak activity for human lymphocytes and this activity was unrelated to endotoxin potency. Responses of blood lymphocytes to Brucella antigens have been detected as early as 3 days following immunisation with killed *Brucella abortus*. The pattern of response was characterised by increasing sensitivity to low antigen concentrations (Andersen et al. 1971). Endotoxins produced by *Shigella* or *E. coli* have, in our hands, been ineffective as stimulators of human lymphocytes.

When lymphocytes are obtained for culture shortly after antigenic stimulation the mitotic response of the host may still be continuing in the blood lymphocytes. Activated lymphocytes are regularly detectable in fresh human blood a few days after a primary or a secondary immunisation, the responses reaching a maximum at 5-7 days (up to 4% activated cells) and disappearing completely by one month (Crowther et al., 1969). Pre-immunisation may also increase the 'irritability' of lymphocytes rendering them more sensitive to all mitogenic stimuli. Lymphocytes from patients recovering from Hong Kong flu have been found to be more affected than normal lymphocytes by the

minor stimuli which constitute the background stimulation (Parker and Lukes, 1971). Blood lymphocytes of sensitised guinea pigs reinjected with a small amount of antigen a few hours prior to harvesting the cells show a heightened sensitivity not only to the antigen concerned but to unrelated antigens previously encountered by the animals (Housley and Gell, 1969).

It is not possible, without elaborate labelling experiments, to translate the number of blast cells seen in cultures into a figure for the number of antigen-reactive cells present at initiation of the culture. Even when allowances are made for clonal expansion of the reactive sub-population the extent to which 'recruitment' of uncommitted cells has occurred is unknown. For example, it has been shown that if the lymphocytes of a donor responsive to diphtheria toxoid are mixed in the proportion 1:2 or 1:3 with lymphocytes of an HL-A identical diphtheria toxoid-unreactive sibling the level of response to antigen in culture is almost identical to that of cell populations derived solely from the reactive donor (Schellekens and Eijsoogel, 1971). There are other complications which make precision of interpretation very difficult. The response may be affected by the way the antigen is 'presented' as already discussed in relation to tuberculin. Associated (98S) hemocyanin (KLH) has been shown to be more effective than dissociated (18S) KLH in stimulating lymphocytes of immune and non-immune donors, stimulation indices for non-immune individuals ranging from 9 to 93 for 98S KLH compared with 1.3 for 18S KLH (Green and Borella, 1971). Improved responses to soluble antigens when complexed with antibody are probably also due to presentation of antigen in suitable macromolecular form (Oppenheim, 1972). Enhanced responsiveness is not easy to demonstrate and occurs only at certain critical antigen-antibody ratios. Studies on the lymphocytes of horses presensitised to ovalbumin show that the cellular response was depressed at some antigen-antibody ratios and enhanced at others, the levels of thymidine incorporation varying between 5 and 216% of that in cultures containing antigen alone. Similar increases and decreases were observed with cells of animals sensitive to sheep red cells when antigen was complexed with antiserum. Cells of non-sensitised animals were not stimulated by immune complexes (Banks, 1973).

9.1.2. Tissue location of antigen-responsive cells in animals at various times after immunisation

Many in vivo and in vitro studies have now shown that antigen-binding and

antigen-reactive cells in immunised animals are not randomly distributed throughout the lymphoid tissues. The distribution depends on the antigen studied and the species. Circulating lymphocytes may initiate antibody responses to sheep red cells on transfer to syngeneic animals but not to bacterial toxoids and other antigens for which spleen cells are required (Strober, 1968). Cells reactive to tetanus toxoid, when perfused through spleen, have been shown to migrate into the splenic tissue and not to re-emerge with the perfused cells (Ford, 1972).

In vitro studies have shown that after a single intravenous injection of sheep red cells into rabbits high levels of rosette-forming, plaque-forming and antigen-reactive cells are found in the blood and spleen at 5–17 days whereas by 84–360 days activity was absent from blood cells. Although rosette-forming and plaque-forming cells had also disappeared from the spleen there were still many cells present which could be stimulated into DNA synthesis by culture with sheep red cells. When bovine albumin (in CFA) was the immunogen, lymphocytes responsive to antigenic stimulation were found in the blood for up to 18 months, although they were always present in greater numbers in spleen and lymph nodes (Gery et al., 1970). Lymphocytes reactive to human IgG, tissue extracts and other antigens also appear temporarily in rabbit blood after immunisation (Benezra et al., 1967). Antigen-reactive lymphocytes have been detected in the bone marrow of rabbits as well as in blood, spleen and lymph nodes (Singhal et al., 1968). In vitro responses of cells in spleens of mice immunised with sheep red cells were found only transiently and were not detectable 8 weeks after immunisation (Radcliffe and Axelrad, 1971). The antigen reactivity induced in animals depended on the dose of antigen and whether it is administered in solution or in adjuvant emulsion. When rabbits were immunised with bovine albumin (20 mg in adjuvant) a marked rise in antigen-sensitivity of blood lymphocytes was observed, lasting for about one month. Under the same conditions immunisation with 0.5 mg of bovine albumin (in adjuvant) produced a peak response at day 16 followed by a steady decrease. The enhanced sensitivity to small concentrations of antigen in culture was most marked in rabbits immunised with the lower dose of antigen. Injection of the antigen in solution not only did not produce enhanced lymphocyte reactivity but reduced the sensitising effect of subsequent immunisation with antigen in Freund's adjuvant (Benezra et al., 1971).

Lymphoid cells in peritoneal exudates of immunised animals, which have long been known to be exceptionally active in MIF tests, have also been

shown to induce more antigen-specific mitotic activation than cells of a draining lymph node, both in respect to the level of response and in sensitivity to low concentrations of antigen (Rosenthal et al., 1971). The differences in antigen sensitivity appeared to be due to a higher affinity of the peritoneal exudate cells for antigen. The proportion of T cells present in lymph node and peritoneal exudate preparations were roughly equivalent and their responses to PHA were similar.

Restriction of immunological reactivity to certain sites following local immunisation is now a well-recognised phenomenon. A corresponding compartmentalisation of antigen-reactive cells has now been shown to occur. When guinea pigs were immunised subcutaneously with DNP-human IgG, antigen-reactive cells were found in the spleen but not in bronchial washings whereas the reverse was true when animals were immunised with antigen in nose drops (Waldman and Henney, 1971). Similarly when rabbits were injected subcutaneously with bovine albumin in incomplete Freund's adjuvant, antigen-reactive cells were regularly found in the blood 14–21 days later whereas reactive cells were rarely found in the blood of animals immunised by oral administration of small amounts of bovine albumin despite the presence of comparable amounts of circulating antibody. The effect of subcutaneous injection of bovine albumin into rabbits already orally immunised was to increase the level of circulating antibody without emergence of antigen-reactive cells into the circulation (Goldberg et al., 1971).

9.1.3. The role of the macrophage

It has been suspected for a long time that antigen attached to the surface of a macrophage is peculiarly effective in inducing an immune response. This has been confirmed by exposing lymphocytes, macrophages and other cells to labelled antigen, washing them and comparing the immunogenicity of a standard amount of bound antigen with that of soluble antigen. Macrophage-bound-antigen was more immunogenic than lymphocyte-bound-antigen which in turn was more immunogenic than soluble antigen. Thymocyte-bound-antigen or hepatoma-bound-antigen were ineffective (Seeger and Oppenheim, 1972). Most of the antigen bound by macrophages initially is endocytosed rapidly (2–5 hr) and catabolised, but the small amount (less than 10%) which remains is relatively stable and the coated macrophages retain their lymphocyte-priming capacity for long periods. The membrane-bound antigen may be removed by trypsin or EDTA treatment (Unanue and

Askonas, 1968; Unanue and Cerottini, 1970). There is also good evidence for synergism between lymphocytes and macrophages in lymphocyte activation by antigens in culture (Seeger and Oppenheim, 1970). A macrophage-rich population produced by subjecting human leucocytes to 2000 r X-irradiation and culturing for 5–7 days has been shown to have the capacity of markedly increasing the response to PPD of lymphocytes of sensitised donors (Schechter and McFarland, 1970). The macrophages needed to be intact, viable cells with the opportunity of direct contact with the lymphocytes and to have been pulsed with antigen sometime during the preceding 72 hr. The lymphocytes formed clusters around the macrophages during the culture period. Macrophages appear to be superior to monocytes in performing this synergistic role (Hanifin and Cline, 1970).

*9.1.4. Relation of *in vitro* responses to delayed hypersensitivity and antibody production*

In vitro responses which have at least some measure of specificity have now been obtained to a variety of bacterial protein antigens, to animal proteins and to some synthetic antigens in a variety of species. Specific responses to *Trichinella* (Kim et al., 1971) and schistosomal egg antigens have also been demonstrated (Colley, 1971). Specific lymphocyte activation was at first expected to be correlated solely with delayed hypersensitivity to the antigen concerned, lymphocyte transformation being equated with 'cellular immunity'. It is now well established that T cells play an important indirect role in antibody responses to certain ('thymus-dependent') antigens and that both T and B cells are antigen-reactive. Enhanced responsiveness to antigen of both T and B cells might therefore be expected to be a frequent finding in animals immunised purely for antibody production.

There have been many comparisons of immune responses with *in vitro* reactivity of lymphocytes to antigen. A good correlation between delayed hypersensitivity and *in vitro* responses to a range of antigens was obtained by Mills (1966). *In vitro* responses of lymphocytes of immunised monkeys correlated with either atopic or purely delayed hypersensitivity (Mackler and Mally, 1971). Good responses in culture were obtained from lymphocytes of rabbits immunised either for delayed hypersensitivity or for antibody production (Jevitz and Ekstedt, 1971). Peripheral lymphocytes of individuals immunised with keyhole limpet hemocyanin (KLH) responded in culture to KLH and the result correlated better with a haemagglutination test (for

serum antibody production) than with delayed hypersensitivity (Curtis et al., 1970).

It has been clearly shown, in hapten-carrier systems, that the requirements for the induction of lymphocyte transformation are related to those for immunogenicity and that the marked specificity of the response is not simply that of the serum antibody produced by the immunisation. Only immunogenic members of the homologous series α -DNP-(lysine)_n provoked incorporation of ^3H -thymidine into lymph node cells from guinea pigs immunised with the conjugate in adjuvant. Conjugants in which $n=8$ or more were immunogenic and capable of inducing lymphocyte activation and release of MIF. This could not be explained if the cell receptor for antigen were simply a bound antibody of specificity similar to that of the serum antibody. The binding energy of antibody to DNP-lysine₃ was almost as great as to DNP-lysine₁₀ yet the former was quite inactive and the latter active in inducing lymphocyte activation. Although most of the antibody was directed against the DNP group the lysine carrier was essential for lymphocyte activation. It was possible to produce some lymphocyte activation with lysine₈₋₁₀ but not DNP-lysine₃₋₆ or lysine₄-DNP-lysine₄. As in Dutton's earlier hapten-carrier experiments in rabbits the hapten itself was not able to induce or to inhibit lymphocyte activation. It is possible that T cells, which are likely to constitute the major responding population, react with sites on the antigen to which antibody is not formed. Receptors on the B cell, on the other hand, are thought to be antibody-like and the lack of response of B cells to DNP-short chain oligolysines might indicate a requirement for antigen-responsive helper T cells (Stulbarg and Schlossman, 1968; Schlossman et al., 1969; Schlossman, 1972). Another interesting series of experiments also demonstrates the importance of the 'carrier' part of the molecule in lymphocyte activation and the relation to immunogenicity. Some strains of guinea pigs are responsive to immunisation with DNP-polylysine (DNP-PLL) and some are not. Addition of DNP-PLL to lymph node cells from genetic responder animals immunised with the conjugates in adjuvant induced significant incorporation of ^3H -thymidine whereas negative results were obtained with lymph node cells from genetic non-responders. Addition of DNP-PLL bovine albumin or DNP-PLL-ovalbumin also failed to stimulate in spite of the fact that cells from the node were producing anti-DNP-PLL antibody. This suggests that the function of the substance produced by the PLL gene in responder animals is to act at a crucial stage to form an antigen-inducer complex (Green et al., 1968).

9.1.5. Nature of antigen-specific activation

As already indicated in section 1, 'suicide' and fractionation experiments have largely confirmed the specific nature of antigen-induced proliferation. 'Suicide' experiments with 'hot' DNA-precursors show that a specific antigen-reactive portion of the population is required and 'suicide' experiments with 'hot' antigen show that elimination of antigen-binding cells also eliminates antigen responsiveness. Although lymphocytes not bearing specific receptors or reactive capacity may be 'recruited' once an activated culture is established, the reaction cannot be initiated with a lymphocyte population which lacks antigen-binding lymphocytes. B lymphocytes have more antigen-binding receptors on their surface than T lymphocytes and most of these receptors are immunoglobulin in nature. Removal of immune B lymphocytes by passing a suspension of immune lymphoid cells through an antigen-adsorbent column has generally been found to have little effect on antigen-reactivity of the population. Also, blocking of the surface immunoglobulin sites of immune rabbit lymphocytes did not diminish the proliferative response to antigen (Mond et al., 1973). Deletion of B cells from a suspension of immune mouse lymphoid cells, by treatment with anti-immunoglobulin and complement, caused only a small reduction in antigen-reactivity. Experiments of this kind indicate that B cells and immunoglobulin receptors have little importance in antigen-reactivity as measured *in vitro*. This is true, however, only for thymus-dependent antigens. Thymus-independent antigens do stimulate B cells directly but the stimulation sometimes appears to be largely non-specific (Coutinho and Moller, 1973). It is, however, still believed that the principle antigen-recognition sites on B cells are immunoglobulin in nature.

Since it is known that both T and B lymphocytes may proliferate in the presence of antigen and that non-immunoglobulin as well as immunoglobulin receptors are probably involved, it would not be surprising to find that results obtained have varied a great deal with the species and the antigen investigated and the experimental conditions. Results in conflict with those reported above have sometimes been obtained. An increase in the proportion of complement-binding (B) lymphocytes has been reported to occur in cultures of antigen-stimulated immune guinea pig lymphocytes (Elfenbein et al., 1972). Both primary and secondary responses to *Brucella abortus* antigen were reduced by incubation of the spleen cells with anti-immunoglobulin, but not by deletion of T cells with anti- θ and complement, whereas responses

to sheep red cells were always greatly inhibited after incubation with anti- θ . With DNP-hemocyanin as antigen neither primary nor early secondary responses were affected by anti- θ whereas the late secondary response was greatly affected (Takahashi et al., 1971). It cannot be said, however, that these experiments confirm the antibody nature of antigen-combining sites since other reagents reacting with cell surface components will also block responses to antigens. For example, treatment of mouse spleen cells with con A will block the response to erythrocyte antigens (Dutton, 1972). For antigen-specific stimulation, as with mitogen-induced stimulation, it is probably essential that the activant should remain on the surface receptors for some hours to induce an irreversible response. It has been known for some years that most of the material deposited on a cell surface is rapidly removed by endocytosis and it has recently been discovered that a 'capping' process (see § 11.1.3) is a preliminary to this. Although most of the antigen may be removed from B cells by this process a small amount must remain if mitotic activation is to occur and the receptor for this residual antigen may conceivably not be immunoglobulin in nature. In a somewhat analogous process most of the antigen taken up by macrophages is endocytosed and broken down, but the small residue remaining on the cell surface persists for long periods and has an important immunogenic role.

Experiments with tolerant animals have provided further information about the relationship between antigen-binding and antigen-reactive cells and immune responses. The antibody response of guinea pigs to a hapten (DNP), following immunisation with DNP attached to a carrier (guinea pig albumin, GPA), is markedly reduced if the animals are pretreated with the hapten attached to another carrier, a co-polymer of D-glutamic acid and D-lysine (Cohen et al., 1973). The partial tolerance produced was accompanied by a marked reduction in the number of B cells binding DNP-GPA. However, the delayed hypersensitivity skin reactions and the level of lymphocyte stimulation by DNP-GPA was not different from that of immunised animals which had not been pre-treated. This indicated that the antigen-responsive cells in the tissue culture system were largely T cells and that no significant T cell tolerance had been produced. The reduction in the number of antigen-binding B cells may have been a consequence rather than a cause of the tolerance induced in B cells.

There is compelling evidence of a genetic nature that initiation of immune responses to many antigens is not directly dependent upon antigen recognition by antibody-like receptors of an immunoglobulin nature. Specific

immune response (1r) genes exist which control the responses of particular strains of mice and guinea pigs to antigens of highly restricted heterogenicity e.g. synthetic polypeptides with limited numbers of different L-amino acids or weak native antigens differing only slightly from host proteins (see § 9.1.4). Responses to these antigens are genetically linked to the expression of histocompatibility antigens and are initiated by thymus-derived lymphocytes. In mice the 1r gene concerned is closely linked to the H-2 locus but is not linked to immunoglobulin heavy chain linkage. A gene-product, not yet identified, which is present on T lymphocytes is considered to be vitally concerned with the recognition of many antigens (Benacerraf and McDevitt, 1972). Although the antigen-binding receptors on T cells presumably derive their specificity from sequences of amino acids built up in the same manner as an Fab fragment of antibody there is no reason why the receptors should bear any other resemblance to serum antibody. If this is so for T cells it may be asked if there are any compelling reasons why the 'trigger' receptors on B cells should be immunoglobulin-like in nature. At the time when dogmatists stated that all the receptors on lymphocytes were antibody-like it was regarded as inconceivable that nature would have evolved more than one sophisticated recognition mechanism. On this view it would now be reasonable to hypothesise that the antigen-receptors on both T and B cells are not immunoglobulin in nature and that the immunoglobulin antibody is synthesised by B cells as a later event after differentiation. This would at a stroke eliminate from consideration the conflicting data on the lack of a relationship between the immunoglobulin class detected on B lymphocytes and that of the antibody subsequently synthesised, with the necessity for the postulation of immunoglobulin 'switches'. The antigen, if it is to activate the lymphocyte, must remain on the surface for some hours for irreversible activation to occur. Rather than the 'fluid' receptor of immunoglobulin-type which would serve, by 'capping' and endocytosis to remove the antigen from the cell surface before activation had occurred, a more stable receptor which would allow undegraded antigen to remain on the surface for some time would be a better candidate for the 'trigger' site. The function of antigen-binding immunoglobulin on the surface of B cells may be similar to that of cytophilic antibody on macrophages viz. to 'present' antigen to lymphocytes (T and B) in the vicinity in a mixed cell type of reaction. This would account for the 'blocking' effects occasionally obtained with anti-immunoglobulins. It is likely that intimate cell surface contacts between lymphocytes of both types and macrophages are an essential part of the initiation stage of the

reaction and that antibody to any antigen on the surface of these cells would interfere with the response mechanism. Hence antibodies cannot be used to pin-point the 'trigger' sites. Inevitably we must conclude that we do not yet know the nature of the antigen-trigger sites on B and T cells.

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Biological properties of activated lymphocytes

Lymphocytes which have been activated by mitogens, foreign cells, antigens or antibodies may be shown to have undergone a change which alters their properties and which may correspond to a further stage of differentiation. They are more radioresistant than small lymphocytes and they are better able to support the growth of many viruses. They become endowed with new functional capacities. For example, activated T cells are more effective stimulators of allogeneic lymphocytes, they may exert a cytotoxic effect on various cultured and tissue cells, they may activate macrophages and they may promote the activation and differentiation of B lymphocytes to antibody-forming cells. Some of these phenomena require the direct participation of viable lymphocytes and cannot be reproduced with culture supernates or cell extracts. In yet more complex events both a whole viable lymphocyte and an additional factor or cell (e.g. an antibody or a phagocytic cell) is required. Most experiments have been conducted with mixed T and B cell populations from blood or tissues but it is now believed that activated T cells have been responsible for most, but not all, of the phenomena. As dissection of the various reactions progresses it is becoming apparent that sub-populations within the broad T and B divisions have distinctive biological properties and it is already known that a third type of lymphocyte or lymphocyte-like cell (K cell) is involved in certain types of cytotoxic reaction. Lymphocyte activation is known to be accompanied by the release of active substances into the culture medium. The term 'lymphokine' has been used to describe a biologically active substance produced by sensitised lymphocytes after incubation with antigen (Dumonde et al., 1969). It is a term sometimes used more loosely to describe any non-antibody material released by lymphocytes during incubation. Lymphokines or lymphocyte activation products (LAP) are distinct from other leucocyte products with biological

activity such as Lawrence's 'Transfer Factor', obtained by direct extraction of immune lymphocytes.

10.1. Lymphokines

Many early studies showed that the migration of cells from explants of lymphoid tissue from hypersensitive animals was inhibited in the presence of antigen. An antigen-induced inhibition of the migration of peripheral blood leucocytes or peritoneal exudate cells from immunised animals was later demonstrated. These observations were developed into a reproducible laboratory test by the introduction of an apparatus which allowed measurement of the rate of migration of cells from a capillary tube (David et al., 1964). With this test it could regularly be shown that the migration of peritoneal exudate cells obtained from guinea pigs, immunised in such a way as to develop delayed hypersensitivity, was specifically inhibited by antigen. Lymphocytes from the lymph nodes of sensitised guinea pigs without added macrophages migrated normally in the presence of antigen and it was presumed that the antigen was acting directly on the peritoneal macrophages. Subsequent investigation revealed, however, that both lymphocytes and macrophages were required for inhibition to occur. The lymphocytes had to be obtained from an immunised animal and provided the specific part of the reaction while macrophages from normal guinea pigs were as good as 'immune' macrophages (Bloom and Bennett, 1966). Thus two stages are involved in the migration inhibition test: a) the action of antigen on sensitised lymphocytes leads to liberation of a factor now called MIF (migration inhibition factor), b) the attachment of MIF to the surface of macrophages renders them 'sticky' and impedes their migration. Since any material toxic to macrophages will inhibit their migration and antigen-antibody complexes prepared in antibody excess are also inhibitory, controls to check the validity and specificity of the measurement are essential, but have not always been included. Lymphoid cells from different tissues of a sensitised guinea pig differ markedly in their ability to produce MIF. Peritoneal lymphocytes are very effective, lymph node cells from suitable nodes are active, whereas thymus and bone marrow cells are very much less active (Winkelstein, 1972).

Guinea pig MIF has been purified 1300-fold by fractional salt precipitation and chromatography on DEAE cellulose and Sephadex (Dumonde et al., 1972). Activity is associated with a glycoprotein fraction of mol. wt. between

56,000 and 82,000 with smaller amounts in the 20,000–56,000 range. MIF migrates with the pre-albumin fraction. It is destroyed by trypsin and neuraminidase but unaffected by ribonuclease. It is stable to heating at 56 °C but not to 80 °C for 30 min. It is actively synthesised within the cell and its formation is inhibited if puromycin is present to prevent protein synthesis during the culture period. It is produced mainly, if not exclusively, by activated T cells. At least one major fraction of MIF activity has been identified (by Sephadex fractionation and isoelectric focussing) among the 14 or so labelled proteins synthesised by activated guinea pig lymphocytes in the presence of labelled amino acids (Sorg and Bloom, 1973).

It was once thought that MIF was liberated only by specific antigen acting on the lymphocytes of a hypersensitised animal and not during the lymphocyte activation induced by allogeneic lymphocytes or by PHA, con A or other non-specific mitogens. Recently there have been several clear demonstrations of the release of MIF and other soluble factors by the action of PHA or con A on non-immune lymphocytes (see Remold and David, 1972 for references). The factors responsible, when examined by Sephadex fractionation (Sephadex also serves to remove con A), disc electrophoresis and isopycnic centrifugation are indistinguishable from the MIF liberated from antigen-stimulated lymphocytes. A substance closely resembling MIF is also produced by continuously dividing lymphoid cells (see section 10.4) and even by fibroblasts. It appears possible that MIF and some other lymphokines may be produced simply as a consequence of mitotic activation of lymphocytes and further that non-immunocompetent cells may be able to produce similar substances.

Lymphocyte culture supernatants have other biological activities. Factors acting on macrophages such as a macrophage spreading inhibitor, a macrophage aggregator and a phagocytosis-promoting factor have been described and a factor which increases incorporation of labelled nucleosides into the RNA of monocytes. Activated lymphocytes also produce a factor, possibly identical with MIF, which markedly increases the levels of some macrophage enzymes (Nathan et al., 1971). This enhanced metabolic activity may be part of an important defence mechanism since it has been shown that the action of an antigen on lymphocytes from a donor sensitised to this antigen greatly enhances the bactericidal activity of macrophages present (see Koster and Mackaness, 1971, and Simon and Sheagren, 1972 for references). The enhancement, which is not correlated with the presence of antibodies but correlates with MIF production, is non-specific, e.g. the addition of

bovine gamma globulin to lymphocytes of sensitised guinea pigs will produce a factor which enhances the bactericidal activity of macrophages for *Listeria*. Lymphocytes from peritoneal exudates have been shown to be superior to lymph node lymphocytes in inducing this macrophage activation, as they have been in the production of MIF. Many other cell types are also affected by lymphokines. For example, an inflammatory factor in lymphocyte supernates will produce a local increase in vascular permeability when injected; another factor is mitogenic to lymphocytes; a cytotoxin produced by activated lymphocytes is destructive to cell monolayers or inhibits their growth; soluble chemotactic factors released will attract neutrophils or eosinophils (Colley, 1973); acid production by some cells may be enhanced by the action of antigen or PHA on lymphocytes; a lymphokine may cause histamine to be released from platelets. Also histological changes occur in the paracortical region when lymphokines enter a node via the afferent lymph. All these phenomena and the properties of MIF and other factors are discussed in detail in several reviews (Dumonde et al., 1969; Bloom, 1971; Pick and Turk, 1972). It is not yet known whether many different factors are involved or whether the various effects are produced by a single substance which possesses many biological activities.

The demonstration of the production of lymphocytomitogenic substances by activated lymphocytes has an important bearing on the specificity of the cellular response to a stimulant. Are all the activated lymphocytes in a culture responding to the attachment of antigen to their cell surfaces or have some been non-specifically 'recruited' by the proximity of blast cells or the liberation of mitogenic substances in the medium? It is well known in the tissue culture field that cells grow more rapidly in 'conditioned' compared with fresh medium and the beneficial effect appears to be due to metabolic intermediates in the 'conditioned' medium. A low degree of activation could well be improved by 'conditioned' medium without the specificity of the response being affected. As far as possible augmenting effects due to nutritional factors have been eliminated in tests for mitogenic substances and there is good evidence that activated lymphocytes produce mitogenic factor(s) (see Smith and Barker, 1972 for references). However, under normal culture conditions the effect is very weak and 'recruitment' is probably slight. Recruitment has been shown not to occur in rat MLR cultures (see § 7.2.2) but may be significant in PPD-stimulated cultures (see § 9.1). The mitogenic factor appears to affect B cells rather than T cells as might be expected since it is thought to be produced by activated T cells

(Dutton et al., 1971). Some proliferation of B lymphocytes may therefore occur in mixed populations even when a T cell stimulant is used. Mouse B lymphocytes, which do not respond directly to con A, have been reported to become mitotically activated in the presence of factors released by thymocytes (Andersson et al., 1972).

It is paradoxical that activation of lymphocytes should render them susceptible to growth of viruses within them (§ 10.3) and at the same time greatly enhance the production of interferon. The interferon is produced by human lymphocytes stimulated by PHA, antigens or allogeneic lymphocytes (Wheelock, 1965; Strander and Cantell, 1967; Greene et al., 1969; Gifford et al., 1971). The molecular weight of the interferon produced by PHA-activated lymphocytes is approximately 18,000 as judged by Sephadex G-100 chromatography and is similar to that produced by cells infected with Newcastle disease virus (Merigan, 1971). Interferon production is maximal some days after the peak of blast transformation.

10.2. Cytotoxicity

The many early claims to have demonstrated specific 'cell-mediated' cytotoxicity 'in vitro' were based on observations of destruction of target cell monolayers after the addition of large numbers of lymph node or spleen cells from immunised animals. Many of these effects were not truly 'cellular' but were due to liberation of antibody by the immune lymphoid cells during the incubation period; others may be seen, in retrospect, to have been due to the combined action of antibody, macrophages and lymphocytes. Very often the best designed experiments gave negative results, e.g. Medawar (1948) cultured fragments of lymph node or spleen from homografted rabbits in intimate contact with skin from the original donor with minimal disruption of either tissue. Under culture conditions far more physiological than those used by later workers he could detect no adverse effect on either the mitotic or migratory activity of the cultured epidermal cells.

We have now a very good idea of the way in which the various cellular and humoral components combine to produce the cytotoxicity. This is due in no small part to the persistent efforts of Professor Perlmann and his colleagues who have also produced a valuable review of the earlier work (Perlmann and Holm, 1969). Cells may be killed in many ways other than by lymphocytes such as by the classical action of antibody (to a cell surface antigen) and complement or by the combined action of antibody and macro-

phages or of antibody and granulocytes. Purified macrophages are also capable of marked extra-cellular killing (Lohmann-Matthes et al., 1972a) and human monocytes, although not initially cytotoxic to heterologous red cells, become so after incubation in culture and conversion to macrophages (Seljelid and Munthe-Kaas, 1973). It has already been mentioned that activated lymphocytes may, either directly, or by the release of a supernatant factor, enhance the activity of macrophages (see Evans et al., 1972). This review is concerned solely with lymphocyte-mediated cytotoxicity i.e. with the direct cytotoxic action of activated T lymphocytes, with the cytoxin or growth inhibitor found in lymphocyte supernates and with the cytotoxic effects of K 'lymphocytes' on target cells sensitised with specific IgG antibody.

10.2.1. Measurement of cytotoxicity

Cell death induced by antibody and complement is usually measured by observing the morphological change and loss of dye-exclusion under a phase contrast microscope. This is technically unsatisfactory for measurements of cell-mediated cytotoxicity when large numbers of blood lymphocytes or spleen or lymph node cells are added to relatively small numbers of target cells. Several workers have found it satisfactory when using glass-adherent target cells e.g. mouse fibroblasts or lung carcinoma cells, but detachment from the glass does not necessarily signify cell death. It is much more convenient to label the target cells with a radioactive substance and measure the release of the labelled substance which occurs when the cell dies. Cell death, whether produced by antibody and complement or by cellular means, is accompanied by leakiness leading to passage of intracellular constituents into the medium. Thus a variety of labels e.g. radioactive phosphate (labels RNA, DNA, phospholipid and acid-soluble small molecules), radioactive amino acids (label proteins) and radioactive thymidine (labels DNA which sheds off high M.W. fragments when the cell dies) have been used, but the most widely used label is now ^{51}Cr -chromate. This appears to label the cytoplasmic proteins which are released into the medium when the cell dies. The cells may best be labelled by adding a large amount of ^{51}Cr -chromate ($100 \mu\text{Ci}$) to a concentrated cell suspension (5×10^6 viable cells per ml) for a short time (30–60 min at 37°C) as recommended by Brunner et al. (1970). Cells may also be labelled by overnight incubation with ^{51}Cr -chromate ($1\text{--}3 \mu\text{Ci}$ per 10^6 cells in 1 ml of medium). Even over

long incubation periods, however, only a small percentage of added label will be bound by the cells. Growing cells incorporate more label than resting cells but all cells, including small lymphocytes and red cells, may be labelled to any desired level of radioactivity by suitable adjustment of the labelling conditions. If the target cells are from a line maintained in culture the ^{51}Cr -chromate may be added directly to a bottle of growing cells after changing the medium.

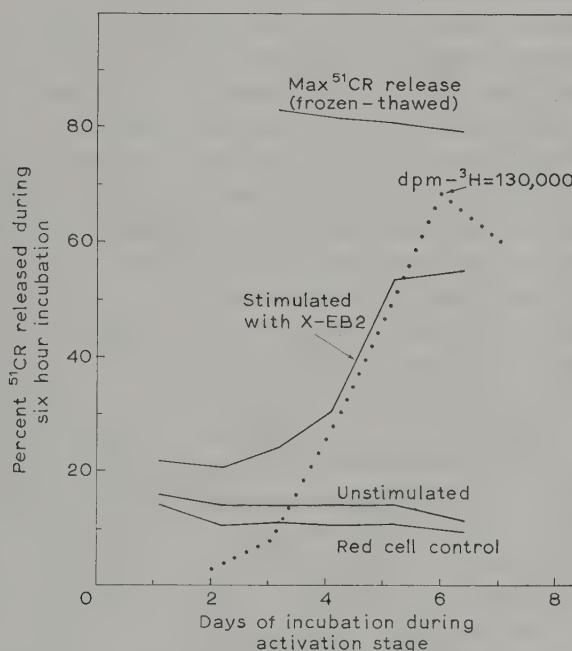


Fig. 10.1. Acquisition of cytotoxicity by human blood lymphocytes during the course of a mixed cell activation. Human lymphocytes (10^6 in 1 ml of medium) were cultured with 10^6 EB2 cells (X-irradiated with 6000 rads) or without stimulant. Some of the cultured cells were tested each day for their capacity to release ^{51}Cr from 10^6 EB2 cells labelled with ^{51}Cr . It will be seen that although some cytotoxic capacity is present in freshly isolated cells and that this has increased by day 3 the maximal cytotoxic capacity is not reached until day 5 or day 6 when large numbers of blast cells are present and there is a high level of ^3H -thymidine incorporation. The cytotoxic effect was measured over a 6 hr incubation period because the reaction had almost reached completion by this time and further incubation merely increased the 'background' of spontaneous ^{51}Cr -release from the target cells.

Labelled cells should be spun down and washed four times in small volumes of medium containing serum, the last wash being performed just before the cells are used. The labelled cells are mixed with the effector cells and, after incubation, the tubes are centrifuged, the supernate (or a measured part of it) removed and the percent release into the medium calculated from measuring the ^{51}Cr present in the supernate and that retained in the cell deposit. An example of the application of the technique will be found above (fig. 10.1). Only short-term incubation (up to 24 hr but usually 4 to 8 hr) is possible because the target cells release ^{51}Cr spontaneously at a fair rate e.g. 10% over 6 hr. Cells which are most readily killed often have a high background release whereas cells from which the label is released only very slowly, e.g. sheep red cells, are poor target cells. It is mainly because of the high spontaneous release of ^{51}Cr that the technique is not sufficiently sensitive to measure low grade cytotoxic effects e.g. of lymphokines.

Growth inhibition of a dividing cell population over a period of 24 hr or more is a more sensitive technique, but is obviously open to difficulties of interpretation of results because factors influencing the rate of growth of the cells may be measured instead of cell killing. In fact no cytolytic effects on a variety of target cells could be demonstrated using the supernates of cultures of activated lymphocytes under conditions in which the lymphoblasts themselves were markedly cytotoxic as judged by ^{51}Cr -release (Holm et al., 1973). It was concluded that the growth inhibitory factors present in the supernates were not primarily cytolytic but inhibited target cell metabolism and proliferation. Sometimes loss of function can be used as an indicator of cell damage e.g. loss of hemolytic plaque-forming capacity of immune spleen cells when incubated with allogeneic lymphocytes (Hirano and Uyeki, 1971). The ^{51}Cr release method has been extended to the detection of cellular sensitivity to soluble antigens by using target cells (mastocytoma) to which has been coupled the soluble antigen by means of a carbodiimide reagent (Henney, 1970).

10.2.2. Cytotoxic potential of lymphocytes activated by mitogens

It has for long been thought that lymphocytes have a direct effector role e.g. that in a skin graft they exert a direct cytotoxic action on the skin cells. There is reason to believe that this is true of T lymphocytes, but only after they have been activated *in vivo* or *in vitro*. The mechanism of the reaction and the factors governing the specificity of the killing events are little under-

stood. Most information has been derived from somewhat artificial systems, since these are the best controlled and are more susceptible to analysis. When it was first shown that the addition of PHA to a mixture of non-immune lymphocytes and allogeneic Chang liver cells or fibroblasts produced cell killing, it was not certain whether the PHA was acting as a 'sticky' substance bringing the lymphocytes into close proximity with the target cells or whether the damaging effect was associated with blastogenic activation. It was subsequently shown by Holm and Perlmann (1967) that lymphocytes activated by antigens (e.g. PPD), staphylococcal filtrate or allogeneic leucocytes also acquired cytotoxic capacity whereas treatment with non-mitogenic agglutinating agents such as polylysine was ineffective, thus establishing that metabolic activation of the lymphocytes was an essential part of the initiation of the cytotoxic process. Agents inhibiting respiration and glycolysis of the lymphocytes prevented acquisition of cytotoxicity. Once activation had occurred it was not abolished by X-irradiation, or by agents inhibiting DNA synthesis. Although the cytotoxicity was non-specific in that a variety of cells of various types and species were susceptible to killing, whatever the stimulating agent used to induce the lymphocyte activation, it was nevertheless shown to be a property associated only with immunocompetent lymphocytes. Leukaemic lymphocytes, Burkitt lymphoma cells and a variety of other cell types did not possess this capacity. Cell-cell contact was essential for killing to occur. Cell supernates were not cytotoxic. Thymocytes, although capable of some degree of mitotic activation, did not develop into 'killer' cells. Subsequent studies have in general confirmed most of these observations, but the simple view that T lymphocyte cytotoxicity is a direct function of the degree of metabolic activation of these cells has had to be modified in many respects. Activation of lymphocytes with a mitogen does not always produce cytotoxic lymphocytes. The cytotoxic effect, when present, may only be demonstrable with certain target cells. The mitogen, when present at the effector stage, may modify the target cells to make them more or less susceptible to killing. Even when non-specific stimulants are used, the killing reaction may not be entirely non-specific. In mixed cell activations, reactions varying greatly in degree of specificity have been reported. There are species variations both in relation to mitogen and mixed cell reactions. It has sometimes been possible, under exceptional culture conditions, to produce cytotoxic cells from thymocytes of some species. It is convenient to consider the mitogen studies first and the mixed cell studies afterwards.

Human lymphocytes activated with PHA have received most attention. They have been shown to be cytotoxic to some target cells (Chang human liver carcinoma cells, various other tumour-derived cells of human origin, rat and mouse fibroblasts and chicken red cells) but other target cells (human red cells, lymphocytes or Burkitt lymphoma cells for instance) are not readily lysed (Perlmann and Holm, 1969). However, human blood lymphocytes (even autologous cells) are lysed if they are first treated with PHA for a short period. Attempts to produce similar cytotoxic reactions with mitogen-activated animal lymphocytes have been largely confined to the rat and the mouse. Significant cytotoxic reactions have again sometimes been obtained only when mitogen was present at the killing as well as the initial activation stage (Asherson et al., 1973). This double requirement for mitogen may raise doubts about the biological significance of the reaction, but it should be emphasised that, even with mitogen present, only activated T cells have rapid killing capacity. When the cytotoxic properties of PHA-activated human and mouse lymphocytes are compared, some kind of specificity is observed. Mouse lymphocytes activated by PHA are more cytotoxic to mouse L fibroblasts than to human Chang cells whereas the reverse is true of human lymphocytes, suggesting that allogeneic cells are more readily killed than xenogeneic (Stejskal et al., 1973). As in the human system mouse PHA blasts are not cytotoxic to all target cells. No killing of mouse embryo monolayer cells was obtained with mouse PHA blasts under conditions in which lymphocytes activated by the monolayer cells themselves were cytotoxic (Berke et al., 1969).

Animal lymphocytes activated by other mitogens have frequently been shown to have cytotoxic properties, but again there have been failures, some of which could be due to a requirement for lectin at the effector as well as at the activant stage. Mouse lymphocytes activated by con A (lymph node cells but not bone marrow cells or thymocytes) develop cytotoxicity to mouse fibroblasts (Stavy et al., 1972). Con A activated hamster lymphocytes have also been shown to have cytotoxic activity (Singh and Tevethia, 1973). Spleen cells were more effective than lymph node cells and it was important to use low concentrations of con A as high concentrations were ineffective or inhibitory. The lytic reaction did not require the presence of con A. In some systems con A has been found not to produce cytotoxic effects and even to inhibit PHA-induced cytotoxicity. Results with PWM more closely resemble those obtained with PHA. Rat lymphocytes exposed to PWM underwent morphological changes indicative of activation, but were not

cytotoxic to mouse or rat embryo monolayer cells unless PWM was also present at the effector stage (Ginsburg, 1971).

In contrast to peripheral T cells, lymphocytes obtained directly from the thymus are not cytotoxic to target cells following treatment with a mitogen. In studies on human foetal cells, for example, Stites et al. (1972) showed that spleen and peripheral blood cells were cytotoxic to chick red cells in the presence of PHA whereas thymocytes were not. It is known that only a small percentage of thymocytes undergo mitotic activation in response to stimulants and it may be that the major inactive population blocks the cell-cell interactions of the minor population in cytotoxicity reactions. Cultured thymocytes have, however, sometimes been found to develop cytotoxic potential and it seems likely that there is a requirement for a further differentiation stage of the minor population which is only achieved under very good culture conditions. Successful maturation of mouse thymocytes to cytotoxic cells has been reported by Wagner et al. (1972). In mixed cell cultures (see § 10.2.3) mouse thymocytes, particularly those from cortisone-treated animals (i.e. in which the proportion of the 'mature' minor population is increased), will develop into active killer cells (Mosier and Cantor, 1971).

10.2.3. Cytotoxic potential of lymphocytes activated by allogeneic cells

Cytotoxic lymphocytes are always generated when human peripheral blood lymphocytes are stimulated in a mixed cell reaction (Balfour et al., 1972). They are cytotoxic to Chang cells and to cells from human lymphoid cell lines but not to allogeneic small lymphocytes or to lymphoblasts produced in a mixed cell reaction. Blast cells produced by activation of human adult or cord blood lymphocytes during culture with human lymphoma cells show similar properties, neither small lymphocytes or blast cells being killed under conditions in which lymphoma cells are readily killed. These mixed cell-generated cytotoxic reactions do not require addition of any mitogenic or 'sticky' substance and appear to be much more closely related to *in vivo* reactions than those hitherto considered. However, even in mixed cell systems mitogens alter the properties of target cells. Treatment of small lymphocytes with PHA or culturing them for 2–3 days with PHA renders them susceptible to killing. Both syngeneic and allogeneic cells are similarly affected and the major part of the reaction appears to be quite non-specific. Using smaller amounts of a purified PHA to produce the target lymphoblasts

it appears to be possible to eliminate the non-specific auto-killing while retaining an allogeneic killing reaction, the specificity of which is related to the HL-A type of the 'stimulator' cells (see Miggiano et al., 1972 and ch. 7).

Human lymphocytes activated by culture with cells from a continuously growing human lymphoid cell line become cytotoxic to the cells from the same line and to other target cells (see § 10.4 and § 7.1.8). Cytotoxic activity is gradually acquired during culture, the curve roughly coinciding with the rise in DNA synthesis (fig. 10.1). Red cells and normal lymphocytes are not killed by the activated lymphocytes whereas most lymphoid cell line cells, or mouse lymphoma and Chang liver cells are susceptible to killing. There was some suggestion in these experiments that cells of the lymphoid line used for the activation were more readily killed than cells of another line, but target cell susceptibility was not correlated with HL-A disparity of donor and target cells. Cells from an autochthonous line could be used both for the activation and killing stages suggesting that the whole reaction may have some importance as an immunological surveillance mechanism. Cells from certain lymphoid lines were much more readily killed than those from some other lines whether normal allogeneic lymphocytes or cells from any of a variety of lymphoid lines were used for the activation stage. In the human system there appear to be properties of the target cell, unrelated to antigenic structure in any obvious way, which determine susceptibility to killing. No antibody or supernatant material or lectin was required to assist the activated lymphocytes in the killing of the target cells. The purer the lymphocyte preparation the better the killing, so that it is unlikely that macrophages play any important part in the killing in this system. The reaction is almost certainly due to a T lymphoblast acting directly on the target cell, the killing resulting from a surface-surface interaction. The killing reaction is rapid, reaching apparent completion a few hours after mixing. The normal lymphoblasts appear to survive the killing reaction i.e. it is not a suicide event, and they are capable of killing a fresh inoculum of target cells. It might be expected, therefore, that a comparatively small number of activated blood lymphocytes could entirely prevent the growth of a cell line culture. This does not in fact appear to be the case. Cultures of cell line cells fail to grow if two or three times their number of normal lymphocytes are added but a small number of lymphocytes has little effect on the growth of the cell line cells. This suggests that the activated lymphocytes are able to kill only a few target cells.

In the rat and the mouse the cytotoxic potential of lymphocytes activated

by foreign cells in an *in vivo* or an *in vitro* situation has been much studied. Rat lymphocytes grown for several days over mouse embryo monolayers become activated to large pyroninophilic cells which are cytotoxic to the monolayer cells and to fresh embryo monolayers from the same strain of mouse (Ax et al., 1968). Rat lymphocytes cultured on monolayers of embryo-derived cells of the same outbred rat strain also became activated and developed cytotoxicity (Ginsburg and Lagunoff, 1968). The rate of the ^{51}Cr -chromium release was proportional to the number of large pyroninophilic cells present and the specificity of the killing reaction was related to the antigenic composition of the monolayer cells on which the lymphocytes had been trained (Berke et al., 1969). Using a similar system Lohmann-Matthes et al. (1972b) found that optimal sensitisation of lymphocytes required 48 hr of close contact with allogeneic fibroblasts. After this time the lymphoblasts could be transferred to syngeneic monolayers for differentiation to reach completion. It was during this period that the lymphoblasts developed cytotoxicity. Maximal cytotoxicity required a 5-day incubation period. Mouse lymphocytes may become sensitised in a similar fashion to hamster tumour cells (Beverley and Simpson, 1972). Sometimes a one-way mixed lymphocyte reaction has been used as a way of generating cytotoxic cells as in the human system. For example, lymphocytes of CBA (H-2k) mice cultured with mitomycin-treated BALB/c (H-2d) spleen cells for several days developed cytotoxicity to ^{51}Cr -P815 mastocytoma (H-2d) cells (Wagner et al., 1972). One-way mixed cultures containing C3H lymphocytes plus C3H.DBA/2 F₁ hybrid lymphocytes developed cytotoxicity after several days to L5178-Y (DBA strain) lymphoma cells (Hayry and Defendi, 1970). Sometimes lymphoma cells have been used for the activation phase. Mouse spleen cells (2×10^7) incubated for 5 days with allogeneic lymphoma cells (1×10^4) developed cytotoxicity towards ^{51}Cr -labelled lymphoma cells (Wunderlich and Canty, 1970).

An elegant series of experiments has been performed in which allogeneic mouse lymphocytes have been sensitised to DBA antigens and tested for cytotoxicity against ^{51}Cr -DBA-mastocytoma cells. Lymphoid cells from spleen lymph nodes or blood of C57BL/6 mice which had been injected with a DBA lymphoma or mastocytoma developed maximal cytotoxicity to ^{51}Cr -mastocytoma cells 10–11 days after immunisation (Brunner et al., 1970). The cytotoxic activity of the circulating lymphocytes reached a peak 20–60 days after immunisation while the activity of spleen cells dropped to low levels in the same period. Although the cytotoxic effect was thought to be

specific for DBA antigens there was considerable variation according to the type of DBA cell used as a target cell such that mastocytoma>lymphoma>lymphocyte. It was thought that antigen density differences could account for the result. The reaction required direct contact between effector and target cell and was inhibited by antibody to cell surface antigens. Strong evidence for identifying the cytotoxic cell as a T cell was obtained by transferring C57 thymocytes into lethally irradiated DBA mice and showing that the injected cells after growth (in the presence of DBA antigens) in the recipient spleen developed cytotoxicity to DBA mastocytoma cells (Cerottini et al., 1970). By prior fractionation of the thymocytes over a bovine albumin density gradient it was shown that the thymic progenitors of the cytotoxic cells were a minor population of medium density containing high concentrations of H2 and low concentrations of θ antigens (Shortman et al., 1972). Cytotoxic cell progenitors in spleen differed from those in thymus in having a higher buoyant density typical of that of the peripheral small lymphocyte. A similar conclusion was reached from experiments in which intact or thymectomised CBA or A . CBA F₁ mice were injected with DBA/2 mastocytoma. The spleen cells of normal but not of thymectomised mice at 11 days were cytotoxic to ⁵¹Cr-mastocytoma cells (Miller et al., 1971). Since reconstitution was possible with thoracic duct lymphocytes from a normal syngeneic mouse and since thymocytes also developed cytotoxicity in a suitable *in vivo* environment it was concluded that T lymphocytes react with cells bearing foreign histocompatibility antigens and give rise to progeny which develop cytotoxic activity.

Direct cell-cell killing by thymus-derived lymphoblasts may appear as anything from a highly specific, antigen-dependent killing to a quite non-specific and even, perhaps, non-immunological process according to the system used and the technique employed. The most artificial systems are those in which a sticky substance such as PHA has been added. It has already been mentioned that lymphoblasts from a mixed cell reaction will kill PHA-treated autochthonous as well as allogeneic lymphocytes. Non-immune lymphocytes can also be induced to exert cytotoxicity on autochthonous and allogeneic fibroblasts by PHA, ALS and antigen-antibody complexes (Lundgren et al., 1968). In these circumstances the added substances have an effect other than mitogenic activation. Mutual *in vitro* cell destruction was also noted by Granger and Koller (1968) when non-immune or immune mouse or rat small lymphocytes were added to genetically dissimilar target cells with PHA or xenogeneic antibody. A similar

reaction has been described by Tridente and Yung (1973). If human or mouse lymphocytes are treated with PHA for a brief period and then cultured with allogeneic lymphocytes, the percent inhibition of the PHA response (representing the damage to the PHA-treated target cells) appears to be a measure of the degree of histoincompatibility of the two populations. Another cytotoxic reaction of the same type was reported by Moller and Lapp (1969). Lymphoid cells from tolerant mice of strain A, actually carrying (A:CBA)F₁ skin grafts for 1-6 months, were found to be competent to cause destruction of both (A:CBA)F₁ and CBA fibroblast target cells in tissue culture in the presence of PHA. In experiments of this type the strange self-destruction reactions obtained might be explained by unphysiological cell-cell interactions induced by the 'sticky' agents added. In systems in which activation is produced by mixed cell reactions this objection cannot be raised. Yet the blast cells generated in a human mixed lymphocyte reaction are cytotoxic to a wide range of allogeneic and xenogeneic cells. Moreover, lymphocytes activated by mixed cell reactions *in vivo* have been shown to exert a cytotoxicity of low specificity (Singh et al., 1972). Spleen cells from F₁ hybrid mice undergoing graft-versus-host reactions were cytotoxic to syngeneic, allogeneic or xenogeneic target cells. The reaction required direct cell-cell contact. Specific or partially specific effector cell reactions have been reported to arise from some *in vitro* mixed lymphocyte reactions in the mouse (Hayry and Defendi, 1970; Svedmyr and Hodes, 1970). From careful studies of the cytotoxic effects of human lymphocytes activated by lymphoid cell line cells of various HL-A types we have had to conclude that the ease with which target cells are lysed by activated lymphocytes does not appear to be related in any obvious way to their antigenic constitution. Regardless of HL-A type some lymphoid cell line cells are killed more readily than others and fibroblasts, red cells and normal lymphocytes are very difficult to kill. According to Brondz and Golberg (1970), the immune lymphocyte has a complex of receptors to a whole set of immunising antigens and is able to interact with the target cell only if the matching between the complex of lymphocyte receptors and target alloantigens is close enough. This expresses a concept which approximates to what most immunologists would find most likely and most acceptable, but which does not much help in explaining the killing reactions observed in practice in human mixed cell systems.

10.2.4. Some features of the cytotoxic process

The generation of cytotoxic lymphocytes in a mixed cell system is a slow process taking several days. The cytotoxic event, by contrast, is rapid and measurements are usually made over a period of hours. In rodent and human systems the cytotoxic potential of the lymphocyte population is proportional to the number of lymphoblasts in the early stages of culture. At later stages smaller cells (possibly the progeny of blast cells) also show cytotoxic activity. The reaction is typically first order both with respect to target and effector cells indicating that killing is a one-hit phenomenon (see Berke et al., 1969 and Henney, 1973). The latent interval preceding events initiating the cytolytic process is very short. Release of small molecules from the target cell begins almost immediately and is followed later by the release of large molecules just as it is when cells are killed by antibody and complement (see ch. 8). There is an inverse correlation between the intracellular levels of cyclic AMP and cytolytic activity (Henney et al., 1972). Adenyl cyclase stimulating agents (histamine, isoproterenol and prostaglandins) raise the cAMP level and inhibit cytotoxic activity. The importance of permitting or enhancing membrane-membrane interactions between effector and target cells has already been stressed. Agents such as cytocholasin which appear to inhibit these interactions are inhibitory (Plaut et al., 1973), while lectins sometimes enhance cytotoxicity. The importance of an initial membrane interaction is also supported by the finding that potential killer cells in a population may sometimes be preferentially eliminated by removing target cell-adherent lymphocytes (Golstein et al., 1972). These are some of the clues we now have to the mechanism of the killing reaction. This information has been supplemented by morphological studies.

Activated lymphocytes are much more motile than small lymphocytes and may be seen to crawl over target cells. Lymphocytes activated by PHA rapidly infiltrate monolayers of target cells and, after 18 hr, target cells covered with lymphocytes become detached from the surface (Biberfeld, 1971). Lymphocytes may attach to target cells and undergo vigorous movements while still attached or remain quietly adherent for long periods. They may then detach and migrate rapidly and apparently randomly. Occasionally the target cell retracts pseudopodia or detaches from the surface. Events which are frequently indistinguishable from these may, however, also be seen in syngeneic lymphocyte-target cell mixtures or in allogeneic mixtures in which no target cell killing can be demonstrated by

isotope-release methods. From morphological observation it is very difficult to deduce which of the many cell-cell interactions seen relate to the cytotoxic events.

In Summary it may be said to be well established that peripheral lymphocytes acquire cytotoxic capacity when activated by antigens, foreign cells or common stimulants. The cell responsible is a thymus-derived lymphoblast. Resting small lymphocytes do not have this property. Mitotically activated thymocytes do not initially possess it either, but may acquire it after a maturation period in culture. The killing is a result of an unknown process consequent upon direct cell-cell contact between effector and target cell. The intimate cell-cell contacts necessary for cell killing occur spontaneously in some human and mouse systems in which lymphocytes have been activated by allogeneic cells. These reactions mimic events which could well occur *in vivo*. In other systems a mitogen has to be added at the killing stage and may act by artificially inducing effector cell-target cell associations or by modifying the membrane of the target cell. In neither of these types of killing is an antibody or a supernatant co-factor involved and it is unlikely that co-operating cells (B lymphocytes or macrophages) are required either. The killing is relatively rapid and is not a suicidal event. The chief enigma at present is the degree of specificity of the killing, and the nature of the 'education' process when specificity is found. Specificity is generally not found with activated human lymphocytes as effector cells whereas it has been found more often than not in mouse systems although few systematic studies have been made. Some of the discrepancy may be due to the fact that the sensitisation stage with human lymphocytes has usually been performed *in vitro* while that with mouse lymphocytes has been *in vivo*. However, mixed cell reactions *in vitro* have been used successfully in mouse systems to produce specific killer cells while under very similar conditions killing of low specificity has been obtained with human lymphocytes.

10.2.5. Antibody-assisted lymphocytotoxic reactions

It has been known for a long time that the combined action of antibody to surface antigens and complement is lytic to cells. Attachment of antibody to a cell surface also opsonises the cell so that phagocytes more readily attach to it and ingest it. The opsonic effect of IgG antibody for mononuclear phagocytes depends on the Fc piece of the molecule, whereas IgM antibodies

are opsonic for mononuclear and polymorphonuclear phagocytes only in the presence of complement. Early investigations of the effect of antibodies on lymphocyte cytotoxicity revealed that inhibitory or 'blocking' effects were often obtained. These observations were made in systems in which it is now known that the killing was brought about by activated T cells, the cell-cell interactions involved being impeded by antibody to sites on effector or target cells. By contrast, marked enhancement of the cytotoxicity of non-immune lymphoid cells on some target cells (chicken red cells, PPD-coated chicken red cells or Chang cells) was noted following the addition of sublytic amounts of antibody to sites on the target cells (see Perlmann and Holm, 1969). Lymphocytes from tolerant animals were also effective once the target cells were sensitised with antibody (Brurenik et al., 1970). The role of the lymphocytes apparently implicated in these reactions appeared to be different to that of the lymphocytes involved in direct cell killing reactions in several respects. Antibody priming was essential whereas blastogenic activation of the lymphocytes was not required. The effector cell population was distinct from that responsive to PHA (MacLennan and Harding, 1970). It was suspected that B lymphocytes rather than T were involved because spleen cells from thymectomised animals were as active as those from normal animals (Van Boxel, 1972). The distribution of the effector cells in rat and mouse tissues (high activity in spleen and peritoneal exudates, much less in lymph nodes, slight activity of thoracic duct cells compared with marked activity of blood cells) confirmed that they could not be T cells. The reasonable assumption that this non-T cell cytotoxicity of a lymphocyte-like cell was mediated by a B lymphocyte, proved, on subsequent study, to be false (see Greenberg et al., 1973). Removal of immunoglobulin-bearing cells from mouse spleen suspensions did not remove the effector cells and populations enriched with immunoglobulin-bearing cells did not show enhanced activity. When mouse spleen cells were fractionated by velocity sedimentation the cytotoxic activity of the fractions did not correlate with the presence of small or large immunoglobulin-bearing cells. Thus it was established that, although the effector cell might resemble a lymphocyte morphologically, it did not possess the functional, antigenic or physical characteristics of a T or a B cell. In its tissue distribution in mice and in its possession of receptors for the Fc piece of IgG, the effector cell closely resembles a monocyte and it is probable that pro-monocytes and other cells of the monocyte series are responsible. The closely similar distribution of monocytes and effector cells of mouse spleen after fractionation by density

gradient or velocity sedimentation provides strong supporting evidence (Greenberg et al., 1973). The cells differ from monocytes in not being phagocytic but probably acquire this property at a later stage of development. Phagocytosis is evidently not necessary for killing to occur; in fact even phagocytes appear to be capable of extracellular killing.

Human blood contains cells of a similar type which are potent cytotoxic agents against target cells coated with IgG antibody e.g. sheep fibroblasts coated with rabbit IgG antibody (Moller and Svehag, 1972) or human cells bearing HL-A antibodies (Hersey et al., 1973). It is probable that they, too, will be identified as cells of the monocyte series. In both mouse and human systems these cells are now called 'Killer cells' or 'K cells'. This is a general term, deliberately imprecise and already there is evidence that more than one cell type may be implicated (Zighelboim et al., 1973). The extent to which this type of killing occurs *in vivo* is uncertain. As in the activated T cell killing there is a great deal of variation of the level of killing with the type of target cell selected for study.

10.2.6. Cytotoxic effects of soluble substances released by lymphocytes

In many systems in which lymphoblasts exert a readily measurable cytotoxic effect it is impossible to reproduce this effect with the supernatant fluid from the culture in which the active lymphoblasts were generated or with the medium in which effector and target cells were incubated. Using morphological or growth-inhibition or other criteria (e.g. by measuring the release of label from ^3H -thymidine-labelled target cells or the inhibition of protein synthesis or by Coulter counter evaluation of cell death) the release of cytotoxic or cytoinhibitory substances has, however, been clearly demonstrated in a variety of culture systems (Bloom and Glade, 1971). A toxic substance has been found in the medium from mixed cultures from some donors. It has been found in the supernates of PHA-stimulated lymphocyte cultures and is released by the action of PPD on lymphocytes from a tuberculin-sensitive individual (Granger and Williams, 1968; Granger et al., 1969). Guinea pig lymph node and spleen cells stimulated with con A appear to release material cytotoxic for mouse L-fibroblasts (Schwartz and Wilson, 1971). Using the formation of pocks on the chorioallantoic membrane of embryonated eggs as an indicator of cytotoxicity, Pinto et al. (1972) found that syngeneic chicken lymphoid cells acquired pock-forming capacity after incubation with the supernates of antigen-stimulated lymphocyte cultures.

The pock stimulating factor was heat-stable and essentially non-dialysable. It is not clear what relation lymphotoxin killing bears to whole cell killing. Whole cell killing is thought by some to be merely a more efficient means of delivering the lymphotoxin to the target cell, death being brought about by the local release of the lethal substance or (to meet the specificity requirement) a direct transfer of it to the target cell. The many indicator systems for measuring growth inhibition or cell killing may be measuring different substances or the same substance at different levels of concentration.

The possible involvement of lymphotoxins in anti-bacterial immunity should not be overlooked. The inhibition of multiplication of virulent tubercle bacilli in mouse peritoneal macrophages brought about by incubation with splenic lymphocytes from immunised mice may be mediated by such a substance (Patterson and Youmans, 1970). Activation of macrophages greatly enhances their capacity to kill bacterial as well as foreign animal cells and this is an important way in which lymphokines may assume an effector role.

10.3. Growth of viruses and mycoplasmas in lymphocyte cultures

Virus-lymphocyte relationships are of interest from several points of view:

- a) Viruses of many types have been shown to grow much more readily in activated than in resting lymphocytes. This enhanced growth appears to be associated with metabolic events accompanying activation (see § 13.6).
- b) Paradoxically, the process of activation which renders the lymphocyte more susceptible to viral replication within it, also enables it to produce an interferon-like substance.
- c) 'Cell-mediated immunity' is known to play a vital role in combating certain viral infections notably pox, herpes and papovavirus infections (Merigan, 1974). Immunity is related to T lymphocyte function.
- d) Viruses which grow in T or B lymphocytes may damage normal immunological defence mechanisms. They may kill the lymphocytes in which they grow or they may 'transform' them into abnormal proliferating lymphoid cells with consequent disorganisation of lymphoid tissue and interference with normal patterns of lymphocyte migration.
- e) Viral-transformed lymphocytes may have abnormal surface characteristics and, as a result, they may be eliminated by one of the cytotoxic mechanisms described elsewhere in this chapter. These mechanisms, however, may themselves be impaired if the virus will grow in the particular type of lymphoid cell

concerned. f) Lymphocytes may transitorily acquire specific resistance to the growth of a virus.

The discovery that viruses will grow in PHA-activated lymphocytes, but not in small lymphocytes, was first made with herpes simplex virus. Similar results were later obtained with mumps, vesicular stomatitis, vaccinia, yellow fever, polio and other viruses (see § 13.6). The phenomenon appears to be a general one, occurring with RNA and DNA viruses and with lymphocytes of many species stimulated with a variety of mitogens. Poliovirus and vesicular stomatitis virus (VSV), for example, will grow in lymphoblasts produced by antigen-stimulation or ALS as well as in PHA blasts (Kleinman et al., 1972). Herpes simplex grows in human lymphocytes stimulated with PHA, PWM, con A or ALS, the virus titre correlating better with the level of DNA synthesis than the number of blast cells present (Kleinman et al., 1972). Electron microscope studies have confirmed that viral replication in PHA cultures is limited to the blast cells. The most detailed studies have been made with VSV. It was shown that the VSV replication titre in PHA-stimulated preparations of unpurified human leucocytes was approx. two logs higher than in unstimulated cultures. Furthermore what replication of VSV did occur in untreated cultures was in the monocyte-macrophage population (Wheelock et al., 1971). When purified human lymphocytes were exposed to various doses of PHA for 16 hr, then infected with VSV, and the virus yield and enhancement of ^3H -uridine incorporation measured over the subsequent 24 hr, it was found that even a two-fold stimulation of ^3H -uridine incorporation was accompanied by significant VSV replication. Maximum VSV yields were obtained from cells undergoing 10–14-fold stimulation in response to 5 µg PHA (approx. 3 logs increase in VSV titre). Further increase in the PHA concentration increased the ^3H -uridine incorporation but reduced VSV replication (Wheelock et al., 1971).

The growth of VSV or Newcastle disease virus in lymphoblasts has been adapted by Jimenez and Bloom (1971) as a virus-plaque assay for antigen-sensitive cells. There is good evidence, at least in the case of VSV, that it is activated T lymphocytes in which growth of virus occurs. Con A and PWM produced 33-fold and 17-fold increases respectively of the growth of VSV in mouse spleen cells whereas *E. coli* lipopolysaccharide (LPS), which increased ^3H -thymidine incorporation to the same degree as PWM, produced only a 2–3 increase in VSV titre (Kano et al., 1973). Part of the LPS increase may have been due to macrophage stimulation. The numbers of virus-infected (plaque-forming) cells were 6% (with con A), 3% (with PWM) and

0.2% (with LPS). Populations of spleen cells depleted of T cells with anti θ or 'nude' mouse spleen cells did not produce virus-plaques after stimulation with con A or PWM. Mouse thymocytes, thought to contain fewer than 1% B lymphocytes, could be stimulated by con A to produce as many as 3% virus-plaques.

Many of the viruses which will grow in activated lymphocytes in vitro produce a demonstrable inhibition of lymphocyte growth (see Wheelock et al., 1971 for references). Rubella virus (an RNA virus) causes a marked inhibition of the DNA synthesis of PHA-stimulated human lymphocytes (Olson et al., 1968; Knight and Najera, 1969). The inhibition was obtained when the small lymphocytes were treated with virus and washed before addition of PHA, suggesting that the virus had attached to or entered the small lymphocytes. The course of the virus infection may vary. In most cases (e.g. with herpes simplex or VSV) the end result is death of the lymphoblast. In other cases viral transformation may occur with the production of an abnormal proliferating cell. It has been known for some time that the ease with which human lymphoid lines may be established is improved by adding X-irradiated EB virus-bearing cells from another lymphoid line.

Lymphocytes which have been infected with and modified by virus in such a way as to alter their surface characteristics may be reacted against by mechanisms described for the destruction of foreign cells. This is most likely to be successful if the effector cell itself is not susceptible to the virus concerned. For example, the EB virus appears to infect and transform B lymphocytes, but the infected cells are susceptible to killing by activated T lymphocytes. Most of the viruses discussed grow well in activated T lymphocytes and may defeat this effector mechanism. However, lymphocytes appear to be able to inactivate some viruses directly. It has been shown that the myxoviruses Sendai, parainfluenza 1, influenza and VSV are inactivated in cultures of normal human or mouse lymphoid cells in the presence or absence of mitogens (Zisman and Denman, 1973). Fractionation of the cells showed that some lymphocyte populations but not macrophages or cells producing antibody to sheep red cells were responsible for the virus inactivation. Studies have also been made of lymphocyte induced inactivation of 17D yellow fever virus. Blood lymphocytes tested pre-vaccination and during the first 5 days following vaccination of the donor were found to support virus replication in cultures treated with virus and PHA. In contrast, lymphocytes removed from the circulation between the 7th and 14th day after

infection failed to produce infective 17D virus under the same culture conditions. By the 25th day, however, peripheral lymphocytes had regained their capacity to support the replication of 17D virus (Wheelock et al., 1971). There were no differences in PHA responsiveness or interferon production of the blood lymphocytes or in the capacity to support the growth of an unrelated virus (VSV). No free 17D virus neutralising antibody was produced. Resistance was thought to be due to a cytophilic antibody or a specific intracellular resistance.

Viral antigens are able to induce immunospecific lymphocyte activation in the same way as other protein antigens (see ch. 9). Inactivated mumps virus has been shown to provide a potent stimulus for lymphocytes of individuals primarily immunised to the virus with persistence of reactivity for up to 40 years and good correlation with delayed hypersensitivity (Smith et al., 1972). The response was related to antigen dose and required the presence of a radioresistant adherent cell population.

Mycoplasmas, even when non-viable, can inhibit the response of lymphocytes to PHA (Copperman and Morton, 1966). The inhibition could be reversed by removing the mycoplasma hominis as late as 48 hr after inoculation. Mycoplasma arginini inhibits lymphocyte proliferation indirectly by removing arginine from the medium. At least one mycoplasma can produce a non-specific lymphocyte activation (see ch. 6).

10.4. Lymphoid cells grown in continuous culture (LCL cells)

During the eleven years since Pulvertaft's original observation that lymphoma cells from a Burkitt tumour proliferated rapidly in vitro and continued to grow over a long period, innumerable other human lymphoid lines have been established. There are now very large numbers of these lines in use in laboratories throughout the world (see § 3.1.13). It is usually about 50 days before the culture population reaches establishment level, and the monoclonal population resulting may be regarded as having undergone abnormal proliferation. The morphology, growth characteristics and immunological and biochemical properties of these LCL cells have been extensively studied. Microscopically, cells of most lines superficially resemble PHA-blast cells, but cells which look like small lymphocytes, plasmablasts and macrophages are also present (see § 5.1.7). Cells of many lines grow in clumps, which are usually loose gatherings of cells readily dispersed by

shaking. The majority of the cells do not stick to glass. Motility is much less marked than that of normal lymphoblasts and different in character. Pseudopodia are not regularly thrown out and withdrawn in the way that they are from normal lymphocytes and the cells do not crawl over the surface of other cells in the way that normal lymphocytes do. There is nothing resembling a uropod present.

It has been of great interest to determine similarities and differences between the properties of LCL cells and the normal lymphocytes of the original donor and the extent to which the biochemical characteristics and antigenic expression of the cell line cells remain constant over long periods. Extensive studies of the enzymic expression of LCL cells have been made. Acid phosphatase is commonly present but alkaline phosphatase and peroxidase are not detectable by histochemical tests. Iso-enzyme profiles have been used as genetic markers and their persistence studied. Characteristic phosphoglucomutase polymorphism was found to be retained over long periods (Conovers et al., 1970). A similar constancy has been found in the expression of surface antigens. The HL-A serotype of cells of LCL initiated one year or more previously is similar to that of the fresh lymphocytes of the original donor (see Mackintosh et al., 1972, for references). Some 'extra' reactions are revealed with certain typing sera. The quantity of HL-A antigens per cell is much greater for the LCL cells than for small lymphocytes and even when allowance is made for the greater surface area of the LCL cells it is found that the antigen density per unit area, as determined by absorption experiments, is greater by a factor of about 12 (Rogentine and Gerber, 1970). LCL are in fact an excellent source of HL-A antigens for isolation studies (Reisfeld et al., 1970; Miyakawa et al., 1971). There are antibodies in normal serum to unidentified antigens on LCL cells and to EB virus-coded antigens, but these do not account for the 'extra' reactions obtained with some typing sera. The similar typings in different laboratories of some Burkitt lymphoma lines maintained for very long periods *in vitro* are evidence for the stability of the HL-A antigenic expression of these cells. The 'extra' reactions obtained may merely reflect an extra sensitivity of the cytolytic test consequent on the quantitatively greater expression of antigen on these cells, or may represent the emergence of new antigens (possibly embryonic antigens or cell division antigens) associated with the high metabolic activity of the LCL cells. Moore and Woods (1972) also found the HL-A antigenic pattern of LCL to be remarkably stable despite alterations in chromosome constitution.

Often, when the full complement of HL-A antigens was not detected on fresh lymphocytes, the 'missing' antigens were detectable on the LCL cells but in other instances the number of antigens detectable on the LCL cells was greater than expected on a two-locus hypothesis.

All of more than 100 lines tested by Moore et al. (1968) were shown to produce immunoglobulins or free heavy or light chains. These proteins are indistinguishable antigenically from normal immunoglobulins of serum. Occasionally a line is encountered which does not produce immunoglobulin (Moore and Minowada, 1972). Different lines produce and secrete into the medium IgG, IgM or IgA of either kappa or lambda light chain specificities (Fahey et al., 1966; Matsuoka et al., 1967; Osunkoya et al., 1968). Most lines produce about 3 µg per g per day. Two lines producing γ and α heavy chains have been described (Takahashi et al., 1968). By immunofluorescence it was shown that all immunoglobulin-producing cells contained both of the heavy chains and this result was confirmed by cloning experiments. Immunoglobulins were found to be produced by all of 22 lines derived from patients with hypogammaglobulinaemia, myeloma and Waldenstrom's macroglobulinaemia (Stites et al., 1971). This must mean that at least some of the blood lymphocytes of hypogammaglobulinaemic donors are capable of immunoglobulin biosynthesis in accord with the demonstration that immunoglobulins are synthesised in short-term blood lymphocyte cultures from these patients. Since the production of immunoglobulins by LCL cells is proportional to their growth rate it is quite conceivable that lymphocytes which, in a resting stage, are not producing detectable amounts of immunoglobulin can be shown to produce them when induced to proliferate continuously. There have been few successful attempts to establish LCL from cells of myeloma patients. One produced only free lambda chains and these were identical to those found in the patient's urine (Matsuoka et al., 1967). Two lines have been established from blood and bone marrow cells of a patient with an IgE myeloma. One line produced IgE-lambda identical to that in the patient's serum and the other IgG-kappa (Nilsson et al., 1970). LCL have been successfully cloned in soft agar and the immunoglobulin profiles of the several clones have been shown to be identical, indicating that the cell lines are monoclonal with respect to immunoglobulin production. Almost all cells after hydroxyurea blocking are actively synthesising immunoglobulin at the period before DNA synthesis. The LCL maintain their characteristic immunoglobulin production over long periods of culture.

Little attention has been paid to the biosynthesis of other soluble proteins

by LCL cells but $\beta 1c$ globulin (C3) has been shown to be produced by one line (Glade and Chessin, 1968). Interferon is another product. There has been one report that LCL will produce small amounts of antibody after incubation with antigen (Kamei and Moore, 1968). It would be wrong to conclude from this that LCL cells are immunocompetent cells. The production of a MIF closely related in properties to that produced by the action of antigen on sensitised lymphocytes (Tubergen et al., 1972) is also no proof of immunocompetence as fibroblastic lines produce a similar substance. The same may be said of reports of the production of other substances with activity resembling that of lymphokines e.g. lymphocytotoxins (Granger et al., 1970) and weak mitogenic effects. The capacity to produce damage to renal tissue when injected under the kidney capsule, a reaction classified as a GVH reaction indistinguishable from that produced by normal lymphocytes in a graft-versus-host reaction (Gordon et al., 1971) is likewise no indication of immunocompetence when one remembers that these are robust, dividing cells which have the capacity to grow in a malignant manner in newborn rats and immunosuppressed newborn hamsters (Southam et al., 1969).

Cells from human LCL are very potent activators of normal lymphocytes in a mixed lymphocyte-type reaction (§ 7.1.2 and § 7.1.8). The activated lymphocytes are cytotoxic to the LCL producing the activation, to other LCL and to some other types of cell (Hardy et al., 1970). Both the activation and killing reactions may be demonstrated in an entirely autochthonous system, i.e. activated lymphocytes produced by incubation with autochthonous LCL cells acquire the capacity to kill autochthonous LCL cells. It is obvious that this may reflect a possible immunological surveillance mechanism for the removal of aberrant lymphoid cells. It is probable that similar events occur *in vivo*. A malignant melanoma patient has been transfused with nearly 200 g of his own autochthonous cultured lymphoblasts and this procedure neither harmed the patient nor produced diminution of his metastases (Moore, 1970). Presumably the LCL cells were rapidly killed by normal lymphocytes. Only normal lymphoblasts possess cytotoxic capacity of the type described in § 10.2. No cytotoxic effect of similar kind can be obtained by mixing LCL cells with cells of another line or another type. By this dynamic *in vitro* test LCL cells are not immunocompetent.

The Epstein-Barr (EB) or Herpes-like virus (HLV), first demonstrated in Burkitt lymphoma lines, has since been found in many other LCL including those initiated from normal individuals. Of 109 lines examined at Roswell

Park, 49 were EB-virus positive, 15 negative and 45 uncertain. Some LCL have been initiated under conditions which rigorously excluded introduction of virus from other lines (e.g. see Gerber and Monroe, 1968). Evidence for the presence of EB virus or EB viral antigens in some of these lines was later found. It has generally been found to be easier to establish lines from the leucocytes of individuals with antibodies to EB viral antigens in their serum and whose cells may therefore be harbouring the virus in latent form. The antigens of EB-virus-infected lymphocytes from LCL of normal donors appears by membrane immunofluorescence to be the same as that of Burkitt lymphoma cells (Gerber and Goldstein, 1970). In view of the association of a virus with LCL the growth of other viruses in these cells may be impeded. One LCL tested was susceptible to growth of herpes simplex and Coxsackie type A-15, the effects of which were highly cytopathic, but resisted the growth of most other viruses tested (Wallace, 1969). Mumps virus and vesicular stomatitis virus will also grow in Burkitt lymphoma cells (Chung and Murphy, 1970).

The nature of the progenitor cell whose progeny grow continuously into the clone of cells composing a particular LCL is not known. Since most lines are derived from a mixed population of blood leucocytes, only indirect methods may be used to identify the cell responsible. It may be a T or B lymphocyte or a partially differentiated lymphocyte or even a stem cell. On the basis of surface antigens the majority of the lines have been shown to resemble B lymphocytes (e.g. they do not form rosettes with sheep red cells, they have complement receptors and surface immunoglobulin) but a few are T-like. It has been suggested that lymphoid cells endowed with proliferative potential are present at low concentration in the circulating blood of apparently healthy individuals and are selected during long-term culture. In some ways this is an attractive idea but unless the proliferation potential remained latent *in vivo* the cell would be removed by the surveillance mechanism already described. Furthermore, the marked improvement in initiation rate of LCL using co-cultivation with cells containing EB virus may suggest that a normal blood lymphocyte may become infected with virus and transform to a continuously dividing state. On this view a high proportion of blood lymphocytes have the capacity for continuous growth after viral transformation. Alternatively it could be argued that the virus-containing LCL used in co-cultivation experiments benefit initiation by providing a MLR-type stimulus as well as a feeder-type effect and in support of this view improved initiation rates have been obtained simply by adding

a purified PHA as stimulant. However, it is T cells which respond to PHA and in the MLR whereas most lines have the surface characteristics of B cells. The properties of LCL cells and normal lymphocytes are compared in table 10.1.

Table 10.1
Comparison of the properties of small lymphocytes, normal lymphoblasts and LCL cells.

LCL cells	Small lymphocytes	Normal lymphoblasts
Range of morphology but most cells large and lymphoblast-like. High N/C ratio. Cytoplasm contains single and polyribosomes.	Small cells. High N/C ratio. Cytoplasm few ribosomes.	Large cells. High N/C ratio. Cytoplasm single and polyribosomes (T blasts) with some e.r. (B blasts).
Little surface adherence	T cells non-adherent B cells weak surface adherence	Weak surface adherence
Grow in clumps (most lines) or diffusely	Single cells but tendency to attach to other cells such as macrophages, fibroblasts	Single cells but tendency to attach to macrophages, fibroblasts etc.
Grow continuously without stimulation	Enter DNA synthesis only when stimulated	Return to resting state after pulse stimulus
Move slowly	Obvious motility	Highly motile
Monoclonal	Mixed population	Mixed population
Not cytotoxic	Little cytotoxic activity	T blasts cytotoxic to many cells
Very good lymphocyte stimulators	Good lymphocyte stimulators	Very good lymphocyte stimulators
Good target cells	Poor target cells	Poor target cells
Same radiosensitivity as cells of non-lymphoid lines	Highly radiosensitive	Much more radioresistant than small lymphocytes
Frequently express EB virus antigens. May be EB-virus-transformed cells	Do not readily support growth of viruses	Many viruses grow within them
Usually make immunoglobulins and have other B-cell markers	Mixture of T and B cells	Mixture of T and B blasts, proportions depending on stimulant
HL-A type of original cell donor but antigens present in higher concentration with some 'extras'	Characteristic HL-A type sometimes not 'full-house'	Characteristic HL-A type, sometimes express antigens lacking on small lymphocytes

10.5. Radiosensitivity

When an animal is exposed to several hundred rads of whole body X-irradiation there is a dramatic fall in the numbers of viable lymphocytes in the blood and lymphoid tissue. It has long been known that lymphocytes are almost unique among non-dividing cells in suffering death after comparatively small doses of X-irradiation. Relatively enormous doses are required to kill fibroblasts, macrophages, nerve and muscle and non-dividing epithelial cells. It is doubtful if the entire loss of lymphocytes can be attributed to the direct effects of X-irradiation on these cells. Some of the loss, for example, may be due to the liberation of lymphocytolytic steroids from the adrenal cortex. This is particularly the case with the thymocytes of steroid-sensitive species such as the rabbit, rat and mouse.

Even under *in vitro* conditions, however, small lymphocytes are demonstrably radiosensitive. Less than 2% (compared with 70% in controls) of lymphocytes from human blood survived for 3 days in culture following X-irradiation *in vitro* with 1000 *r* and as little as 10 *r* had an effect on the survival time of rabbit thymic lymphocytes (Schrek, 1959). Lymphocytes which have been activated by PHA or by allogeneic lymphocytes appear to be protected from the lethal effects of X-irradiation (Schrek and Stefani, 1964; Schrek, 1968). It is something of a paradox that mitotic activation of a lymphocyte should make it less sensitive to X-irradiation since it is generally true that dividing cells are much more radiosensitive to irradiation than non-dividing cells. The radiosensitivity of the small lymphocyte is greater even than that of dividing cells. Mitotically activated lymphocytes and cells from lymphoid cell lines are approximately as radiosensitive as cells from other dividing cell populations. The radiosensitivity of the small lymphocyte appears to be associated with its metabolically resting state and may be related to its incapacity to repair radiation damage (Vaughan-Smith and Ling, 1974). The cell-damaging effect of the irradiation itself appears to be dependent on metabolic events as it is not expressed at low temperatures (Schrek and Elrod, 1965). In dividing cells the nucleus is the site of lethal radiation damage. It is by no means certain that the same is true of small lymphocytes. The fact that circulating small lymphocytes bear potentially lethal nuclear radiation damage inflicted many years previously (Buckton and Pike, 1967) suggests that nuclear damage may not be lethal to small lymphocytes or that lymphocytes in the active state when radiation is encountered may revert to a small lymphocyte and survive the radiation

damage. The capacity of lymphocytes to respond to stimulants by DNA synthesis and cell division has proved a useful way of measuring the damage inflicted on lymphocytes by irradiation, the response decreasing with time elapsing since irradiation (Ilberry et al., 1971; Vaughan-Smith and Ling, 1974).

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Response to stimulation of lymphocyte sub-populations and lymphocytes from various tissues

11.1 Stimulation of T and B lymphocytes by mitogens

One of the main hopes for the future in this field is that mitogens will be discovered, or tailored, which will activate defined sub-populations of lymphocytes. It has for long been known that the various 'non-specific' mitogens do not behave identically. There are individual variations, species variations and differences between populations of lymphocytes obtained from the various lymphoid tissues. It was once imagined that a mitogen could guide an uncommitted lymphocyte towards 'cellular' or 'humoral' immunity. Differences in the morphology of the blast cells and their progeny which were shown to be related to the stimulant used (e.g. PHA or PWM) were regarded as evidence that the pathway of differentiation of the lymphocyte had been influenced by the particular mitogen. Although an effect of this kind cannot be entirely excluded, there is now very good reason to believe that most of the lymphocytes in peripheral tissues are already at least partially committed in their capacity for differentiation and that the differential effects of mitogens are due to their capacity to stimulate different sub-population of lymphocytes.

Attention has so far been focussed on the major sub-populations, namely T and B, and some progress has been made. Methods of identifying T and B cell mitogens have, as yet, been entirely empirical and the molecular basis of differential stimulation is not known. The total number of receptors for particular mitogens on the surface of T and B cells appears, at least for some mitogens, to be not significantly different. Presumably there are more 'trigger' receptors on the surface of the cells most responsive to the stimulant, but other factors of a metabolic nature may also be involved. The first realisation that most of the lymphocytes responsive to PHA or con A were

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and B populations found in peripheral lymphoid tissues the delineation of response may not be as clear-cut as experiments with the isolated populations would indicate. In the mixtures some degree of indirect stimulation of B cells seems to occur. This may be why the dose-response curve of pure mouse T cells to con A has been found to be less broad than that of T and B mixtures (Andersson et al., 1972). It may, alternatively, indicate that non-T cells aid the effective 'presentation' of stimulant. The response of thoracic duct lymphocytes of rats to PHA was improved by including some unresponsive non-circulating cells (from the blood of rats depleted by thoracic duct drainage for three days) in the culture (Adler et al., 1970).

Indirect activation of B cells, when it occurs, may be induced by diffusible products of T cells or macrophages. Activated peripheral mouse T lymphocytes have been shown to release soluble factors which aid the maturation of B cells (see § 10.1). However, the extent of the B cell recruitment is often overstated. Janossy et al. (1973) found that the selectivity of con A for T cells was maintained in cultures containing varying numbers of T and B cells. Chromosome-marker experiments to identify the source of dividing cells in cultures of cells from chimeric animals, have tended to confirm that it is the T population which is primarily activated by PHA (Davies et al., 1971; Greaves and Janossy, 1972), but it has also been shown that if lethally irradiated CBA mice are reconstituted with bone marrow and thymus cells bearing different chromosome markers, both T and B cell mitoses are found in cultures of their spleen cells stimulated with PHA (Piguet and Vassalli, 1972). A different result has been obtained in the chicken. Con A failed to induce a detectable proliferation of chromosomally-marked B₂ cells of chicken spleen under conditions in which a marked T cell response was obtained (Weber, 1973).

Human lymphocytes have also been separated into their sub-populations and tested with con A and PHA. Rosettes formed between human blood lymphocytes and sheep red cells have been centrifuged through Ficoll-Triosil to separate the rosette-forming (T) cells from the remaining mononuclear cells and their response to PHA tested. The rosette-forming population consisting of virtually pure T cells with few monocytes or B cells present, had a low spontaneous level of ³H-thymidine incorporation but responded to PHA to a greater extent than the unfractionated population (Wybran et al., 1973). More than 90% blast transformation was found in 72 hr PHA cultures prepared from rosette-forming human blood lymphocytes and less than 1% showed any surface immunoglobulin by immuno-

fluorescence (Yata et al., 1973). In contrast, the residual population, depleted of rosette-forming cells, had a low PHA responsiveness and 20–50% of the cells showed surface immunoglobulin.

There is clear evidence, in both mouse and human systems, that isolated T cells respond to PHA and con A. Neither B lymphocytes nor monocytes or macrophages are required in any auxiliary role except perhaps in improving the 'presentation' of the mitogen. It is less certain that in cultures of mixed T and B cells that some and perhaps many of the B cells are indirectly activated. There are technical difficulties in reaching a clear solution of this problem. Conventional culture conditions may, and probably do, discriminate against B cells. In cultures of mouse lymphocytes it has been found that the proportion of B cells steadily diminishes (Jones and Roitt, 1972). The loss of B cells may be accelerated by activated T cells exerting a cytotoxic effect on mitogen-coated B cells. On the other hand activated T cells may contribute to an indirect activation of B cells by release of mitogenic or growth-promoting agents. Indirect B cell activation would be expected to enhance immunoglobulin production of the culture along with DNA synthesis of B cells. It is significant that PHA induces little if any increase in immunoglobulin synthesis as measured by radioamino acid incorporation. Another indirect way in which B cells might be stimulated is by modification of the physical nature of the stimulant. It has been reported that B lymphocytes are able to respond to PHA when it is attached to an insoluble (Sephadex) bead or to con A when it is covalently linked to a plastic Petri dish (see reviews given earlier). However, some insoluble absorbents appear to activate B cells to some extent even when treated with inert proteins rather than mitogens, suggesting that this form of activation may not be strictly mitogen induced.

It is probable that SF also stimulates principally the T cells of rabbits and humans. Like PHA and con A it is active against cells obtained directly from the thymus and stimulates a high proportion of the peripheral population. There is, as yet, no direct way of identifying the T cells of rabbits.

11.1.2. Pokeweed (PWM), bacterial endotoxins, dextran sulphate, PPD and other stimulants active against B cells

A tendency for PWM to produce activated human lymphocytes with more rough endoplasmic reticulum than the blast cells found in PHA cultures was noted some years ago (see Chessin et al., 1966 and ch. 5). The difficulties

encountered in confirming this report may be understood if, as we now believe, PWM stimulates both T and B lymphocytes.

In mice there is good evidence that B lymphocytes may be stimulated by PWM in the absence of T cells (Shortman et al., 1973) and, despite earlier reports to the contrary, cells of the spleens of 'nude' mice will respond to some preparations of PWM (see fig. 11.1). A higher proportion of the new protein synthesised by mouse spleen cells is immunoglobulin in nature in cultures containing PWM than in those containing PHA (Parkhouse et al., 1972). It is, however, quite clear in the mouse system, that PWM is not a specific stimulant for B cells. Peripheral T cells and cortisone-resistant thymocytes are also activated. In the rat and the chicken too, both T and B lymphocytes are activated by PWM (Goldschneider and Cogen, 1973; Weber, 1973).

All four lipopolysaccharide endotoxins tested by Greaves and Janossy (1972) were found to be specific stimulants for B cells of the mouse. T cells, either peripheral or thymic, were unresponsive. The endotoxin used with most success has been the lipopolysaccharide from *E. coli* 0111:B4. At a concentration of 10 µg/ml it has been shown to stimulate DNA synthesis of mouse spleen cells, to enhance the synthesis of 19S immunoglobulins and reduce 7S immunoglobulin production (Andersson et al., 1972). Spleen cells of normal or thymectomised mice respond to endotoxins to about equivalent degree. Unfortunately endotoxins cannot be regarded as general reagents for stimulation of B lymphocytes. Only about 20% of the B cells of a mouse spleen are thought to be responsive (Janossy et al., 1973). Negative results have been reported with mouse lymph node cells (Dumont, 1973). There are cells in rat and rabbit spleens and in rat lymph nodes (see § 4.1.3) which respond to endotoxins but their identity has not been established and human peripheral blood cells are unresponsive. It is possible that only a B sub-population is responsive, that this may be localised partly in the spleen and that endotoxins may be active only against B cells of some species. As with other stimulants indirect effects on the other populations may occur. When endotoxin acts on human or mouse lymphoid cells it has been shown to cause the release of factors which are mitogenic for mouse thymocytes (Gery et al., 1972).

Dextran sulphate has been shown to activate spleen cells of mice of several strains (Ruhl et al., 1974). The highest rate of stimulation (^{3}H -thymidine stimulation ratio of 20–24) was found in 48 hr cultures of spleen cells from 'nude' mice stimulated with 100 µg/ml of dextran sulphate (mol. wt. 5×10^5)

Spleen cells of all strains of mice tested were responsive but there were strain variations ('nude') > C57 > DBA > C3H > BALB/c > A/J. Cortisone-resistant thymus cells were only weakly responsive. Spleen cells of C57 mice were also shown to respond to other substances of a polyanionic nature such as poly A-poly U, poly I, poly I-poly C, polyethylene sulphate, pentosane sulphate and less strongly to heparin, poly C and poly A but not to dextrans of mol. wt. 6×10^4 and 2×10^6 .

Tuberculin PPD, which acts as a specific antigen, stimulating only the lymphocytes of sensitised animals when used in low concentrations, will activate the spleen cells of unsensitised mice when present in high concentrations. The responsive cells appear to be B cells (see ch. 9). Many thymus-independent antigens have been shown to induce B cell stimulation. Pneumococcal polysaccharide type SIII is one of these (Coutinho and Moller, 1973). Spleen cells from normal mice and 'nude' mice responded to this substance with enhanced DNA synthesis and immunoglobulin synthesis. Cortisone-resistant thymocytes did not respond. Other thymus-independent antigens thought by these authors to act as B cell stimulants are polymerised flagellin, polyvinylpyrrolidone, levan and dextran.

It is difficult to make generalisations about the effectiveness and selectivity of B cell stimulants. Most information is available about mouse spleen. In addition to bacterial lipopolysaccharides, various anionic substances of high molecular weight containing repeating determinants have been found to be effective. Non-immunogenic polyanions are also known to increase the serum immunoglobulin levels two- or three-fold when injected into mice. However, there have been many unsuccessful attempts to stimulate B cells with substances of this type. Human blood lymphocytes, for example, do not appear to respond to endotoxins or to polynucleotides; mouse lymph node cells have been found not to respond to endotoxins and there have been reports of marked strain variations in responses of mouse spleen cells to polynucleotides. Stimulation with dextran sulphate seems to occur only if a preparation of suitable molecular weight and degree of sulphation is used. Other charged dextrans, e.g. dextran phosphate, have not yet been shown to possess activity. Dextran sulphate, like the endotoxins, probably stimulates only a sub-population of B cells and most work with both substances has been done with mouse spleen cells. It is likely that cross linking of sensitive surface receptors with a molecule of appropriate backbone with suitable disposition of anionic groupings will trigger B lymphocytes, but there appear to be, as yet unexplained, tissue variations of B cell responses.

11.1.3. Response to mitogens of lymphocytes from blood, appendix, spleen, thymus, tonsil and bone marrow

In so far as they are part of the circulating population, lymphocytes in lymph nodes, spleen and other lymphoid areas would be expected to respond to lymphocyte stimulants in exactly the same way as peripheral cells. Those lymphocytes in spleen and lymph nodes which are not part of the recirculating population and particularly, lymphocytes obtained from the thymus, might be expected to respond differently. The response to a stimulant is obviously likely to depend on the proportion of T and B cells in the lymphoid tissue examined. In the mouse from 80–88% of the thoracic duct lymphocytes are thought to be of T-type whereas T lymphocytes compose only 50–55% of the splenic population. The proportions of T and B cells in lymph nodes lie between these two ranges. Not all differences in the responsiveness of lymphocytes from various sources can be explained simply in terms of the number of T or B cells present in the culture, possibly because sub-populations of different degrees of responsiveness to PHA and con A, for example, are present in different proportions in the various tissues.

Differences in the responses of cells from mouse lymph nodes, spleen and thymus to PHA and con A have already been discussed (see § 11.1.1). More comprehensive studies have been made in rabbits. There are lymphocytes in rabbit blood, spleen, lymph nodes, thymus, appendix, Peyer's patches and Sacculus rotundus which respond to PHA, con A and SF (table 11.1). Con A and SF are particularly good stimulants for rabbit lymphocytes. Responsive

Table 11.1

Stimulation of lymphocytes from rabbit tissues. Lymphocytes from the various tissues were cultured at 2×10^6 per ml in 1 ml cultures. The figure entered = ^{3}H -thymidine incorporated into the acid-insoluble fraction over the period day 4-day 5 ($\text{dpm} \times 10^{-3}$). Abbreviations: L.N., lymph node; B.M., bone marrow; App., appendix; S.R., = Sacculus rotundus; P.P., = Peyer's patches; AAS, = anti-allotype serum. From data provided by Mrs. Sue Menzies.

Stimulant	Blood	Spleen	L.N.	B.M.	Thymus	App.	S.R.	P.P.
none	0.9	1.9	2.1	1.6	0.9	0.9	0.3	1.1
PHA	7.2	11.4	4.7	2.4	3.2	10.6	8.9	3.6
con A	18.0	6.2	13.1	1.6	5.1	13.7	7.2	7.1
AAS	3.9	0.4	2.4	0.3	0.4	1.0	1.1	1.3
SF	16.8	4.7	18.8	0.3	3.7	12.2	3.1	3.2

cells are abundant in the thymus at birth when there are very few lymphocytes present in the spleen or appendix and negligible numbers of SF-responsive cells. As the number of lymphocytes in appendix and spleen increases soon after birth the number of SF-responsive cells increases correspondingly, suggesting, but not proving, that these tissues have been seeded with SF-responsive T cells. There are differences in the kinetics of the responses to mitogens with cells of the various tissues e.g. spleen cells regularly respond more quickly than blood lymphocytes (see Knight and Ling, 1968). This is probably due to the fact that the mitogen-responsive cells in spleen are part of a proliferating population whereas those in peripheral blood are in a resting (G_0) phase. In the mouse it has indeed been shown that the PHA-responsive population in the spleen is in a proliferative phase (Moorhead and Claman, 1974). In view of earlier claims of a bursa-equivalent role of gut-associated lymphoid tissue in the rabbit, it is noteworthy that lymphocytes responding to con A, PHA and SF, which are presumably T cells, are present in large numbers in all the lymphoid tissues of adult rabbits with the exception of the bone marrow. Anti-allotypic sera, which react with immunoglobulin determinants on the lymphocyte surface, will activate lymphocytes of all tissues except thymus and bone marrow (table 11.1 and ch. 8). This is in accord with the accepted view that anti-immunoglobulin sera react only with B lymphocytes but it is probably misleading. In the human and mouse species anti-immunoglobulin sera used in surface-binding tests do distinguish B cells from T, but in these species anti-immunoglobulin sera do not produce lymphocyte activation (see ch. 8). In the rabbit a high proportion of lymphocytes of blood and tissues bind anti-immunoglobulin and anti-allotype antibodies and also undergo a marked mitotic activation. Under good culture conditions the number of blast cells found in rabbit blood lymphocyte cultures containing anti-allotype antibodies may approach the numbers obtained with the T cell stimulant con A. It seems improbable, on purely quantitative grounds, that two distinct populations are being stimulated by two such potent stimulants. It is more likely that, in the rabbit, as in other species, the major population of lymphocytes in blood and tissues is a con A-responsive T cell population which happens also to have 'trigger' sites bearing antigenic determinants also present on immunoglobulins of this species.

Lymphocytes in the thymus of all species have distinctly different properties from those in peripheral tissues (see ch. 2). Only a minor population has properties resembling those of peripheral T cells and even these have not

reached quite the same stage of differentiation. It is the cells of the minor population which respond to con A, PHA, PWM, SF or allogeneic lymphocytes and many of the unresponsive cells die during the culture period. The responsive cells are larger than peripheral small lymphocytes. The number of unresponsive cells in the thymus decreases after cortisone administration and increases after orchidectomy. In species where survival of peripheral lymphocytes is relatively low e.g. chicken, mouse, very low level responses of thymocytes to mitogens may be found simply because the death rate, even over a two day period, is considerable. The survival rate of thymocytes is always lower than that of peripheral lymphocytes in all species and, because a major part of the thymocyte population is unresponsive, the overall response at equivalent cell concentrations is always less than that of peripheral lymphocytes. Thus human thymocytes are typically much less responsive to PHA than lymph node lymphocytes which respond approximately as well as blood lymphocytes (Winkelstein and Craddock, 1967). Little is known about the location of the responsive cells. Using pig thymus tissue in organ culture most of the PHA-responsive cells have been found in the medulla (Weber, 1966).

Human tonsillar and appendiceal lymphocytes are often available from tissue removed at operation. The background activity of human tonsillar cells is higher than that of blood lymphocytes and this is reflected in the greater numbers of blast cells seen in cultures not containing stimulant. Since the tonsils are frequently infected this could reflect stimulation from bacterial or viral antigens. However, cultures set up in conventional media containing antibiotics do not usually appear to be contaminated after several days of culture and the cells look healthy. Freshly separated cells have a very high rate of glycolysis and it may be advisable to use a lower lymphocyte concentration than is customary for blood lymphocytes. Tonsillar lymphocytes respond to PHA, SF, con A and leucocyte antibodies. In the experience of Oettgen et al. (1966) tonsillar lymphocytes respond as well as peripheral to PHA, to the streptolysin S mitogen and several antigens. The percentage of blast cells present in 'unstimulated' cultures of human appendiceal lymphocytes has been estimated to be 0.83 ± 0.44 compared with 59.5 ± 9.2 after PHA stimulation, the corresponding figure for blood lymphocytes under similar conditions of culture being 66 ± 12 (Astaldi, 1968).

11.1.4. 'Spontaneous' DNA synthesis of lymphocytes from blood and tissues

If cultures are set up with lymphocytes from the various lymphoid tissues of normal non-immunised animals there is considerable variation in the level of DNA synthesis in cultures not containing stimulant. Generally blood lymphocytes have the lowest level of 'background' stimulation and bone marrow and thymus are the highest, although moderate to high 'background' counts are obtained from spleen, lymph node (varying according to location – mesenteric nodes usually contain many active cells) and gut-associated lymphoid tissue. Lymphocytes from tissues of one-year old, neonatally thymectomised rats show increased 'spontaneous' activity (Dabrowski and Dabrowska, 1972). What is usually recorded as the 'background' count in the control cultures at the end of the period of incubation may be made up of two components: a) the DNA synthesis, which reflects a continuation of the proliferative activity of the cells when they were freshly isolated, b) the response to stimulants e.g. foetal calf serum, in the medium. Generally, in short term cultures of tissue cells, component (a) accounts for most of the background. With tissues like thymus and bone marrow the DNA synthesis over the first 24 hr following isolation is extremely high and thereafter falls steadily.

Non-lymphoid as well as lymphoid cells are in DNA synthesis in cell suspensions prepared from some tissues e.g. bone marrow, mouse spleen. In lymph nodes and thymus virtually all the active cells are lymphocytes. A few dividing cells released from almost any tissue in the body might theoretically appear in the blood but, in practice, all the 'background' DNA synthesis of normal adult human leucocyte preparations appears to be due to lymphocytes. Any infection or immunisation will increase the numbers of activated lymphocytes present in the blood for a period of days or weeks (Gump and Fekety, 1967). The same phenomenon is seen in immunised animals. An immune response to any antigen, provided it is of sufficient intensity, seems to be accompanied by an increase in the number of active forms of lymphocytes in the blood. Increased 'spontaneous' DNA synthesis of blood leucocytes has been recommended as an early indicator of homograft rejection. There is an increase in the numbers of activated lymphocytes in the blood in Hodgkin's disease, which, it is thought, may be an indication of a reaction to the infections to which these patients are prone (Crowther et al., 1969). Up to 4% of the blood lymphocytes were classified as 'large lymphoid cells'

and proved, on electron microscopic examination, to have the characteristics of T blasts (many polyribosomes, poorly developed endoplasmic reticulum) and about 10–20% of the cells were 'medium' lymphocytes with enough endoplasmic reticulum to assign them to the plasma cell series. Abnormal DNA-synthesising cells may also be present in blood, e.g. in acute leukaemias, but in chronic lymphocytic leukaemia (CLL) the percent of cells in DNA synthesis is reduced because the CLL cells themselves are never in DNA synthesis. The activated lymphocytes (atypical mononuclear cells) present in the blood in infectious mononucleosis have been shown to have the characteristics of T cells (Sheldon et al., 1973), probably at a late stage of differentiation. The 'background' DNA synthesis of the mononuclear cell fraction of cord blood is about ten times that of adult cells, presumably indicating a proliferative stage of seeded out T and B lymphocytes prior to the establishment of a stable peripheral population. The level of DNA synthesis falls rapidly during the first few days of culture. Relatively large numbers of lymphocytes and monocytes are present in cord blood and in the blood of infants during the first few weeks of life. Red cell precursors and other cells not normally found in adult blood may also be present.

11.1.5. Ontogeny of mitogen responsiveness

Since the state of maturity of a lymphoid tissue will affect both the numbers of T and B lymphocytes it contains and their degree of differentiation it is to be expected that mitogen responsiveness will also be affected. In the earliest stages of ontogeny of lymphoid tissue lymphocytes are to be found only in the thymus and foetal liver. In species such as the mouse, which are immature at birth, there are few lymphocytes in the spleen and other peripheral lymphoid tissues of the newborn and those present are almost entirely B cells; θ -positive cells are virtually absent. In the rat and rabbit, too, there are very few lymphocytes in peripheral tissues at birth which respond to T cell stimulants. The responses remain low for about a week and then rise rapidly, reaching a maximum by about six weeks of age.

The thymus is usually the first tissue in which mitogen responsiveness can be clearly demonstrated and the nature and number of responding cells varies with age. In both young and old rats the mitogen-responsive cells are confined to a minor population of large cells and there is frequently a higher proportion of large cells in the thymus of rats less than one week of age (Knight et al., 1973). In the human thymus, an early foetal period has

been described, before the 20th week, during which medium lymphocytes number at least one third of the total number and a good response to PHA and PWM is obtained. This is followed after the 20th week by a phase in which only 10% of the thymic lymphocytes are medium-sized and responses to PHA and PWM are less pronounced (Kay et al., 1970; Papiernik, 1972). Thereafter PHA and PWM-responsive cells are present in blood and spleen. The onset of responsiveness of human lymphocytes to allogeneic cells seems to occur at an even earlier time than mitogen-responsiveness. Cells responsive in an MLR are present in the human foetal liver as early as at five weeks of gestation. Cells obtained at 5–20 weeks of gestation from foetal thymus, spleen or blood responded to PHA and cells from thymus, spleen, blood or liver responded to allogeneic cells (Carr et al., 1972a).

Although all investigators have agreed that human cord blood lymphocytes respond to the common mitogens and to allogeneic lymphocytes there has been some disagreement as to whether responses are higher or lower than those obtained with adult blood lymphocytes. In one study (Ayoub and Kasakura, 1972) cord blood lymphocytes were found to be less responsive to PHA than those of adults, but according to Carr et al. (1972b) cord blood lymphocytes respond better than adult at low doses of PHA and as well as adult at intermediate or high PHA levels. The reaction kinetics differ from those observed with lymphocytes of adult donors in that the minimum G_1 period is about 4 hr shorter and some cells enter the S period very soon after PHA is added (Weber et al., 1973). These differences may be accounted for by the predominance of large and medium sized cells in cord blood compared with the predominance of small lymphocytes in blood of adults.

In the mouse the responsiveness of thymocytes to various stimulants changes remarkably as the animal matures. By 15 or 16 days of gestation the thymus is densely populated with lymphocytes whereas there are virtually no lymphocytes in the peripheral tissues. Lymphocytes from the 18 or 20 day old foetal thymus of BALB/c mice responded markedly to PHA, gave a mild response to con A and PWM and a marked response in the MLR. At birth a mild response of thymocytes to PHA was observed and a moderately good response to con A, PWM and in the MLR. Thymocytes from adult BALB/c mice, in contrast, responded very well to con A, PWM and in the MLR but gave a low grade response to PHA (Mosier, 1974).

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The mechanism of lymphocyte activation

As a model system for the study of cells passing from a quiescent to an active state, the lymphocyte activation system has many advantages. Potent mitogens are available in large quantities; they are stable substances susceptible to chemical and physical analysis, and they can be labelled. The lymphocytes themselves are single cells which survive comparatively well when cultured in simple media, and they are easily obtained in sufficient quantity for many biochemical techniques to be applicable. They consistently remain in a quiescent, non-dividing, state until a mitogen is added, and the response may be followed from the point of addition of the mitogen, the time of which is accurately known. The main disadvantage is that while lymphocytes appear morphologically to be a rather homogeneous population, it is now apparent that they are both functionally and biochemically rather heterogeneous, and the full extent of this heterogeneity may not yet be appreciated.

Two main types of approach have been explored in efforts to bring us closer to an understanding of the mechanism of activation. Attempts have been made to characterize the stimulant receptor sites by direct or indirect techniques, and to determine the fate of the stimulants after they bind to the receptors. Alternatively, the biochemical changes occurring in the period immediately after the addition of the stimulant have been studied in some detail to attempt to identify the key metabolic effects of the stimulants on the lymphocytes. These studies have been supplemented by kinetic and drug-inhibition experiments which have, in some respects, been illuminating. More recently they have been complicated by the suggestion that stimulation, even by strong non-specific agents such as PHA, is not a response of a single lymphocyte, but requires interaction between different cells, and possibly different types of cells.

12.1. Cellular requirements for activation

The early studies on lymphocyte activation were carried out with mixed cell populations from blood or lymphoid organs, but it was realised from the earliest studies that, by morphological criteria, the response of lymphocytes was rather heterogeneous, and that even when the most effective stimulants were used there were always some lymphocytes which remained unaffected. The possibilities that different lymphocytes were activated by different specific and non-specific stimulants were considered by several early workers, but research in this area has been much more intensive since the formalization of the now generally accepted division of lymphoid cells into two main classes, T lymphocytes and B lymphocytes. The basis of this classification and the different responses of B and T lymphocytes to mitogens are discussed in detail in chs. 2 and 11.

Studies on more purified lymphocyte populations have suggested that the original idea that a lymphocyte would combine with its stimulant and then undergo activation may also be an oversimplification. There is some evidence that activation by either non-specific or specific mitogens requires interaction between different cells, either direct interaction between lymphocytes brought into contact by agglutinating stimulants, or interaction involving macrophages in the case of non-agglutinating stimulants. The erythrocytes contaminating most lymphocyte cultures may also play a role in the response to some mitogens.

12.1.1. Lymphocyte-lymphocyte interactions

The formation of aggregates occurs after the addition of almost all mitogens to lymphocytes, but there is conflicting evidence about the importance of this for activation. It has not proved possible to demonstrate activation of lymphocytes cultured individually, but this probably reflects the difficulties experienced in culturing most types of mammalian cells in thin suspension. Lymphocytes immobilised in chicken plasma clot cultures were found to enlarge after the addition of PHA (Coulson and Cohn, 1973). The degree of morphological transformation was rather lower than usual, and few cells in mitosis were seen, but this could be due to the unusual culture conditions rather than a requirement for cell interaction. In contrast, when the formation of aggregates in cultures stimulated by pokeweed mitogen was prevented by agitation of the cultures, the response to the mitogen was very greatly

reduced (Peters, 1972). The formation of the aggregates occurred over a period of several hours after the addition of the mitogen, and agitation for only the later part of culture, after the aggregates had formed, did not affect the response adversely. Agitation was not enough to prevent aggregation after the addition of agglutinating stimulants such as PHA. However, cell interaction could be prevented by culturing lymphocytes at low densities in Petri dishes in a medium containing 0.3% agar. In these conditions the lymphocytes were unable to increase their rate of uridine incorporation into RNA in response to PHA (Peters, 1972). Lymphocytes preincubated with PHA before addition to the agar containing medium, or lymphocytes cultured in the same conditions at higher cell concentrations, did show normal responses.

The inhibition of lymphocyte activation by hyaluronic acid (Darzynkiewicz and Balazs, 1971) may also be due to inhibition of cell interaction. This agent was much more inhibitory at low cell densities, and against those stimulants such as pokeweed mitogen and antigens which cause relatively weak cell-cell interaction. Other mucoproteins would also be expected to hinder this process, and some of the complex effects of serum may be exerted in this way.

It has frequently been suggested that some material initiating, or essential for, activation may pass from cell to cell, and Hulser and Peters (1972) have shown that low resistance junctions exist between different cells in the aggregates formed after addition of PHA. These workers were able to insert glass microelectrodes into bovine lymphocytes immobilized in agar without apparently causing serious damage to the cells. They found that lymphocytes had a rather low membrane potential and an average membrane resistance, neither of these parameters being significantly affected by addition of PHA. To look for intercellular communication one microelectrode was used to record the membrane potential of a cell, and a second was inserted into a nearby cell and used for injecting current pulses. No evidence was found for intercellular communication between unstimulated lymphocytes. However, communication was observed between the cells in the aggregates formed after PHA stimulation, even when the cell whose membrane potential was being measured was separated from the cell to which the current pulse was applied by several other cells. This intercellular communication was not simply a function of agglutination as no communication was found when the agglutinates were formed by an anti-pig lymphocyte serum, which agglutinated but did not stimulate the bovine lymphocytes. Evidence for intercellular

communication could be found very rapidly after the addition of PHA. The shortest interval observed was only 1 min, and the rate limiting step seemed to be the time required for the PHA to reach the cells.

The consequences of this intercellular communication have not yet been studied. While it seems a reasonable hypothesis from the data above that lymphocyte stimulation should be regarded as a cooperative response rather than the response of a single cell, this cannot yet be taken as established. We do not yet know what types of molecules or information can be transferred from cell to cell and what effects such transfer would have. Solution of this problem may greatly increase our understanding of all control mechanisms.

12.1.2. Lymphocyte-macrophage interactions

With the introduction of column filtration techniques for the routine preparation of lymphocyte cultures with low levels of contaminating phagocytic cells, it was found that the response to many of the weaker lymphocyte stimulants was lost or greatly reduced (Oppenheim et al., 1968). Activity was restored by the addition of phagocyte-rich populations. This was true not only for those specific stimulants whose action had an immunological basis, but also for some of the weaker non-specific stimulants, such as staphylococcal filtrate. Even the response to suboptimal concentrations of PHA and anti-lymphocyte serum was reduced, and while most workers have found that the response to optimal doses of these strong stimulants was not affected, Levis and Robbins (1970a) claimed that highly purified cultures showed a marked dependence on the presence of macrophages for their response to even optimal concentrations of PHA. The numbers of macrophages required for activation are very small, often no more than 1% of the number of lymphocytes in the cultures.

The situation is complicated by the dependence of lymphocyte activation on the cell concentration. The response to PHA is relatively independent of this parameter, but the responses to weaker stimulants, such as antigens, are greatly enhanced by increasing the cell concentration (Moorhead et al., 1967; Hersh and Harris, 1968). The important concentration is not the nominal concentration of the cells when in suspension, but the actual local concentration in the cultures. Most workers allow the cells to settle out during culture, and the local concentration will thus depend critically on the shape of the culture vessel. The reason that mitogens such as PHA, which agglutinate lymphocytes, are relatively independent of the nominal cell con-

centration may simply be that the agglutination always maintains the cells in a high effective local cell concentration.

As cell concentrations are increased, it becomes progressively more difficult to demonstrate any requirement for macrophages (Oppenheim et al., 1968). It is, however, very difficult to discriminate between the alternative hypotheses that macrophages are required only to facilitate lymphocyte-lymphocyte interaction at low cell concentrations, and that there is an absolute requirement for contact with macrophages which the lymphocytes are more likely to establish with the few macrophages inevitably present when the cells are cultured at high density. There is very good cytological evidence that lymphocytes do form aggregates around macrophages, particularly in the presence of a stimulant (McFarland et al., 1966). The lymphocytes attach to the macrophages by means of their uropod, and this contact is often maintained for a prolonged period. At later times after stimulation enlarged and DNA synthesizing cells are much more frequent in such aggregates than elsewhere. Similarly, many workers have observed that the most rapidly growing cells in PHA stimulated cultures are found within cell aggregates, while a higher proportion of those outside aggregates show fewer signs of activation.

The formation of such aggregates has been studied in detail by Salvin et al. (1971), using normal and sensitized macrophages and lymphocytes from inbred guinea pigs. They found that the presence of the antigen (PPD) did not affect the motility of normal or sensitized lymphocytes. However, sensitized, but not normal, lymphocytes did adhere to either sensitized or normal macrophages. The antigen also reduced the motility of sensitized macrophages and, in the presence of sensitized lymphocytes, normal macrophages. The effect of sensitized lymphocytes and antigen on normal macrophages was very similar to the effect of migration-inhibition factor, which is released by lymphocytes after stimulation (see § 10.1). This paper also confirmed earlier studies using mixed cultures from human identical twins which showed that for activation to occur *in vitro* in response to an antigen it was necessary for the lymphocytes to be sensitized to the antigen, but irrelevant whether or not the macrophages were sensitized (Levis and Robbins, 1970b).

12.1.3. Lymphocyte-erythrocyte interaction

Although many workers have found that lymphocyte stimulation occurs perfectly normally with lymphocyte populations which naturally contain

very few erythrocytes, such as thoracic duct lymphocytes or lymph node cells, or which have had their erythrocyte contamination greatly reduced by experimental manipulation, there have been a number of reports that the stimulation of human blood lymphocytes by PHA can be augmented by the addition of erythrocytes (Tarnvik, 1970; Yachnin et al., 1971; Johnson et al., 1972). Augmentation is found only with PHA preparations containing the erythroagglutinating form of PHA, PHA-H, and not when mitogens which do not agglutinate erythrocytes, such as PHA-L, staphylococcal filtrate or pokeweed mitogen, are used.

The extent of the augmentation observed is rather variable, probably as a function of the success of different investigators in removing residual erythrocytes from the lymphocytes, but in some circumstances the augmentation of DNA synthesis may be by more than a factor of 10, and the stimulation is essentially dependent on the addition of erythrocytes. The augmentation can be seen throughout the response, and at all PHA and lymphocyte concentrations tested, but is clearly much more marked when the PHA and lymphocyte concentrations are low. While the augmentation increases as the erythrocyte concentration is raised up to an erythrocyte:lymphocyte ratio of 100:1, as few as one erythrocyte per 10 lymphocytes may double the lymphocyte DNA synthesis under some conditions.

The augmentation does not require intact erythrocytes, but can also be caused by heat-killed erythrocytes or erythrocyte membranes. One explanation proposed for these results is that PHA adsorbed on erythrocyte membranes is in a particularly favourable steric configuration or local concentration to stimulate lymphocytes, although direct attempts to stimulate lymphocytes with PHA attached to erythrocyte membranes have been successful only at very high PHA or erythrocyte concentrations (Kay, 1971; Johnson et al., 1972). In so far as these data support the concept that cell membrane interaction is crucial for the initiation of lymphocyte stimulation, they suggest that the membrane alone may be the important feature, and that exchange of information or material between cells is not required.

12.2. The binding of stimulants to lymphocytes

The initial interaction between lymphocytes and mitogens is the binding of the mitogen to the receptor on the lymphocyte surface. Inhibition of mitogen binding invariably prevents activation, so this interaction is presumably necessary. In consequence, a great deal of effort has been expended in the

study of this process, with the main aims of identifying the lymphocyte receptors, and determining the fate of the stimulant, and what it does to cause activation.

It is perhaps appropriate to stress that the binding of at least the lectins to lymphocytes is in no way unusual. Receptor sites for con A and PHA, and most probably other lectins, are present in similar numbers on all nucleated mammalian cells examined, and there is no evidence yet to indicate that the lymphocyte receptors are qualitatively any different from those of other cells. Even 'cap' formation occurs when mouse fibroblasts are incubated with con A (Comoglio and Guglielmone, 1972). The effects of lectins on lymphocytes are, however, unique. While PHA and con A do affect the metabolism of other mammalian cells, these effects can in the main be ascribed to trivial causes, such as agglutination. There is no other cell whose metabolism is so remarkably stimulated by its interaction with these agents.

12.2.1. Stimulant receptor sites

When the stimulation of lymphocytes has an immunological basis, the stimulant presumably reacts initially with the appropriate specific receptor on the cell membrane. Thus antigens will interact with antibody, anti-immunoglobulins with surface immunoglobulins, and anti-lymphocyte serum or globulin with the immunogenic determinants on the cell surfaces. In the mixed lymphocyte reaction the initial interaction presumably involves the recognition of histoincompatibility determinants.

Information on the initial interaction of non-specific stimulants with lymphocytes is derived almost exclusively from the study of the plant lectins. Many of these stimulants also agglutinate both erythrocytes and leucocytes. Erythroagglutination by these, and by other, non-mitogenic, lectins can in many cases be specifically inhibited by simple sugars and oligosaccharides, and their binding to erythrocytes is thought to result from the attachment of active sites of these lectins to such saccharide groups on the erythrocyte surface (reviewed by Sharon and Lis, 1972). Agglutination is probably due to the interaction of different active sites of the same lectin molecule with different erythrocytes.

The reasonable supposition that the interaction of lectins and lymphocytes operates through a similar mechanism has received considerable support. The situation is most clear in the case of con A (Powell and Leon, 1970) and the lentil mitogen (Young et al., 1971). The mitogenic effects of

both these agents on lymphocytes are strongly inhibited by sugars which also inhibit erythroagglutination by these two lectins. The most effective inhibitory sugar is methyl- α -D-mannoside, with methyl- α -D-glucose, D-mannose and D-glucose somewhat less effective.

The evidence in the case of PHA is somewhat less clear. Erythroagglutination by PHA is not affected by mannose or glucose but can be inhibited by N-acetyl-D-galactosamine (Borberg et al., 1966), and some workers have observed partial inhibition of lymphocyte stimulation by this sugar (Borberg et al., 1968), especially when early parameters of the response are examined (Fisher and Mueller, 1969; Pogo, 1969). This inhibition is generally found to be rather weak, and is sometimes not detectable (Lindahl-Kiessling and Peterson, 1969), probably because it is masked by other, much more potent, inhibitors present in the culture medium. The mitogen from *Wisteria floribunda* is also inhibited by N-acetyl-D-galactosamine, and in this case the inhibition is rather more effective (Toyoshima et al., 1971). Erythrocyte agglutination by pokeweed mitogen is inhibited by both N-acetyl-D-galactosamine and N-acetyl-D-glucosamine, but it is not known whether the mitogenicity is similarly affected (Landy and Chessin, 1969).

This specific inhibition of the actions of lectins by sugars led to the hypothesis that the sugars were acting as competitive inhibitors of the lectin binding to oligosaccharide receptors. This hypothesis is supported by observations that con A and the lentil mitogen will bind the inhibitory sugars directly, and that sugars inhibit the binding of their respective lectins to lymphocytes (Borberg et al., 1968; Powell and Leon, 1970). However, even in the best cases the sugars must be present at very high concentrations to be effective, and they are clearly weak inhibitors. The binding sites themselves are likely to be saccharide groups of greater complexity.

In confirmation of this hypothesis, glycopeptides isolated from erythrocyte membranes, from a number of serum proteins, and from a variety of other sources have been shown to be up to three orders of magnitude more effective as inhibitors of lymphocyte stimulation by erythroagglutinating PHA (H-PHA), con A, and the lentil and *Wisteria* mitogens than simple sugars (Kornfeld and Kornfeld, 1969, 1970; Pardoe et al., 1970; Toyoshima et al., 1971; Kornfeld et al., 1971). These glycopeptides seem to act, in all cases where it has been tested, by inhibiting the binding of stimulants. It does not seem to be established whether these glycopeptides also inhibit the binding of the non-erythroagglutinating form of PHA (L-PHA).

The proposed structures for some of these glycopeptides are shown in fig.

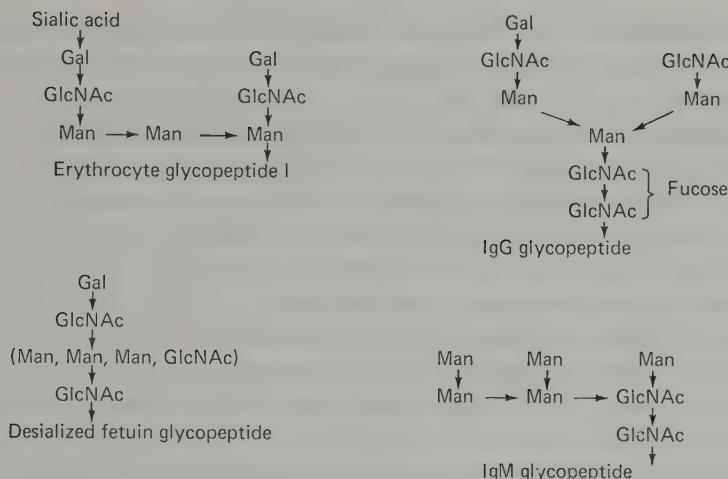


Fig. 12.1. Structures of glycopeptides which inhibit the binding of mitogens to lymphocytes. All four glycopeptides inhibit the binding of the lentil mitogen, and the erythrocyte and fetuin glycopeptides also inhibit H-PHA binding. Gal, galactose; Man, mannose; GlcNAc, N-acetyl-D-glucosamine. Redrawn from Kornfeld et al. (1971).

12.1 (taken from Kornfeld et al., 1971). All the effective inhibitors seem to contain mannose residues, but N-acetyl-D-galactosamine residues are rarely present. The same glycopeptide can prevent the action of both PHA and the lentil mitogen, but these two mitogens seem to have specificity for different regions of the molecule. Removal of the terminal sialic acid residue from the erythrocyte glycopeptide (fig. 12.1) does not affect its capacity to inhibit the activity of either lectin, while removal of the galactose residue results in loss of the inhibitory activity to PHA but not to the lentil mitogen, the specificity of which seems to be directed towards the N-acetyl-glucosamine and mannose residues (Kornfeld and Kornfeld, 1969; Kornfeld et al., 1971).

Thus, lectins with different specificities may well interact with different parts of the same glycopeptide. Direct support for this observation comes from the demonstration that PHA strongly inhibited the binding of ^{131}I -labelled lentil mitogen to erythrocytes (Kornfeld et al., 1971). There was also some reciprocal inhibition, but this was rather weak and variable, probably because the affinity of PHA for the receptor is greater than that of the lentil mitogen.

These demonstrations that there are a number of glycopeptides to which mitogens can bind do not, of course, tell us what the lymphocyte receptors

for these mitogens are. There may, indeed, be a wide range of different receptors on the lymphocyte surface, and not all of these may be involved in mitogenesis.

Lymphocyte PHA receptors are presumably also surface glycoproteins, as treatment of lymphocytes with proteases causes a transient loss of their ability to be stimulated by PHA (Lindahl-Kiessling and Peterson, 1969). The protein part of the glycoproteins may also be of some importance in stimulant binding, as up to 90% of the inhibitory activity of a glycoprotein preparation is lost during the proteolytic digestion used to prepare glycopeptides (Kornfeld and Kornfeld, 1970). The role of the protein is probably to hold the oligosaccharide chains in a favourable configuration for interaction with the mitogen. Glycoproteins which bind PHA and con A can be extracted from lymphocyte membranes with sodium deoxycholate (Allan et al., 1971) and con A and lentil mitogen receptors have been purified by affinity chromatography on columns containing con A or lentil mitogen covalently linked to sepharose 4B (Allan et al., 1972; Hayman and Crumpton, 1972). Such isolated receptors are very potent inhibitors of lymphocyte activation, but they have not yet been extensively characterized.

The demonstration that glycopeptides which act as competitive inhibitors for mitogen binding are present in many other mucoproteins, particularly in a number of serum proteins, provides one possible explanation for the common observation that the mitogen concentration required for stimulation is affected by the concentration of serum or the presence of other preparations containing glycoproteins. The effects of serum in particular may well be complex, but in at least some situations its main effect is on the binding of the mitogen (Powell and Leon, 1970; Kay, 1971a).

The numbers of lectin molecules that can bind to the lymphocyte surface have been determined by several groups. The method that is generally used is to label the lectin with either ^{125}I or ^{131}I , a procedure which seems to have little effect on its biological activity, and then determine the uptake of radioactive lectin at a number of different concentrations, and extrapolate the number of molecules bound at infinite concentration. Human lymphocytes have been found to bind up to 2.7×10^6 molecules of H-PHA per cell (Kornfeld, 1969) and either 2.4×10^6 or 6.6×10^6 molecules of lentil mitogen per cell (Kornfeld et al., 1971; Stein et al., 1972a). Mouse spleen cells, bone marrow cells, thymocytes and lymphocytes and rat lymph node cells have all been found to bind about 10^7 molecules of con A per cell (Edelman and Millette, 1971; Stobo et al., 1972; Betel and Van den Berg, 1972). At these

very high concentrations it has been calculated that the lectin molecules must be quite closely packed on the lymphocyte surface (Betel and Van den Berg, 1972). However, all workers agree that optimal lymphocyte stimulation occurs when the concentration of the stimulants on the surface is much lower than the maximal level. The numbers of immunoglobulin molecules on mouse spleen cells that can bind to anti-immunoglobulin are much lower, probably no more than 4×10^4 per cell (Yahara and Edelman, 1972).

The binding sites for lectins and for anti-lymphocyte serum seem to be fairly homogeneous, resulting in fairly sharp dose response curves to these mitogens, in sharp contrast to the much broader dose response curves found on antigenic stimulation, where the receptors have a very wide range of affinities for the antigen (Foerster et al., 1969).

12.2.2. Requirements for stimulant binding

The binding of lectins to lymphocyte receptors seems to be a simple reaction analogous to their binding to sugars, carbohydrates and glycoproteins. It occurs quite normally if the lymphocytes have been killed by heating to 70 °C for 30 min (Kay, 1971a) or when lymphocyte ATP synthesis is completely blocked by metabolic inhibitors (Lindahl-Kiessling and Mattsson, 1971).

In normal culture conditions the binding of most stimulants to lymphocytes occurs very rapidly. This is demonstrated by the numerous observations, with many different stimulants, that marked activation occurs if lymphocytes are incubated with mitogens for short periods and then cultured in the absence of stimulant, and by the observation that mitogens affect lymphocyte metabolism in many different ways very soon after their addition. More recent and detailed studies have confirmed that, at least for PHA, con A and anti-immunoglobulin, the binding is essentially complete within minutes of addition of the stimulant (Kay, 1969; Powell and Leon, 1970; Mendelsohn et al., 1971; Yahara and Edelman, 1972). The rate of binding of PHA and con A is dependent on the temperature of incubation, occurring much more slowly at 4 °C, and also on the concentration of the lectin (Kay, 1971a; Lindahl-Kiessling and Mattsson, 1971; Betel and Van den Berg, 1972).

All purified lectins that have been studied contain divalent cations, usually Ca^{2+} and Mn^{2+} , (ch. 6.1) and these have often been shown to be important for their biological activity. Chelation of divalent cations with EDTA prevents the agglutination of erythrocytes by PHA (Tunis, 1965) and the lentil

mitogen (Paulova et al., 1971), and inhibits the binding of PHA to lymphocytes (Kay, 1971b). The binding of both H-PHA and L-PHA was inhibited, and the inhibition could be reversed by either Ca^{2+} or Mg^{2+} . A similar, although less pronounced, inhibition of con A binding to lymphocytes by EDTA has been reported (Lindahl-Kiessling, 1972). These effects are probably due to a requirement of lectin binding for divalent cations rather than a direct inhibitory effect of EDTA, as PHA from which divalent cations have been removed by acidification and extensive dialysis is also unable to stimulate lymphocytes (Lindahl-Kiessling, 1972).

12.2.3. The location of the stimulant

While it has become accepted that lymphocyte stimulants bind initially to the cell membrane, argument has long continued as to whether activation requires the subsequent entry of the stimulant into the cell. The ability of homologous lymphocytes and antigens such as heterologous erythrocytes to bring about lymphocyte activation suggested that internalization of the stimulant would not be required, but it is possible that the antigenic determinants actually responsible for such stimulation might be cleaved from the stimulating cell surface and taken up by the responding cells. More recently, the demonstrations that lymphocytes can be activated by PHA or pokeweed mitogen covalently bound to sepharose beads many times the size of a lymphocyte (Greaves and Bauminger, 1972) and con A cross-linked to plastic Petri dishes (Andersson et al., 1972) or bound to acrylic polymer particles (Betel and Van den Berg, 1972) have largely confirmed the view that the effects of the stimulant can be exerted at the plasma membrane level.

At the same time evidence has accumulated that at least some stimulants can enter the lymphocyte, although it is probable that such uptake is quite irrelevant to activation. Early studies on the localization of stimulant tagged with fluorescent marker, mercury or radioisotopes or using fluorescein-tagged antisera to stimulants gave very variable results. Different groups found the stimulant to be localized in the nuclei, the cytoplasm, near the cell membrane, or in the mitochondrial fraction. Many of these studies can be criticised because of the low purity of the stimulant preparations used, their low specific activity, or the resolution of the detection method used, and the possibility that the activity of the stimulant may have been affected by the labelling procedure used. However, more recent work using highly purified stimulants has shown that while binding occurs initially at many sites around

the periphery of the cell, the mitogen subsequently becomes concentrated in a 'cap' at one pole of the cell, and is then internalised by pinocytosis (Smith and Hollers, 1970; Biberfeld, 1971; Taylor et al., 1971). The formation of the 'cap', which seems often to be localized in the area of the uropod, is dependent on active energy metabolism by the cell and, most probably, its motility. This 'cap' formation shows that the receptors are initially dispersed on the cell membrane, but can move relative to one another under the influence of the stimulants, perhaps because multivalent stimulants can bind to more than one receptor. It further confirms that the membrane itself has a fluid rather than a rigid structure.

Some of the mitogen that binds to the cell membrane is shed from the cell surface while still complexed with its receptors (Jones, 1973). This shedding of surface membrane components is an entirely normal phenomenon, although it occurs more rapidly in some types of cells than in others, and is more prominent in thymocytes than in spleen cells. The rate at which it occurs is accelerated early during the activation process, an increase which is perhaps a consequence of increased motility. Some of the mitogen enters the cells by endocytosis, and seems to be concentrated in cytoplasmic vacuoles which are probably lysosomes (Biberfeld, 1971; Barat and Avrameas, 1973). It may well be degraded by lysosomal enzymes after fusion of the endocytic vesicles with lysosomes, although this has been demonstrated only for ingested anti-immunoglobulin (Engers and Unanue, 1973). Such a fate would be expected to befall any protein deposited on the surface of a nucleated cell. There is no convincing evidence that any mitogen enters the cytoplasm intact, or that this entry into the cell is relevant to activation. In normal culture conditions, there is probably always mitogen attached to receptors on the cell surface.

12.2.4. Continuous requirement for the stimulant

A key requirement for the determination of the mechanism of action of lymphocyte stimulants is to establish whether the stimulant simply imparts a trigger effect, and is not required thereafter, or whether it needs be present continuously. The simplest approach to this problem, to remove the stimulant after incubation with lymphocytes for a short period and then determine whether they become activated, has given surprisingly variable results in the hands of different workers. The results obtained in the case of PHA have varied from normal stimulation after exposure for 5 min (Richter et al.,

1966) to complete and rapid reversal whenever the PHA is removed (Tormey and Mueller, 1965). Such variation is very difficult to explain, even when due allowance is made for the different parameters of stimulation used by the different workers, and the different stages of the response which were examined. Part of the explanation may lie in the different affinities for their receptor of different PHA preparations (§ 6.1). The great majority of workers have found considerable activation after exposure to a variety of stimulants for less than 1 hr but these results indicate that the stimulant has become firmly bound to the lymphocytes, rather than that activation involves a trigger effect. It has been shown that PHA can remain attached to lymphocytes for at least 72 hr after its removal from the culture medium (Naspitz and Richter, 1968).

Evidence that PHA, although bound to lymphocytes rapidly, was still required for activation was first provided by Kay (1969), who compared the effects of washing cells free of PHA to adding anti-PHA antiserum. The PHA bound to the cells very rapidly, but its effect on DNA synthesis could be completely prevented by the addition of anti-PHA after 1–2 hr, and partially reduced for up to 6 hr (fig. 12.2). At later times the stimulation was not affected by anti-PHA. In these experiments the rate of ^{3}H -thymidine

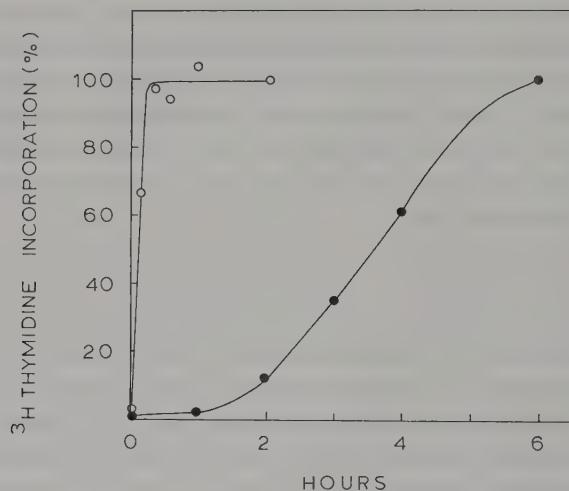


Fig. 12.2. Effect of removal of PHA by washing (○) or by addition of anti-PHA (●) at different times after addition of PHA on the rate of incorporation of ^{3}H -thymidine into DNA at 48 hr. Redrawn from Kay (1969).

incorporation into DNA was measured after incubation for a total of 48 hr, so that only the more rapidly responding lymphocytes would be involved. Addition of anti-PHA for up to 12 hr after PHA completely suppressed DNA synthesis at 72 hr, and addition for up to 48 hr was partially inhibitory at this time (Younkin, 1972). In this case the cells involved would be mainly those cells which responded to PHA more slowly, together with some cells in their second division cycle. Lymphocytes thus seem to become committed to DNA synthesis even in the absence of surface-bound PHA at least 24 hr before the initiation of DNA synthesis. There is an earlier period in which the presence of PHA is required, which varies from cell to cell in much the same way as the G_1 period after stimulation (see ch. 14). Rather similar results have been obtained by the addition of blocking serum bearing the appropriate Ig alleles at different times after stimulation of rabbit peripheral blood lymphocytes by anti-allotype antiserum (Sell et al., 1972).

Perhaps the most convincing evidence on this point has come from studies on activation by con A and lentil mitogen, as these mitogens have been shown to be removed completely from the lymphocytes by addition of sugars such as methyl- α -D-mannoside. Addition of these sugars up to 6 hr after con A completely suppresses ^3H -thymidine incorporation after 72 hr, and addition after 22 hr caused partial inhibition (Powell and Leon, 1970). Almost identical results were obtained with the lentil mitogen (Stein et al., 1972 b).

If, after transient stimulation, cultures are incubated for several days in the absence of stimulants, DNA synthesis declines, few blast cells remain and a population of predominantly small and medium sized lymphocytes which is not, however, identical in morphology to the starting population is obtained (Hardy and Ling, 1973). If the stimulant used is PHA enough may remain bound to the cells to allow some DNA synthesis to continue for up to a week, and for many of the cells to complete more than one division cycle (Polgar et al., 1968; Munakata and Strauss, 1972). This reversion must be due to a requirement by the cells for continuous stimulation rather than an intrinsic limitation of their capacity for growth, as they can readily be stimulated again by readdition of the stimulant.

Comparatively little work has been done on the requirement for the continuous presence of PHA or parameters of lymphocyte stimulation other than DNA synthesis. Mendelsohn et al. (1971) studied the effect of removal of PHA by fetuin 2 hr after its addition on the uptake of the synthetic amino acid α -aminoisobutyric acid, and found that while the elevated rate of uptake

already achieved was maintained for at least a further 3 hr, no further increase occurred after the removal of PHA. The fetuin was shown to remove most, but not all, of the PHA bound. Addition of anti-PHA 24 hr after PHA did not affect the rate of ^3H -uridine incorporation into RNA for 8 hr after its addition. At later times the rate did fall below the control level, but it remained well above the unstimulated level for more than 24 hr (Hausen and Stein, 1968). The synthesis of interferon in response to PHA stimulation (§ 10.1) requires the continuous presence of PHA in the culture medium (Friedman and Cooper, 1967; Epstein et al., 1971) but the relationship of this phenomenon to other aspects of lymphocyte activation is not well defined at present.

In summary, there seems little doubt that the initiation of DNA synthesis requires the presence of the stimulant for a period which is both prolonged and also variable from one cell to another. However, the cell becomes committed to DNA synthesis, independent of the further presence of the stimulant, several hours before the actual initiation of DNA synthesis, and other metabolic changes, once brought about, may be maintained after the stimulant is removed.

12.2.5. Relationship between stimulant binding and activation

While the binding of mitogens to the lymphocyte surface seems to be a prerequisite for activation, evidence is accumulating that this binding is not, in itself, the cause of activation. Activation is certainly not a simple consequence of binding lectins to the cell membrane, as there are many lectins which bind to the lymphocyte surface, and cause lymphocyte agglutination, but do not lead to activation. The lymphocytes so treated retain their capacity to be stimulated by other mitogens. It is, of course, possible that these ineffective lectins bind to different receptors on the lymphocyte surface from the mitogens, but there are inactive lectins which have similar specificities to both con A and PHA, as judged by their reaction with sugar haptens.

In addition the abilities of some mitogens to discriminate between T lymphocytes and B lymphocytes cannot be ascribed to the presence or absence of the appropriate receptors on the cell surface. Mouse lymphocyte populations rich in T lymphocytes and those rich in B lymphocytes bind similar amounts of the radiolabelled PHA and con A though mainly T cells are activated (Stobo et al., 1972; Greaves et al., 1972) and of LPS which affects predominantly the B lymphocytes of mouse spleen (Moller et al., 1973).

There were no significant differences between the proportions of lymphocytes able to bind the stimulants PHA, con A and lentil mitogen in mouse cell populations from a number of sources (Greaves et al., 1972). Although the percentage of θ -positive cells in the different populations examined varied from less than 2% to virtually 100%, in every case nearly all the lymphocytes could bind all the stimulants, as judged by microagglutination or immunofluorescence. The demonstration that B lymphocytes can be activated by con A and PHA, normally considered T cell mitogens, if these lectins are attached to insoluble matrices (Andersson et al., 1972; Greaves and Bauminger, 1972) demonstrates that the B cells must have the appropriate receptors.

One possibility is that cross-linking of the receptors by mitogens may be important for activation. Monovalent Fab' fragments of goat anti-rabbit immunoglobulin bind to rabbit lymphocytes but do not activate them, while the corresponding divalent F(ab')₂ fragments are mitogenic. Activation of lymphocytes to which the Fab' fragments were bound was achieved by the addition of a bivalent antibody directed against the univalent Fab' fragments (Fanger et al., 1970). Similarly, both human and rabbit lymphocytes can be activated by divalent, but not monovalent, fragments of anti-lymphocyte serum (Woodruff et al., 1967; Sell et al., 1970), but Sell et al. did find that monovalent fragments of sheep anti-rabbit immunoglobulin, and rabbit anti-rabbit allotypic immunoglobulin, were mitogenic.

However, cross-linking of receptors by lectins, and consequent 'cap' formation seems to be neither necessary nor sufficient for activation. Essentially no 'cap' formation is seen with mitogenic concentrations of pokeweed mitogen (Loor, 1973) and from structural studies on this mitogen (§ 6.1.4) it seems likely that it may not be able to cross-link receptors. In addition, some concentrations of con A within the mitogenic range not only do not result in 'cap' formation, but also seem to inhibit the movement of other receptors within the cell membrane (Yahara and Edelman, 1973). In contrast, 'cap' formation does occur with the non-mitogenic lectin from the mushroom *Agaricus bisporus* (Ahmann and Sage, 1972), and B lymphocytes are able to 'cap' con A as efficiently as T lymphocytes in conditions which do not result in B lymphocyte activation (Greaves et al., 1972).

Further hypothesis about the relationship between stimulant binding and activation becomes increasingly speculative. One possibility is that the surface receptors are very heterogeneous, and that only a minor sub-population are involved in activation. This sub-population could be qualitatively and

quantitatively different for the different types of lymphocytes. Another possibility is that there is a secondary receptor which is activated by the interaction of the stimulant with the first receptor. A detailed model based on this supposition has been formulated by Moller et al. (1973) to account for their observations on the activation of T and B lymphocytes by con A in different experimental conditions, on the assumption that B lymphocytes contain fewer molecules of the secondary receptor.

12.3. Changes in cell membrane function

All lymphocyte stimulants that have been studied bind initially to the cell surface, and in at least some cases there is good evidence that they do not need to enter the cell. The attachment of such stimulants has many effects on the properties of the cell membrane. An increased adhesion of cells to one another (§ 12.1.1) and to their glass culture vessels (Killander and Rigler, 1965) occurs almost immediately after the addition of PHA. The net charge of the cell membrane is altered by PHA (Vassar and Culling, 1964), although this does not occur if non-erythroagglutinating PHA is used (Caspary and Knowles, 1970), and the numbers of its hormonal binding sites (Krug et al., 1972) change. The metabolism of some phospholipid components of the membrane changes (§ 13.5) and there is morphological evidence of both qualitative and quantitative changes in pinocytosis (Biberfeld, 1971).

Amongst the most interesting of the early changes after stimulation of lymphocytes are the alterations in the transport properties of the cell membrane. Small molecules whose rate of entry into lymphocytes are increased after stimulation include nucleosides (Peters and Hausen, 1971a), sugars (Peters and Hausen, 1971b), the non-utilized amino acid α -amino isobutyric acid (Mendelsohn et al., 1971), phosphate (Cross and Ord, 1971), choline (Peters and Hausen, 1971a), K^+ (Quastel and Kaplan, 1971), Na^+ (Dent, 1971) and Ca^{2+} (Allwood et al., 1971). This list is almost certainly not exhaustive, and will doubtless become longer as more compounds are studied. These changes have generally been studied in PHA stimulated lymphocytes, but some similar changes occur with other stimulants and in the mixed lymphocyte reaction (Van den Berg and Betel, 1971; Whitney and Sutherland, 1972a). These changes are not unique to lymphocyte stimulation, and very similar changes occur when the growth rates of other types of mammalian cells are increased (Cunningham and Pardee, 1969; Hershko et al., 1971).

In many cases the increased uptake of these small molecules seems to be a primary effect of the lymphocyte stimulants on the cells. The increased rates of uptake have frequently been demonstrated within minutes of the addition of the stimulant, and often are not affected by inhibition of protein and RNA synthesis. Unlike later events in stimulation, such as the synthesis of DNA, the increased rates of uptake of these small molecules are not inhibited by high concentrations of stimulants, and, indeed, they are frequently studied using stimulant concentrations which would be toxic at later times. Con A has been shown to have a direct stimulatory effect on the microsomal Na^+/K^+ -independent ATPase of rat lymphocytes (Novogrodsky, 1972). The function of this enzyme(s) is uncertain, but it may be a reflection of an active transport system. In all cases yet studied except one, investigation of the kinetics of these uptake processes before and after stimulation has shown that the K_m for the substrate does not change, but the V_{max} is increased. This shows that the nature of the uptake process does not change after stimulation, and rules out the possibility that the increased uptake is due to some non-specific increase in permeability, due to loosening of the membrane architecture or increased pinocytosis consequent upon binding of the stimulant. These kinetics could most simply be explained by an increase in the number of membrane carriers of the normal type, but they could also be due to an increase in the maximum rate of transport at each of a constant number of sites. The exception is the uptake of Ca^{2+} , where it appears that the K_m decreases, while the V_{max} remains unchanged (Whitney and Sutherland, 1973a). In this case it seems likely that the mitogen increases the affinity of the membrane carrier system for this ion.

The impression of a general increase in the rates of uptake of all small molecules is not, however, fully borne out by a closer examination. While allowances must be made for variation in studies using different types of cells and different culture conditions in different laboratories, there seems to be a clear difference between the very marked but very transient increase reported for phosphate uptake, which rises to 8 times the initial rate after 10 min, but returns to the control rate after 30 min (Cross and Ord, 1971), and the increase in α -amino isobutyric acid uptake, which begins only after 30 min and rises gradually over several hours (Mendelsohn et al., 1971). The increase in α -amino isobutyric acid uptake is also dependent on protein synthesis (Van den Berg and Betel, 1973a) and thus may not be a primary consequence of stimulation. The metabolizable amino acids are more difficult to study because of their small intracellular pool and rapid saturation,

but Peters and Hausen (1971a) found the uptake of leucine to be stimulated much less by PHA than the uptake of uridine, choline or glucose. The uptake of the non-metabolizable amino acid cycloleucine, which is taken up by the same transport system as leucine, is also increased very much less than the uptake of α -amino isobutyric acid (Van den Berg and Betel, 1973 b). These results, and others utilizing natural amino acids, suggest that the amino acids whose uptake is increased after lymphocyte stimulation are those which enter the cell by the Na^+ -dependent transport system.

The significance of these effects is two-fold. At the practical level, they alter the rate of saturation of the intracellular pools of affected metabolites by exogenous radioactive precursors. The consequent increase in the rate of incorporation of the precursor into its products has frequently been thought, mistakenly, to indicate increased synthesis of the product. The clearest example of this is the increased labelling of RNA by exogenous ^3H -uridine (§ 13.1.3), but there are probably many other isotopic studies to which similar considerations apply.

Secondly, at a more theoretical level, the increased membrane transport may provide a link between the binding of the stimulant to the cell surface and the subsequent changes in cell metabolism. It is known from studies on

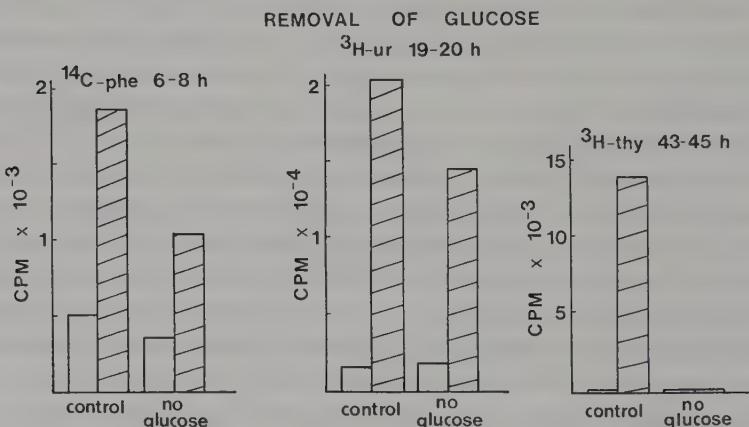


Fig. 12.3. Effect of removal of glucose from the culture medium on the stimulation by PHA of the incorporation of ^{14}C -phenylalanine between 6 and 8 hr after the addition of PHA, of ^3H -uridine 19–20 hr after the addition of PHA, and ^3H -thymidine 43–45 hr after the addition of PHA. Open columns show incorporation by unstimulated lymphocytes, hatched columns incorporation in the presence of PHA.

nuclear transplantation and cell hybridization that the synthesis of DNA and RNA by the nuclei of animal cells is controlled by this cytoplasmic environment but it is not yet known which components of the cytoplasm are responsible. If one of the small molecules whose uptake is increased by growth stimulation were important in this respect, the link would be provided.

It is known that not all the uptake processes stimulated by PHA are essential for lymphocyte growth. Uridine, for example, is not a necessary ingredient of the culture medium, and inhibition of its uptake by dipyridamole does not inhibit stimulation (Peters and Hausen, 1971a). Phosphate can be omitted from the medium without affecting the response, while omission of glucose (fig. 12.3) or several of the amino acids abolishes or greatly reduces the initiation of DNA synthesis, but allows alterations characteristic of the earlier stages of the response, such as increased protein synthesis to occur (Kay, 1973). Addition of enzymes which destroy amino acids in the culture medium has frequently been reported to prevent the induction of DNA synthesis by mitogens. The enzyme used most frequently has been asparaginase (Ohno and Hersh, 1970), but others known to be effective include glutaminase (Hersh, 1971), arginase (Barile and Leventhal, 1968) and phenylalanine ammonia lyase (Abell et al., 1972). However, the effect of these enzymes on the early stages of the response has often not been studied, and at least in the case of asparaginase some of the early changes are not prevented.

Inhibition of K^+ uptake by ouabain allows the very early increase in 3H -uridine incorporation into RNA to occur, but abolishes even the earliest increase in protein synthesis (Kay, 1972), as well as the subsequent initiation of DNA synthesis (Quastel and Kaplan, 1968). Protein synthesis by unstimulated lymphocytes is not affected by this drug for several hours, so that in this case the effect of this inhibition does seem specific for the stimulation. This increase in K^+ uptake may, however, be secondary to other changes. Many of the transport processes activated very early in lymphocyte stimulation are of the type known to be dependent on Na^+ , and accompanied by Na^+ uptake in other cells (Schultz and Curran, 1970). The activity of the Na^+/K^+ -dependent ATPase may be increased to restore the intracellular Na^+/K^+ balance, the maintenance of which may be necessary for the increase in protein synthesis. The increases in Na^+ (Kay, 1973) and Ca^{2+} (Whitney and Sutherland, 1972 b, 1973 b) uptake seem also to be necessary for stimulation, and the increase in Ca^{2+} uptake may be necessary for other changes in cell membrane function such as the increase in α -amino isobutyric acid

uptake. The importance of other metabolites remains to be investigated.

Increased pinocytosis after lymphocyte stimulation has been reported to lead to increased uptake of macromolecules such as neutral red (Hirschhorn et al., 1968) and horse radish peroxidase (Robineaux et al., 1969), although Lucas et al. (1971) and Mendelsohn et al. (1971) found that the rate of inulin uptake was not increased. However, increased uptake of macromolecules from plasma is unlikely to be important for stimulation, as many workers have shown that stimulation can occur when plasma is omitted. One further possibility is that the important transport process to be increased after stimulation is the secretion from the lymphocytes of an inhibitor of growth (Fisher and Mueller, 1968).

In summary, the binding of lymphocyte stimulants to the cell membrane results in a complex sequence of changes in the uptake of a variety of small molecules. In some cases this may be a direct effect of the stimulant. At least some of these changes are quite irrelevant to lymphocyte stimulation. Others seem more important but the hypothesis, propounded originally by Nowell (1960), that PHA may facilitate the entry of 'blastogenic factors' by affecting the cell membrane, remains to be proven.

12.4. Cyclic AMP

The actions of some, but not all, polypeptide hormones on their target cells seem to be mediated by a 'second messenger', cyclic 3'5'-AMP (cAMP) (Robinson et al., 1968). This compound is synthesized from ATP by the enzyme adenyl cyclase, which appears to be located in the plasma membrane, in response to the binding of the hormone to its receptors on the membrane. The breakdown of cAMP to AMP, which is equally important in the determination of its intracellular concentration, is catalysed by a phosphodiesterase located, at least in lymphocytes, in the nuclear membrane (Coulson and Kennedy, 1971).

Many groups of workers have investigated the possibility that the stimulation of lymphocytes by PHA might be mediated by an effect on adenyl cyclase. The most frequent approach has been to add to lymphocytes either cAMP or its analogue dibutyryl cAMP, which penetrates some mammalian cells more easily, or, alternatively, substances which would be expected to increase the intracellular cAMP, either by stimulating adenyl cyclase or inhibiting the phosphodiesterase. Such additions have proved nearly uniformly inhibitory to lymphocyte stimulation by PHA, and have had, in

general, only marginal effects on unstimulated lymphocytes. An alternative approach, which has given much more variable results, has been to attempt to measure the activities of adenyl cyclase or the endogenous cAMP levels in stimulated and unstimulated lymphocytes. Nearly all groups have concluded that PHA and other lymphocyte stimulants cannot be acting simply through cAMP, but it has often been suggested that this compound may have some role.

Initially most workers were testing the hypothesis that lymphocyte stimulation would proceed through the stimulation of adenyl cyclase, by analogy with the effects of polypeptide hormones. However, study of the control of growth of animal cells in culture has shown that the growth rate correlates inversely with the endogenous cAMP level (Otten et al., 1971) and is inhibited by exogenous cAMP (Burk, 1968). While this adds interest to the observations that cAMP inhibits lymphocyte stimulation, the apparent lack of specificity of this effect for cyclic nucleotides for this effect suggests a note of caution.

12.4.1. *Exogenous cAMP*

Lymphocyte stimulation by PHA is inhibited by dibutyryl cAMP at concentrations above 10^{-5} M and cAMP at above 10^{-3} M. It is also inhibited by agents expected to increase the intracellular cAMP level such as the phosphodiesterase inhibitor theophylline, and aminophylline, isoproterenol and prostaglandins, which stimulate adenyl cyclase activity (Hirschhorn et al., 1970; Novogrodsky and Katchalaski, 1970; Smith et al., 1971b). DNA, RNA and protein synthesis are all affected. Stimulation by antigen (Smith et al., 1971b) and allogeneic lymphocytes (Estes et al., 1971) and the lysis of mouse mastocytoma cells by specifically sensitized lymphocytes (Henney and Lichtenstein, 1971) are all similarly affected.

This inhibition does not seem to be a specific property of cAMP. All other cyclic nucleotides tested have had similar effects, and AMP, ADP, ATP and adenosine are all inhibitory at about the same levels as cAMP. None of these compounds have been reported to be toxic to lymphocytes at the levels used, and while this point has often not been tested rigorously, the effects of dibutyryl cAMP have been shown to be reversible. These nucleotides do not inhibit the binding of the PHA to lymphocytes (Smith et al., 1971b).

Low grade stimulation of ^3H -uridine and ^3H -thymidine incorporation has frequently but not universally been recorded after addition of very low con-

centrations of cyclic nucleotides to unstimulated lymphocytes. The stimulations are usually very much lower than those seen with even weak mitogens. The increase in thymidine incorporation in particular is generally marginal, but uridine incorporation increases by as much as 50–100%. These stimulatory effects do seem to be specific for cyclic nucleotides, but in most cases it has not been determined whether the effects are on precursor uptake or RNA synthesis. Gallo and Whang-Peng (1971) have reported an augmentation of up to 50% of the responses of lymphocytes to PHA and allogeneic lymphocytes by dibutyryl cAMP at concentrations just below those which are inhibitory. In this case the percentage of lymphocytes showing morphological transformation increased, as well as the rate of DNA synthesis. Averner et al. (1972) found cAMP to stimulate ^{14}C -uridine incorporation into RNA by lymphocytes incubated with or without PHA by 50–100% without affecting the uptake of the isotope into the acid soluble pool. Their results have, however, a number of unusual features, perhaps related to their use of horse lymphocytes. While the stimulation of ^3H -thymidine incorporation into DNA was of the normal magnitude, incorporation into RNA was increased only by a factor of three after PHA stimulation. In addition, they found no inhibition of the PHA effect in the presence of cAMP concentrations found by others to be strongly inhibitory.

12.4.2. Adenyl cyclase and cyclic AMP phosphodiesterase

In contrast to the general agreement about the effects of direct addition of cAMP and its analogues to lymphocytes, attempts to determine the changes in adenyl cyclase activity during stimulation have met with very variable results. Different groups have concluded that incubation of lymphoid cells with PHA for short periods stimulates (Winchurch et al., 1971; Krishnaraj and Talwar, 1973) inhibits (Makman, 1971) or has no effect on (Novogrodsky and Katchalski, 1970) adenyl cyclase. In one study in which the effects of PHA and con A were compared, con A was found to give a much smaller, and only marginally significant, increase in adenyl cyclase activity, while the two mitogens had comparable effects on thymidine incorporation (Krishnaraj and Talwar, 1973). Others have found that much or all of the adenyl cyclase activity in preparations from blood lymphocytes was derived from contaminating platelets (Wolfe and Shulman, 1969; McDonald, 1971), although lymphocyte activity may have been lost due to sonication or excessive membrane fragmentation (Smith et al., 1971a). While there is

general agreement that addition of fluoride or prostaglandins to the assay greatly stimulates adenyl cyclase, the observation that PHA has a similar albeit smaller direct effect (Smith et al., 1971a) has not been confirmed (Makman, 1971; McDonald, 1971; Krishnaraj and Talwar, 1973).

It is very difficult to compare these results with one another. The cells used in these experiments have been obtained from different lymphoid organs and species, and incubated under different conditions with different concentrations of PHA. Even quantitative comparisons of the results are not possible because of the varying ways in which they are expressed. Further, no quantitative studies on the activity of cAMP phosphodiesterase have yet been published, and this will be as important as the cyclase in determining the intracellular level of cAMP.

The changes in endogenous cAMP concentrations during stimulation by PHA were determined by Smith et al. (1971a), using a radioimmunoassay which depends on the ability of cAMP from cell extracts to compete with the binding of radioiodinated cAMP to anti-cAMP antibody. They found an early 25–300% increase in cAMP which was apparent within 1 min of PHA addition and maintained for 1 hr. By 6 hr the level in stimulated lymphocytes had fallen to below the control level, and it remained low until at least 20 hr. Most of these experiments were performed with unusually high concentrations of blood lymphocytes and very high PHA concentrations, but similar changes, including the late fall in cAMP, seemed to occur in more normal conditions. Cyclic AMP levels of granulocytes were not affected by PHA, although those of platelets were also slightly increased. Lymphocyte cAMP was also increased by isoproterenol, norepinephrine, prostaglandins and aminophylline.

These results have been very widely quoted, but they have not been substantiated by subsequent work. The early increase in cAMP concentration has been reported to occur only if unpurified PHA is used, while neither purified PHA nor con A had any effect (Hadden et al., 1972). In another study, the fall in cAMP later in culture was found only if supraoptimal PHA concentrations were used (Webb et al., 1973). Prostaglandin also increased the cAMP level in rat lymph node cells, as determined by incorporation of ^3H -adenine (Novogrodsky and Katchalski, 1970) but PHA had no effect in this system, in line with its lack of effect on adenyl cyclase.

The conclusions to be drawn from this work are at present far from clear. Neither cAMP itself nor agents which lead to an increase in its concentration in the lymphocytes cause a degree of stimulation even remotely comparable

to that caused by PHA. Indeed, elevated cAMP levels seem to inhibit the proliferative stages of the response, much as they inhibit the growth of other cells in culture. The transient increase early in the response may prove part of the early sequence of changes, although it would seem doubtful at present that PHA could act simply by activating adenyl cyclase, in the way that some other polypeptide hormones seem to.

12.4.3. Cyclic GMP

More recently attention has turned to another cyclic nucleotide, cyclic GMP (cGMP), whose action in many systems counteracts that of cAMP (Kram and Tomkins, 1973). The concentration of cGMP is much lower than that of cAMP, but Hadden et al. (1972) found it to increase between 10- and 50-fold within 20 min of addition of either PHA or con A to lymphocytes (fig. 12.4). If this observation proves to be repeatable, then it clearly suggests that this nucleotide could provide the missing link between mitogen-binding at the surface membrane and the various intracellular metabolic changes which follow. Cholinergic agents such as acetylcholine, which increase the intracellular cGMP concentration in other tissues, have been found to enhance the cytotoxic action of sensitized lymphocytes (Strom et al., 1972); but in the only other study to date in which cGMP levels have been measured after PHA stimulation (Webb et al., 1973), no marked changes were found.

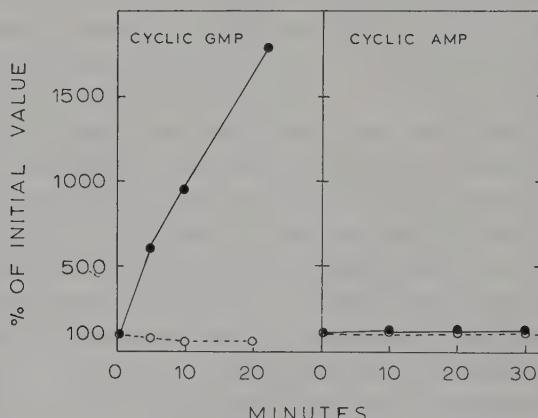


Fig. 12.4. Changes in the concentrations of cyclic GMP and cyclic AMP during incubation of lymphocytes alone (○) or following the addition of PHA (●). Redrawn from Hadden et al. (1972).

However, in this study no measurements were made within the first hour of the addition of the mitogen. Further reports on this topic will doubtless become available in the near future.

12.5. Gene activation

Given the enormous impact on biology in the 1960s of the elegant demonstrations that in bacteria gene activation can be controlled by low molecular weight metabolites, which initiate messenger RNA synthesis by binding to specific protein repressors and preventing their attachment to DNA, it is in retrospect inevitable that most attempts to explain changes in the behaviour or metabolism of mammalian cells should have been in terms of similar mechanisms, in spite of the major differences in organisation of bacteria and mammalian cells. Attempts to explain lymphocyte stimulation have been no exception to this rule and most hypotheses have been based on the premise that the key events would be initiation by the stimulants, directly or indirectly, of transcription at new sites on the genome. The early demonstration that the initiation of DNA synthesis was preceded by increased incorporation of precursors into RNA seemed only to confirm this view. In consequence, a great deal of effort has been devoted to the study of the nuclear deoxyribonucleoprotein (DNP), and the changes reported have led many to believe that the key early event in lymphocyte stimulation is an alteration in the DNP structure resulting in the activation of many previously inactive genes. This belief is still widely held, and has survived the realisation that the early increase in incorporation into RNA is very largely a function of a change in the rate of uptake of the precursors and the failure of the admittedly insensitive methods used to date to detect the synthesis of new species of RNA after stimulation (§ 13.1).

While our present understanding of the role of RNA synthesis in the control of cell function is primitive, it is highly probable that at some stages in the complex sequence of events between stimulation and cell division there will be changes in the rates of transcription of individual genes. The point at issue is whether these changes would occur gene by gene, as part of a programmed sequence, in which case only a small proportion of the genome need be involved and the changes might be gradual and difficult to detect by available methods, or whether stimulation involved an early general genetic derepression, resulting in massive random transcription which somehow leads to initiation of growth. As an intermediate view, the general derepres-

sion might merely make genes accessible to the transcription machinery, with some more specific mechanism selecting which were actually to be transcribed.

The most impressive and coherent body of information on changes in the physical state of lymphocyte nuclear DNP after stimulation has come from the use of quantitative histochemical techniques. The main changes reported have been an increased availability of DNA phosphate groups for binding to acridine orange, a decrease in the number of histone basic groups released to bind to acidic dyes after hydrolysis of the DNA, and a reduction in the resistance to thermal denaturation of the DNP complex. These changes have in general been taken to indicate a loosening of the bonds between DNA and histones, which might make the DNA more accessible to RNA polymerase.

An increase in the binding of acridine orange to DNA in fixed preparations of lymphocytes after stimulation was first reported by Killander and Rigler (1965). The amount of acridine orange bound increased 3-fold within 10 min, and then reached a plateau value which was maintained for 12–48 hr (fig. 12.5). This change was not due to a change in the amount of DNA in the preparations, to any autofluorescence induced by PHA or to any alteration in the kinetics of the staining procedure, but was thought to be due to decreased interaction of the DNA phosphates with the basic amino acids of histones. A similar explanation has been proposed for the increased binding of colloidal iron to lymphocyte DNA that occurs soon after the addition of PHA (Auer, 1972).

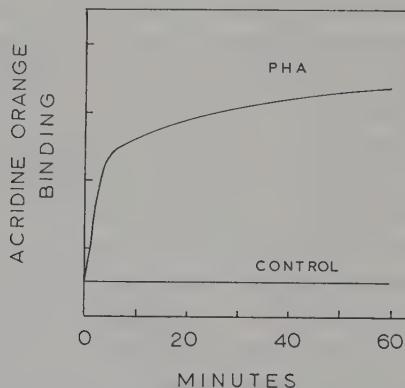


Fig. 12.5. Changes in acridine orange binding to the nuclear DNP in the first hour after the addition of PHA. Redrawn from Killander and Rigler (1965).

A decrease in the staining of histones of mouse thymus or lymph node cells by either ammoniacal silver or alkaline fast green after hydrolysis of DNA was apparent within 1 hr of injection of antigen, while no such change was seen in kidney nuclei (Black and Ansley, 1965a, b). Similar changes were found after incubation of thymocytes with antigen in vitro (Black and Ansley, 1967a) or after incubation of kangaroo lymphocytes with PHA (Burton, 1968). There were some suggestions of qualitative alterations in the histones in these experiments, but no clear indication whether the decreased staining was due to an actual decrease in the amount of histone per nucleus, or simply to the blocking of the basic groups of the histones with which the dyes used interacted. These changes were investigated in greater detail by Zetterberg and Auer (1969), who studied the binding to histones of the dye alkaline bromphenol blue. Bromphenol blue will bind to both lysine and arginine residues of histones after hydrolysis of DNA by trichloracetic acid, but picric acid binds strongly to the guanidino groups of the arginine residues, liberating only the lysine residues. The amount of picric acid bound can be determined independently. Zetterberg and Auer found that after incubation of lymphocytes with PHA for 2 hr there was a 25% decrease in bromphenol blue binding after trichloracetic acid hydrolysis, in close agreement with the earlier findings. However, after picric acid hydrolysis no change in the binding of bromphenol blue was observed, while the amount of picric acid bound decreased by up to 80%. They concluded that the DNP contained many fewer arginine residues bound to DNA phosphate after brief incubation of lymphocytes with PHA.

Decreased interaction of histone basic groups with DNA phosphates would be expected to alter the physical properties of the DNP. Evidence for such an alteration was obtained by the demonstration that the DNP of fixed lymphocytes was less resistant to thermal denaturation after stimulation by PHA (Rigler and Killander, 1969; Rigler et al., 1969). Fixed lymphocytes were incubated at various temperatures in buffer containing formaldehyde, and then cooled rapidly. The preparations were then stained with acridine orange. Denaturation of DNA during heating was then detected by virtue of the different emission spectra of acridine orange bound to single and double stranded DNA.

Studies on the properties of chromatin isolated from thymocytes before and after treatment with antigen have also suggested that alterations occur in its physical properties (Black and Ansley, 1967a, b; Agrell and Mollander, 1969). The changes reported included reduced binding of acidic dyes to

histones, an alteration in the kinetics of histone-DNA fibre formation after dilution from high salt concentration, and a small and transient decrease in the resistance of the DNP to thermal denaturation. However, Black and Ansley (1967a) did not observe changes in the properties of individual isolated histone fractions, and suggested that the changes were due not to changes in the histones, but to some alteration in their binding within the DNP complex.

These changes together make a formidable case for the hypothesis that a very early event in lymphocyte stimulation is an alteration in the nature of the nuclear DNP complex. Similar changes have been reported in the nuclei of other cell types prior to their activation, as, for example, during the activation of chick erythrocyte nuclei after the formation of HeLa cell-chick erythrocyte heterokaryons (Bolund et al., 1969). The reverse sequence of changes have been found to precede the cessation of RNA and DNA synthesis during maturation processes such as spermiogenesis or erythropoiesis. There are, however, a number of observations which complicate this apparently simple picture. The first is that these changes are not confined to those lymphocytes which subsequently respond to stimulation, but affect the whole population in a uniform way. Indeed, similar changes are found in granulocytes after the addition of PHA to leucocyte cultures, although the PHA has no growth stimulating effects on these cells. This observation has led to the suggestion that the change in the nature of DNP is not itself enough to cause gene activation, but may just be a necessary preliminary step.

The second complication is that at least the increase in acridine orange binding occurs when lymphocytes are incubated with PHA at 4 °C (Darzynkiewicz et al., 1969b). At this temperature enzymic processes would be expected to be inhibited, so the alterations in DNP structure giving rise to the increased acridine orange binding cannot require processes such as the acetylation and phosphorylation of histones discussed below.

The third, and most serious, complication is the discovery that most if not all of these changes are highly dependent on the cell concentration on the slides examined (Auer et al., 1970; Bolund et al., 1970; Auer, 1972), and that the primary effect of PHA stimulation is to increase this cell concentration by increasing the adhesiveness of cells to the glass slides (§ 12.3). When the slides contained stimulated and unstimulated lymphocytes at the same cell density, there was no difference in their ability to bind acridine orange (fig. 12.6). A high density of either erythrocytes or HeLa cells will also increase

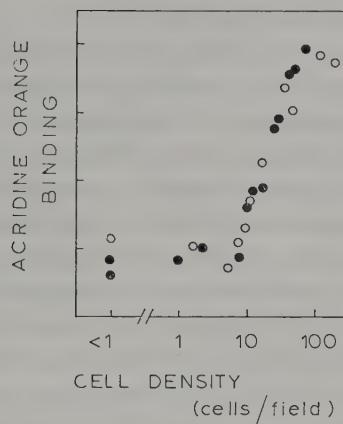


Fig. 12.6. Acridine orange binding to nuclear DNP of lymphocytes incubated without PHA (○) or with PHA for 1 hr (●), expressed as a function of the cell density on the glass coverslips. Redrawn from Bolund et al. (1970).

the acridine orange binding capacity of lymphocytes present on the slides at low density. This effect is due to the secretion of macromolecular exudates of a chemically rather ill-defined nature onto the surface of the slides by cells, and such exudates alone were shown to increase the acridine orange binding capacity of cells at low densities. This clearly greatly complicates the interpretation of these observations, and it is not at present clear whether they should be regarded as further evidence for the central role of cell-cell interaction and adhesiveness in lymphocyte activation, or as a series of unfortunate artefacts.

A further change suggested to indicate an alteration in the structure of lymphocyte DNP after addition of PHA is its increased capacity to bind ^3H -actinomycin (Darzynkiewicz et al., 1969a, b; Ringertz et al., 1969). Actinomycin binds readily to isolated chromatin, but there is little difference in the kinetics of its binding to chromatin isolated from cells with very different rates of RNA synthesis. However, it binds very much less readily to DNP in intact lymphocytes and this binding is increased within 20 min of the addition of PHA. This increase differs from the increase in acridine orange binding in that it does not affect granulocytes, and affects only a proportion of lymphocytes similar to the proportion which show increased incorporation of ^3H -uridine into RNA. Much of the increase in ^3H -actinomycin binding occurs in the first few hours after stimulation, and while there

is some further increase after 24–48 hr, this is very much less marked than the increase in the rate of ^3H -uridine incorporation in this period.

It is, of course, important to establish that these effects are not due to changes in the rate of uptake of ^3H -actinomycin, a point which has been considered in detail by Bolund (1970). While he found some suggestive evidence that the binding to chromatin could be limited by the rate of penetration of the isotope into the cell, he concluded that this was not the only factor involved, as the amount of ^3H -actinomycin taken up by all lymphocytes was much greater, and much more uniform, than the amount bound to the DNP. Marked increases in the rate of uptake of ^3H -actinomycin by lymphocytes 20–66 hr after the addition of PHA have been reported by Agarwal and Loeb (1972), but these authors did not study the early period of the response.

Other changes reported to follow lymphocyte stimulation which might indicate changes in the nature of the DNP are increases in the acetylation and phosphorylation of histones. An increase in the rate of incorporation of ^{14}C -acetate into lymphocyte histones within 15–30 min of stimulation by PHA was reported by Pogo et al. (1966). The isotope was found mainly in N-acetyl lysine residues in the arginine-rich histones, and neither ^{14}C -acetate incorporation into histones nor its stimulation by PHA was dependent on concurrent protein synthesis. Incorporation of ^{14}C -acetate into granulocyte histones was depressed by PHA, as was the incorporation of ^3H -uridine into RNA by these cells.

A similar, but smaller, increase in ^{14}C -acetate incorporation into histones was seen by Cross and Ord (1970), but Monjardino and MacGillivray (1970), who measured ^{14}C -acetate incorporation into whole histone rather than separated fractions, observed a slight decrease in most experiments. They attributed the stimulation seen by Pogo et al. (1966) to a contaminant present in some preparations of PHA. A rapid increase in the incorporation of ^{14}C -acetate by lymphocytes has been demonstrated autoradiographically by Mukherjee and Cohen (1969) and Darzynkiewicz et al. (1969b). Only a fraction of the lymphocyte population was labelled, and this fraction was increased by PHA. Double label experiments showed that the lymphocytes showing increased incorporation of ^{14}C -acetate were the same as those showing increased incorporation of ^3H -uridine into RNA. In both these papers it was shown that the stimulation of ^{14}C -acetate incorporation was not dependent on protein synthesis, and thus not secondary to the incorporation into protein of amino acids formed from the ^{14}C -acetate, but in

neither case is there good evidence that the isotope incorporated is actually in histones.

The most serious reservations about all the work on histone acetylation stem from its dependence on determination of rates of uptake of ^{14}C -acetate, and there is a real danger that the effects studied reflect merely the effect of PHA on the rate of isotope uptake. Little is known about the kinetics of this process, but Darzynkiewicz et al. (1969b) have reported that labelling continues to rise during a chase with unlabelled acetate. This shows that the internal pool of this isotope or its metabolites is not readily saturated by the exogenous precursors, the situation in which such artefacts are most likely to arise. It has also been shown that there is an increase in the rate of incorporation of ^{14}C -acetate into lipids within 1 hr of PHA stimulation (Resch and Ferber, 1972). No qualitative or quantitative changes in acetylation of histone lysine residues after stimulation have yet been reported, but such evidence will probably be required to establish the significance of these changes.

An increase in the incorporation of ^{32}P -phosphate into nuclear proteins (Kleinsmith et al., 1966) and histones (Cross and Ord, 1970, 1971) also occurs very rapidly after the addition of PHA to lymphocytes. In this case the increase in the rate of phosphorylation is matched by an increase in the rate of dephosphorylation, and there is no net change in the histone phosphate content. There is a very marked and rapid stimulation of the rate of uptake of ^{32}P -phosphate by PHA, but the specific activity of the histones increases more rapidly than that of the cellular pool of ATP. The possibility that these results are due solely to changes in the rate of isotope uptake is further increased by the observation that there are transient increases in the activities of both histone kinases and histone phosphatases after PHA stimulation (Cross and Ord, 1971).

Although there are no net changes in histone phosphate or histone thiol content during the first hours of stimulation, increases in both occur during the later stages of the response, apparently related to the initiation of DNA synthesis (Cross and Ord, 1970). The major changes in histone phosphorylation during liver regeneration (Balhorn et al., 1972a) and the cell cycle (Balhorn et al., 1972b) seem also to be associated with DNA synthesis, and the association suggested between histone phosphorylation and gene activation remains speculative.

More recently it has been reported that the addition of PHA directly to isolated and purified nuclei causes parallel increases in the rate of incorporation of nucleotide triphosphates into RNA, and ^{14}C -acetate and ^3H -actino-

mycin into ethanol precipitated nuclei (Rubin et al., 1972). The PHA concentrations required to produce these effects were unusually large (500 µg/ml of PHA-P), but Rubin et al. suggest this may be because this system lacks a cytoplasmic component required for the efficient concentration of PHA in the nucleus. The ability of isolated nuclei to use added acetate for histone acetylation is rather surprising, as detergent-purified nuclei would not be expected to contain the enzyme required to make acetyl-CoA, the normal intermediate (Lippel and Blythe, 1972). Characterization of the material into which the acetate was incorporated would strengthen the conclusion that incorporation is due to histone acetylation. In any event, the physiological significance of these effects must be regarded as dubious, in the light of the observations that it is not necessary for PHA to enter lymphocytes to activate them (§ 12.2.3).

The evidence as yet presented that widespread gene activation occurs during the early stages of lymphocyte activation is suggestive, but less than compelling. Some of the alterations reported seem quite likely to be artefactual, while others may be unrelated to stimulation. Even accepted at their face value, the effects reported are difficult to fit together. It might be thought, for example, that the acetylation and/or phosphorylation of histones might reduce their affinity for DNA and thus bring about the structural alterations causing such changes as the increased binding of acridine orange. This cannot be the case, however, as the increased acridine orange binding occurs at 4 °C, when energy requiring enzymatic processes such as histone acetylation and phosphorylation could not occur.

There is indeed no reason to expect that such widespread gene activation should be required as an early event in the activation of lymphocytes. The changes in the rate of protein synthesis begin only slowly, and become progressively more marked over a period of days (§ 13.2). There certainly are marked changes in the state of nuclear DNP occurring over the same time scale, apparent both by morphological and biochemical techniques (Hirschhorn et al., 1971). If the process of lymphocyte stimulation is seen as the activation of an ordered growth programme, the number of genes to be activated in the early stages could well be too small to be detectable by the methods available.

12.6. Lysosomal activation

Lysosomes are a family of granular sub-cellular organelles containing a

characteristic set of degradative enzymes, which allow their ready identification by either biochemical or histochemical methods. Acid phosphatase is the enzyme most widely used as a lysosomal marker. Lysosomes can be identified in lymphocytes by both light and electron microscopy, and their numbers increase markedly after lymphocyte stimulation by PHA, streptolysin S, staphylococcal α -toxin, pokeweed or antigens (Allison and Mallucci, 1964; Hirschhorn et al., 1965; Parker et al., 1965; Brütinger et al., 1969). Increases are first seen within 24 hr of the addition of PHA and become progressively more marked up to 72 hr. The activities of several lysosomal enzymes in lymphocyte extracts also rise after stimulation, but the changes are in general rather small, being comparable to the changes in cytoplasmic enzymes such as lactate dehydrogenase (Hirschhorn et al., 1967; Nadler et al., 1969).

The initial interest in the lysosomal changes following lymphocyte stimulation followed from the suggestion that a rearrangement and loss of lysosomes preceding cell division might indicate the involvement of enzymes released from lysosomes in the mitotic process (Allison and Mallucci, 1964). In the course of their studies these authors noted that between 5 and 24 hr after lymphocyte stimulation the lysosomes were enlarged and their membranes became more permeable to the substrate used for the histochemical identification of acid phosphatase. In some cases discrete lysosomes were not visible at all. These observations, coupled with evidence that two lymphocyte stimulants, streptolysin S and staphylococcal α -toxin, could directly cause the release of lysosomal enzymes, while lymphocyte stimulation could be inhibited by addition together with the stimulant of drugs which stabilize lysosomal membranes *in vitro*, led to the hypothesis that the release of lysosomal enzymes into the cytoplasm was a key event in the initiation of lymphocyte stimulation (Allison and Mallucci, 1964; Hirschhorn and Hirschhorn, 1965). In support of this hypothesis it was shown that 2 hr after the addition of PHA, before there was any change in the amount of the lysosomal enzymes acid phosphatase and β -glucuronidase, there was some redistribution of these enzymes from the granule fraction to the supernatant fraction of lymphocyte homogenates (Hirschhorn et al., 1968; Nadler et al., 1969). No changes in the distribution of a number of supernatant enzymes occurred. However, PHA, unlike the bacterial stimulants, did not have a direct effect on the release of enzymes from isolated lysosomes.

The evidence for this hypothesis is clearly of a highly circumstantial nature. Perhaps the main weakness is that it is not at all clear that the

effects of either the bacterial stimulants or the inhibitory drugs used, chloroquine and a variety of steroids, are specific for lysosomal membranes. They could well have their important effects on other membranes such as the plasma membrane (§ 12.3). The inhibitors in particular may well act in many different ways, and steroids have been shown to inhibit the uptake of many small molecules by lymphoid cells. While the redistribution of lysosomal enzymes from the granule fraction to the supernatant fraction is statistically significant, it is a small effect and has several alternative explanations. The percentage of β -glucuronidase in the granule fraction fell from 69 to 58%, while that in the supernatant rose from 12 to 20%. The change in the distribution of acid phosphatase was even smaller (Hirschhorn et al., 1968). This could be due to an alteration of the distribution of the enzymes in the intact cell, but it could follow from an increased fragility of the lysosomes after stimulation, or even from the different mechanical conditions for homogenization caused by the agglutination and cell-cell interactions of the stimulated cells.

It is difficult to imagine how release of lysosomal enzymes, if it occurred in the intact cell, could bring about the subsequent lymphocyte growth. It has been suggested that the action of released lysosomal proteases could make more areas of the genome available for transcription, by freeing them from inhibitory proteins, and Hirschhorn et al. (1971) have shown that lymphocyte stimulation is inhibited by the protease inhibitor epsilon amino caproic acid. This compound is effective only at enormous concentrations, 50% inhibition of ^3H -thymidine incorporation requiring an inhibitor concentration of 50 mM, which significantly increases the tonicity of the culture medium. Another protease inhibitor, the tripeptide leupeptin, has been shown to inhibit ^3H -thymidine incorporation by PHA-stimulated guinea pig lymphocytes by up to 30%, provided that it is added together with, or shortly before, the PHA (Saito et al., 1973). However, any such mechanism relying on the release of lysosomal proteases or nucleases would not be expected to have the requisite specificity, nor do the changes in enzyme distribution seem early enough to account for some of the other responses of lymphocytes to stimulation such as the early changes in ribosomal RNA metabolism (§ 13.1.5).

More recently, Biberfeld (1971) has produced convincing evidence that the increase in lysosomes after stimulation is associated with the increased endocytosis of stimulated cells. He showed by electron microscopy that ferritin or ferritin-labelled antibodies bound to human lymphocyte plasma mem-

branes appeared rapidly in endocytic vesicles, formed by invagination of the plasma membrane. At later times ferritin was also found in multivesicular bodies, localised mainly in the Golgi region, and later still it was mainly in dense bodies, found almost exclusively close to the Golgi apparatus. Both multivesicular bodies and dense bodies gave strongly positive reactions for acid phosphatase, and can thus be regarded as lysosomes. Some Golgi elements, which did not contain ferritin, also contained acid phosphatase, and the multivesicular bodies may be formed by fusion of these Golgi elements with endocytic vesicles.

Two main types of endocytic vesicles, large and small, were distinguished. Endocytosis in unstimulated lymphocytes mainly involved small endocytic vesicles while after stimulation by PHA many large vesicles were seen. These large vesicles seemed to be formed by the coalescence of neighbouring cytoplasmic projections, and thus depended on the greatly increased numbers of these projections present after stimulation. The altered distribution of lysosomal enzymes after stimulation could perhaps be due to different fragilities of the multivesicular bodies formed from the large and small endocytic vesicles.

12.7. Comparisons with other biological systems

There are many types of cells in an animal which retain the capacity to divide but do not normally do so. The control mechanisms whereby this is achieved are completely unknown, but they are of the greatest importance to the animal, as their failure would result in the catastrophic uncontrolled growth of the cell type concerned. Elucidation of these growth control mechanisms is one of the prime targets of cancer research.

The ideal system in which to study this problem would be one in which the growth rate of a homogeneous population of cells in culture could readily be controlled by experimental manipulation. The cells should be obtainable in large quantities, it should be possible to obtain and characterize mutant cell lines, and the growth stimulant should be a defined and traceable molecule. Several systems in intact animals have been used to study this problem, and one, liver regeneration after partial hepatectomy, has been used extensively. All have serious disadvantages and meet few of the criteria outlined above. Perhaps the most serious problems are the interference caused by alterations in blood chemistry and hormonal balance consequent on experimental manipulation, and the difficulties in establishing the nature

of the growth signal received by the cells.

The lymphocyte activation system clearly has many advantages, and is now widely used as a model system for the study of mammalian cell growth control mechanisms. Nevertheless, it does deviate from the ideal, in that lymphocyte populations are less homogeneous, and their responses to mitogens less synchronous, than one would wish. More seriously, the types of genetic studies that can be done with this system are very limited. The other system that is now widely used is the density-inhibition of growth in cultured mammalian fibroblasts. The main advantage of this system is that the cells used can be cloned, and that mutant cell lines and cell lines transformed by oncogenic viruses, which have altered growth control mechanisms, can be obtained. The disadvantage is that the factor limiting growth in density-inhibited fibroblasts has proved very difficult to define, and it has thus not been possible to study its interaction with the cells. In addition there are always a significant number of dividing cells present in the cultures, and by many biochemical criteria the differences between growing and non-growing populations are less marked than in the lymphocyte system. The advantages of the two systems are clearly complementary, and the frequency with which experiments which are possible in either have given similar results in both lends comfort to those who wish to find a common mechanism, or set of mechanisms, for controlling growth in all animal cells.

While much of the work currently being done uses one or other of these two systems, it is important to emphasize the value of conclusions based on studies in more specialized systems. Perhaps the most crucial is the clear demonstration that the synthesis of both DNA and RNA by the cell nucleus is controlled by the cytoplasm, which has been provided by studies using nuclear transplantation techniques, or on the properties of the heterokaryons formed by fusion of dividing and non-dividing cells (for references see § 14.3).

A major advantage of the lymphocyte activation system is the opportunity it provides to study the fate of the mitogens used. One of the main conclusions is that it is not necessary for the mitogen to enter the cell for it to cause activation (§ 12.2.3). While under normal circumstances some of the mitogen is taken up by endocytosis, this would probably happen to any protein deposited on the cell membrane and seems not to be of any physiological significance. The mitogens thus seem to resemble the protein hormones, whose effects on their target cells also do not seem to require them to enter the cell, rather than the steroid hormones, which are thought to pass

through the membrane, complex with receptor proteins in the cytoplasm, and then pass into the cell nucleus. If the mitogen does not need to enter the cell, then it must act directly at the membrane, or its attachment to the membrane must result in the formation of a 'second messenger' which is released into the cytoplasm to bring about the alterations in cellular metabolism.

There is a great deal of evidence that mitogen binding to the membrane does cause quite marked alteration in membrane properties (§ 12.3 and § 13.5). Probably the most significant change is the increase in the rate of uptake of many small molecules. Similar changes occur rapidly after growth stimulation in all other systems investigated, although there are minor differences from system to system in the precise range of molecules affected, and in the rates at which the different increases can be detected. Whether these increases are sufficient to cause the increase in cell growth rate has yet to be established, although it can easily be shown that some specific increases are not essential. While nuclear activity is known to be controlled by the cytoplasm, it is not at all clear which components of the cytoplasm are responsible, and it is possible that the nucleus could be sensitive to the cytoplasmic concentration of certain key metabolites. Perhaps the most plausible contender for such a role in the case of lymphocyte activation is calcium. The presence of this ion has been shown to be essential for even the early metabolic changes after activation, and it has been shown to play a key role in controlling the metabolism of many other types of cells. It may also be significant that the K_m of the calcium transporting system decreases after addition of PHA to lymphocytes, while for all the other transport systems studied the K_m remains unchanged, and only the V_{max} increases (§ 12.3). It is difficult to imagine that the mitogen could activate lymphocytes by acting directly on the membrane in any other way than by affecting the rate of membrane transport, but this may be due simply to our ignorance of the functions of the cell membrane.

The second, and more popular, possibility is that the combination of one or more mitogen molecules with membrane receptors results in the formation or destruction of a 'second messenger'. A precedent for such an effector has been established in bacteria, where deprivation of a key metabolite, such as an essential amino acid or a suitable energy source, results in the conversion of much of their GTP into guanosine tetraphosphate, ppGpp. This nucleotide restricts the net synthesis of RNA and other metabolic processes required for growth, and thus brings about a coordinated reduction in the bacterial growth rate. Extensive searches in several laboratories for ppGpp

or similar nucleotides in animal cells have been unsuccessful, and the GTP levels do not seem much affected by the growth rate..

In principle a 'second messenger' could be either a positive effector, which accumulated after the addition of mitogen, or a negative effector, whose concentration was reduced. Perhaps the most popular contender for this role has been cyclic AMP, which has been suggested at different times to be both a positive and a negative mediator of lymphocyte activation. In fact, after much research, the evidence for the involvement of changes in the cAMP levels in lymphocyte activation is conflicting and, in total, unimpressive (§ 12.4). It would probably not be taken seriously but for the dramatic effects of this nucleotide in other systems, where it has been shown that it mimics the action of some (but not all) protein hormones, and that in cultured fibroblasts the cAMP level correlates inversely with the growth rate. More recent work suggests that cGMP has effects in general antagonistic to those of cAMP, and that the cGMP/cAMP ratio may be more important than the cAMP concentration. The recent report that the cGMP level rises rapidly after lymphocyte activation (§ 12.4) suggests that this may prove to be the component of the system whose concentration is affected by the mitogens. Both adenyl cyclase and guanyl cyclase are normally located within the cell membrane so it is easy to imagine that their activities could be affected by mitogen binding.

The metabolic consequences of lymphocyte activation are discussed in detail in ch. 13. However, it is perhaps appropriate to point out here that there is no longer any reason to believe that the main intracellular consequence of activation is the derepression of a large number of previously inactive genes, and that much of the evidence which once appeared to point strongly in this direction has proved capable of other interpretation (§ 12.5). There is evidence that steroid hormones, after combination with their cytoplasmic receptors, may pass to the nucleus and act in part by causing derepression in the classical way, but in many other animal systems the growth rate can be controlled at the translational level (see § 13.2). While it would be surprising if there were not induction of transcription of some genes during the course of lymphocyte activation, there is no reason to think that this is any more than one part of the complex response pattern of cells changing from the non-dividing to the dividing state.

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Metabolic changes during lymphocyte stimulation

There are many changes in the metabolic activities of lymphocytes between the initial stimulation by mitogens and the start of DNA synthesis. Biochemical studies have shown that there are alterations, to a greater or lesser extent, in the activities of almost every metabolic pathway that has been investigated. Some of these changes occur more rapidly after stimulation than others, giving the impression of an ordered sequence of events, and many are well established before DNA synthesis starts. Morphological studies show that these metabolic changes result in the enlargement of the cell, and in particular of the cytoplasm, in the conversion of much of the nuclear heterochromatin to euchromatin and in the appearance of prominent nucleoli.

The most rapid consequences of the attachment of mitogens to their receptors on the lymphocyte membrane are discussed in ch. 12. Some of these have been suggested to mediate the subsequent intracellular metabolic events and may be primary effects of the mitogen binding. This chapter emphasises the sequence of later biochemical changes which precedes the initiation of DNA synthesis and discusses, where possible, their interrelationships. This distinction is necessarily rather arbitrary, as there is at present no clear or agreed division between the two categories, and some of the changes described in this chapter may be apparent as rapidly as some described in chapter 12. In any case, the speed with which an alteration in a metabolic pathway can be recognised depends critically on the sensitivity of the detection method used, and there have been several examples of technical improvements which have shown that a particular change begins earlier than was previously thought.

The metabolic changes to which most attention has been paid are alterations in protein and RNA synthesis. Both begin quite rapidly after stim-

ulation and become progressively more marked. Considering the central role that these molecules are thought to play in the control of cell function, it is not surprising that there have been many detailed studies on both the qualitative and quantitative changes in these areas. Changes in carbohydrate and lipid metabolism have also been studied, although as yet in less detail, and more recently the marked acceleration in polyamine synthesis after stimulation has received some attention.

13.1. RNA synthesis

The changes in RNA metabolism that accompany lymphocyte stimulation have been studied intensively as the potential of this class of molecules for controlling growth stimulation is obvious. In theory the overall rate of protein synthesis could be controlled by the availability of ribosomal or transfer RNA, while the synthesis of individual proteins could be limited by the availability of their messenger RNAs. It is, however, important to realise that both parameters could also be controlled in other ways.

The earliest studies aimed simply to determine the changes in the overall rate of RNA synthesis during stimulation. This has proved much more difficult than was anticipated, and even now the answers available are less exact than one would like. Attempts to determine the qualitative changes in the types of RNA synthesized have been much more successful, but as yet relatively little is known about the ways in which the synthesis and degradation of the different classes of RNA are controlled.

13.1.1. Lymphocyte RNA content

Small lymphocytes have been variously estimated to contain an average of 1 to 5 pg RNA per cell (Kay, 1966; Forsdyke, 1967; Hausen et al., 1969). These values are clearly much lower than those found for most other mammalian cells, but low values would be expected in view of the low cytoplasmic volumes characteristic of lymphocytes.

One of the most prominent characteristics of lymphocyte stimulation is a marked increase in cytoplasmic basophilia, indicative of an increase in the amount of cytoplasmic RNA. Most of the RNA in either stimulated or unstimulated lymphocytes is of the ribosomal type, and electron microscopic studies have confirmed that there is a large increase in the numbers of

ribosomes in the cytoplasm of stimulated lymphocytes (Inman and Cooper, 1963; Sören and Biberfeld, 1973).

Many workers have studied the changes in RNA content of lymphocyte cultures as a function of the response to PHA. Cooper and Rubin (1965) found a 5–33% decrease in culture RNA content during the first 2 hr after the addition of PHA but Monjardino and MacGillivray (1970) have shown that such a fall occurs only at high PHA concentrations and is not an obligatory feature of stimulation. PHA is usually found to cause a 0–50% increase in culture RNA content after 24 hr, rising to about double the unstimulated level at 48 hr (Cooper and Rubin, 1965; Kay, 1966; Forsdyke, 1967; Hausen et al., 1969). The changes occurring at later times are more variable, reflecting the variable culture conditions at these times, but under suitable conditions the RNA levels may continue to rise.

Relatively few quantitative cytochemical studies on RNA content have been carried out. Killander and Rigler (1965) studied the changes in individual cells by two different methods, ultraviolet microspectrophotometry and acridine orange microfluorimetry. The ultraviolet absorption method gave results highly compatible with the studies carried out at the culture level. The mean absorption was twice the initial value after 48 hr incubation with PHA. The stimulated cells were quite heterogeneous, but about 80% had values above the normal range for the unstimulated population. The acridine orange method also emphasized the high proportion of cells responding and the heterogeneity of the response, but appeared to indicate a larger overall increase in RNA content. The increase in cytoplasmic RNA was found to correlate closely with the increase in cellular dry mass (Sören and Biberfeld, 1973), so that the cytoplasmic RNA concentration does not change during activation. This was true even for the largest cells found in stimulated cultures, which contained up to 10 times as much RNA as unstimulated lymphocytes.

13.1.2. Incorporation of precursors into RNA

It is probably a truism that all living lymphocytes are engaged in RNA synthesis. If sufficiently sensitive autoradiographic methods are used, a high proportion can be shown to incorporate the pyrimidine nucleosides ^3H -uridine and ^3H -cytidine into RNA. This property seems not to be distributed uniformly between different categories of lymphocytes. Torelli et al. (1964) have shown that different morphological classes of human blood lympho-

cytes may have different rates of incorporation, while Cooper and Rubin (1965) found that the rates of uridine uptake by 5–10% of column-purified human blood lymphocytes were sufficiently high to be outside the normal distribution predicted for a uniform cell population. Uridine incorporation of cord-blood lymphocytes is greater than that of adults (Winter et al., 1965) and rat T lymphocytes have higher rates of uridine uptake than B lymphocytes (Howard et al., 1972).

When lymphocytes are stimulated by PHA the rate at which they incorporate ^3H -uridine and ^3H -cytidine into RNA is greatly increased. This increase begins soon after stimulation and becomes very prominent well before the initiation of DNA synthesis (Epstein and Stohlman, 1964). Similar increases have been reported with many other mitogens and seem to be a universal feature of lymphocyte activation. There have been no reports of stimulations of lymphocyte DNA synthesis not preceded by a stimulation of incorporation of pyrimidine nucleosides into RNA.

While there has been general agreement about the occurrence of this increase, there have been divergent opinions as to its kinetics. The main confusion has concerned the first few hours after the addition of PHA. Most workers have observed some increase in the rate of ^3H -uridine incorporation

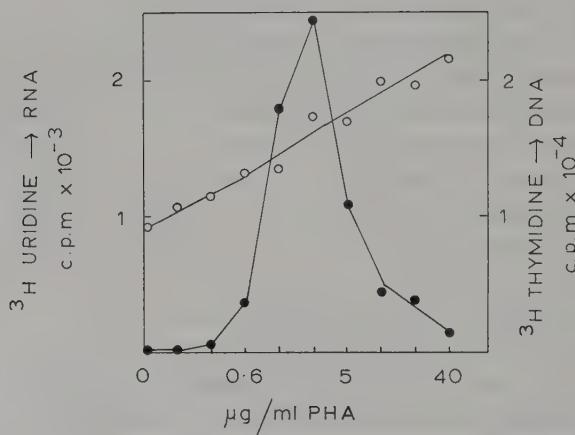


Fig. 13.1. Effect of PHA concentration on the incorporation of ^3H -uridine into RNA in the first 2 hr after the addition of PHA (○) and on the incorporation of ^3H -thymidine into DNA in a 2 hr pulse 2 days after the addition of PHA (●).

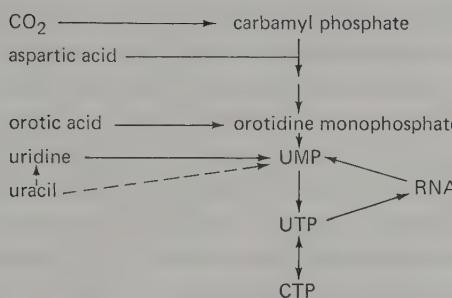


Fig. 13.2 Alternative pathways for the synthesis of uridine nucleotides.

into RNA within 1 hr of the addition of PHA, but in some cases it has been very small, in others as high as four-fold (Cooper and Rubin, 1965; Kay, 1966; Kleinsmith et al., 1966; Pogo et al., 1966; Kay and Cooper, 1969). The explanation for this variation is probably that the optimum PHA concentration for the early stimulation of ^3H -uridine incorporation is much higher than that for subsequent DNA synthesis (fig. 13.1). Workers studying the effects of PHA over extended periods have thus observed rather small early stimulations of ^3H -uridine incorporation, with the major increase beginning quite suddenly after 5–10 hr, and reaching 10–50 times the initial rate after 24 hr. It is possible that the high PHA concentrations which cause large early stimulations are causing an accelerated response to PHA, but more likely that they are bringing about an effect of a different type, such as an increase in the rate of uridine uptake (§ 12.3 and § 13.1.3). These high PHA concentrations are certainly toxic in the long term, but whether they cause accelerated stimulation as judged by other parameters has not yet been determined.

The various pathways by which uridine nucleotides can be produced are shown in fig. 13.2. Lymphocytes, in common with most mammalian cells, do not seem able to utilize uracil to any appreciable extent. They do, however, contain the enzyme system required to synthesize UMP by the endogenous pathway from carbamyl phosphate and aspartic acid (Lucas, 1967; Ito and Uchino, 1971). There is some disagreement about the extent to which this pathway is normally used. Lucas (1967) found that aspartic acid was incorporated into RNA more readily, and orotic acid 100 times more readily, than uridine. In contrast, others have found that orotic acid is

incorporated into RNA much less readily than uridine, while aspartic acid is not incorporated into RNA to any significant extent under conditions in which it was incorporated into protein (Forsdyke, 1968; Kay and Handmaker, 1970). All agree that the incorporation of orotic acid is stimulated very much less than that of uridine after the addition of PHA.

The activity of the endogenous pathway has been measured by determination of the rate of incorporation of $^{14}\text{CO}_2$ into uridine nucleotides, and the activity of the rate-limiting enzyme, the glutamine-dependent carbamyl phosphate synthetase (Ito and Uchino, 1971). Both activities were found to be low in lymphocytes but to be stimulated by PHA after a lag of about 24 hr. The magnitude of the stimulation rises to about four-fold by 48–72 hr. Neither pathway appears to be indispensable. Inhibition of the endogenous pathway by 6-aza-uridine, which prevents the conversion of orotidyl acid to UMP, abolishes ^3H -orotic acid incorporation into RNA but does not affect ^3H -uridine incorporation or the normal course of stimulation (Kay, 1967), while inhibition of uridine uptake with dipyridamole again resulted in normal stimulation (Peters and Hausen, 1971).

The incorporation of other precursors into RNA has been studied less intensively. PHA increases the rate of ^{32}P -phosphate incorporation into RNA, although the stimulation is smaller than that seen with ^3H -uridine (McIntyre and Ebaugh, 1962). The stimulation of the incorporation of the purine nucleosides adenosine and guanosine into RNA is of the same order as the increase in ^3H -uridine incorporation, but unstimulated lymphocytes

Table 13.1

Effect of PHA on the incorporation of ^3H -uridine, ^3H -adenosine, ^3H -guanosine and ^3H -adenine into RNA by human blood lymphocytes. Each isotope was added for a terminal 1 hr pulse.

Isotope	No PHA	20 hr PHA	Stimulation $dpm \times 10^{-3}/10^6$ lymphocytes
^3H -uridine	9.3	159.3	17.1
^3H -adenosine	9.3	95.1	10.2
^3H -guanosine	8.0	225.0	28.1
^3H -adenine	30.6	125.9	4.1

incorporate the purine adenine into RNA much more readily than the nucleoside adenosine, and the incorporation of adenine was stimulated much less by PHA (table 13.1).

13.1.3. Relationship between precursor uptake and RNA synthesis

Initially the changes in the rates of incorporation of precursors into lymphocyte RNA after stimulation were assumed to represent changes in the rates of RNA synthesis. For such an assumption to be valid, two conditions must be met. First, the amount of unlabelled material present in the culture medium competing with the labelled precursor for uptake into the cell and incorporation into RNA must be constant, or any changes in it brought about by stimulation must be known. Second, the rate at which the added precursor saturates the pool of nucleoside triphosphates from which RNA is synthesized must also be independent of stimulation. Ideally the time required for saturation of this pool should be very short relative to the period of the radioactive pulse, and the specific activity of the pool should remain constant throughout the pulse. Should stimulation prove to affect the rate of saturation of the nucleoside triphosphate pool significantly, it is extremely difficult to make the requisite corrections. These require accurate determinations of the specific activities of the pool through the labelling period, an extremely difficult procedure when dealing with the amounts of material available from lymphocyte cultures. Simple determinations of the rate of uptake of the precursor into the pool of triphosphates are not adequate. Furthermore, in all conditions which deviate from the ideal, the assumption that the nucleoside triphosphate pools of all the cells in the culture can be treated as non-compartmentalized becomes critical.

The rate of ^3H -uridine incorporation by lymphocytes incubated with or without PHA in the presence of varying concentrations of unlabelled uridine has been measured by Forsdyke (1968, 1971). He assumes that the rate of uridine incorporation is not affected by the external uridine concentration, and on this basis shows that lymphocyte cultures initially contain significant levels of competing substances, but that these levels decrease after stimulation with PHA. While some such competition might be expected, due to uridine or cytidine in the serum component of the culture medium, Forsdyke also finds such substances in simple media such as Earle's salts solution, a rather puzzling result.

Even more seriously, other work has indicated that the rate-limiting step

for the incorporation of exogenous uridine into RNA is not the rate of RNA synthesis, but the rate of uptake and phosphorylation of the uridine. A high proportion of both ribosomal and non-ribosomal RNA synthesized by all animal cells is known to be metabolically unstable (Darnell, 1968). The RNA synthesized by lymphocytes is entirely normal in this respect (§ 13.1.4). If added ^3H -uridine had free entry to the pool of UTP used for RNA synthesis, the rate at which it is incorporated into RNA would be expected to decline rapidly from the initial rate as the unstable RNA species became saturated. In fact the rate of RNA labelling is approximately linear for several hours (Cooper and Rubin, 1965; Kay, 1966). More recently, it has been shown that the saturation of the UTP pool of unstimulated lymphocytes also takes several hours and that this process is accelerated in PHA stimulated cells (fig. 13.3), even though these cells have a larger UTP pool (Cooper, 1972).

^3H -uridine-labelled nucleotides incorporated into RNA remain in RNA

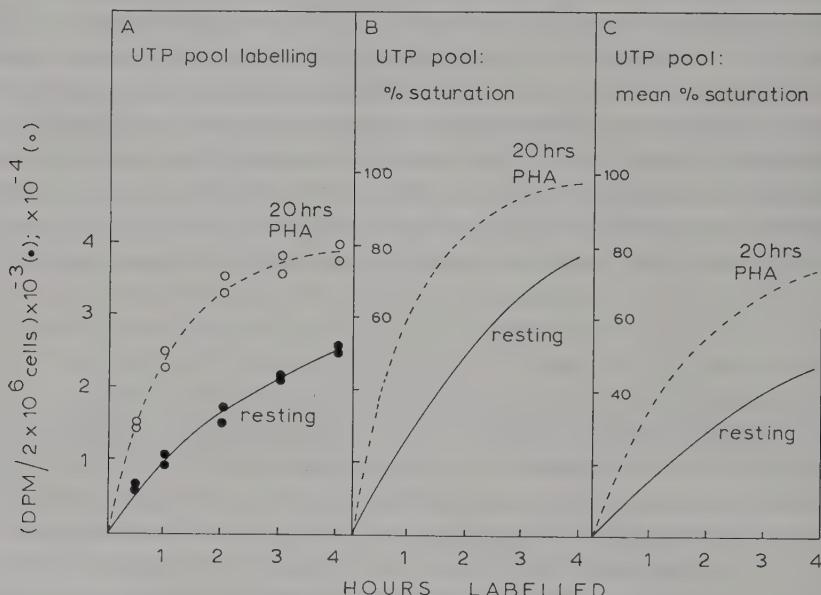


Fig. 13.3. Analysis of the saturation kinetics of the intracellular UTP pool. Resting and PHA stimulated lymphocytes were incubated with ^3H -uridine and radioactivity in the UTP pool was determined at intervals thereafter. A) Rate of labelling of the UTP pool. B) Percentage saturation of the UTP pool. C) Calculation of the mean percentage saturation of the UTP pool. Each point represents the average effective % UTP pool saturation during a labelling period of the duration indicated. Redrawn from Cooper (1972).

during a chase with unlabelled uridine, but not during a chase with actinomycin (Kay, 1966). This probably indicates that most of the nucleotides from degraded RNA are reutilized for RNA synthesis and that added unlabelled uridine cannot prevent this reutilization. As unstimulated lymphocytes are not engaged in net RNA synthesis, new pyrimidine nucleotides need be synthesized only to accommodate any inefficiency in reutilization. After stimulation, accompanied by the onset of net RNA synthesis, many more new nucleotides are required. These are probably made first from nucleosides in the medium or, if this source is depleted, by synthesis by the endogenous pathway (fig. 13.2). Both these pathways of nucleotide synthesis are probably regulated by the pools of pyrimidine nucleotide triphosphates (Lucas, 1967; Ito and Uchino, 1971).

The increase after PHA in the rate of incorporation of uridine into RNA is paralleled not only by an increase in the rate of uridine uptake by the cell, but also by an increase in the uridine kinase activity of the cell extracts (Lucas, 1967; Hausen and Stein, 1968). Almost all the uridine inside the lymphocytes was phosphorylated, mostly as UTP, indicating that phosphorylation occurs immediately following or coincident with uptake by the cell, and that subsequent steps in the formation of UTP are not rate-limiting. While the increase in the amount of uridine kinase may be necessary to permit the increase in uridine uptake after PHA stimulation, Kay and Handmaker (1970) and Peters and Hausen (1971) have presented evidence that the amount of the enzyme is not normally rate limiting. Kay and Handmaker showed that the initial rise in uridine uptake immediately following the addition of PHA could occur even when the increase in uridine kinase activity was inhibited by cycloheximide. They also showed that when drugs which reduced RNA synthesis without directly affecting uridine kinase were added together with PHA, they led to comparable subnormal increases in uridine incorporation and uridine kinase. This implied that the increase in uridine kinase occurred in response to the demand for nucleotides for RNA synthesis, and they suggested that the regulation of the activity and synthesis of the enzyme might be controlled by the pool of nucleotide triphosphates. Peters and Hausen (1971) calculated that the potential extractable uridine kinase activity exceeded the actual rate of uridine uptake by two orders of magnitude. They proposed that the rate of membrane transport might be the rate-limiting step in uridine incorporation, and showed that dipyridamole could inhibit uridine uptake without affecting uridine kinase activity.

It follows that the increase in the rate of uridine incorporation into RNA does not accurately represent the change in the rate of RNA synthesis that occurs following lymphocyte stimulation. If the increased uridine uptake occurs in response to an increased demand for nucleotides, then the increase in incorporation will merely exaggerate the increase in RNA synthesis. The magnitude of the exaggeration will increase with the efficiency of nucleotide reutilization in the unstimulated cells. Other factors, such as the stability of the RNA synthesized, the efficiency of reutilization, or the requirement of processes such as glycogen and lipid synthesis for uridine nucleotides could also change after stimulation, and affect uridine uptake and thus incorporation into RNA. If lymphocyte stimulants affect uridine uptake directly, then changes in incorporation into RNA would occur in the absence of any other metabolic changes. In the intact cell both types of mechanism probably play a part. Similar considerations also apply to the interpretation of ^3H -cytidine incorporation into RNA.

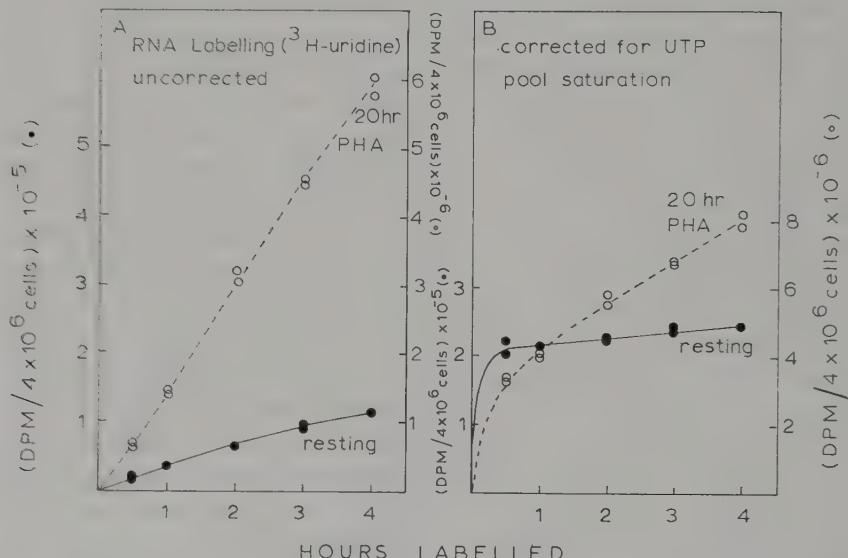


Fig. 13.4. Incorporation of ^3H -uridine into lymphocyte RNA before and after correction for UTP pool saturation. The incorporation of ^3H -uridine into RNA by the same cells as those used for fig. 13.3 is shown in A. When these data are corrected for the mean percentage saturation of the UTP pool, the curves in B are obtained. Redrawn from Cooper (1972).

The only serious attempt to convert measurements of the rate of ^3H -uridine incorporation before and after the addition of PHA into relative rates of RNA synthesis has been that made by Cooper (1972). He corrected each measurement of ^3H -uridine incorporation into RNA for the mean percentage saturation of the UTP pool during the labelling period (fig. 13.4) and found that the approximately linear incorporation was converted into a curve of the type predicted from our knowledge of RNA metabolism. There is a short period of rapid incorporation while the radioactivity enters and saturates unstable RNA, and then a slower rate of incorporation into the more stable RNA species. Even this sophisticated comparison of RNA synthesis between stimulated and unstimulated lymphocytes is dependent on the assumption that the UTP pool of the cells can be treated as a single, non-compartmentalized pool, and that the final specific activities of these pools are the same, before and after stimulation. The latter assumption would be incorrect, and the difference between stimulated and unstimulated lymphocytes exaggerated, if the activity of the endogenous pathway for UTP synthesis became more important after stimulation.

It has been shown recently that when the growth rate of some mammalian cell lines changes the rate of ^3H -adenosine incorporation reflects the change in the rate of RNA synthesis much more accurately than the rate of ^3H -uridine incorporation (Cunningham and Pardee, 1969; Hershko et al., 1971). Table 13.2 shows that ^3H -adenosine and ^3H -uridine uptake and incorpora-

Table 13.2

Effect of incubation with PHA for 22 hr on the incorporation of ^3H -uridine, ^3H -adenosine and ^3H -adenine into the TCA soluble and TCA-precipitable fractions of human blood lymphocytes. Isotopes were added for a terminal 1 hr pulse.

Isotope	PHA	TCA soluble		TCA precipitable	
		dpm $\times 10^{-3}/10^6$ lymphocytes			
^3H -uridine	—	62.5		17.6	
	+	188.4	7.8 \times	199.9	11.4 \times
^3H -adenosine	—	146.2		11.0	
	+	597.6	4.1 \times	158.2	14.3 \times
^3H -adenine	—	1033.8		66.5	
	+	2137.7	2.1 \times	330.4	5.0 \times

tion both increase to about the same extent after the addition of PHA. In contrast, ^3H -adenine is taken up more readily by unstimulated cells, and the stimulation by PHA is less pronounced. ^3H -adenosine is taken up by erythrocytes much more rapidly than the other isotopes. Analysis of the data in table 13.2 shows that the pool of ATP in lymphocytes must contain more than one compartment, and that adenine and adenosine must have different access to these compartments. A higher proportion of the adenosine incorporated after PHA stimulation enters RNA (table 13.2). These differences cannot be explained by differences in the kinetics of uptake of these two precursors. Both label the ATP pool with similar kinetics, saturation occurring over a period of several hours. It is not clear whether compartmentalization occurs at the level of the individual cell, or whether it reflects the heterogeneity of the lymphocyte population. Plagemann (1971) has recently provided evidence for the compartmentalization of the pool of uridine nucleotides in hepatoma cells. Such compartmentalization would render impossible the determination of accurate specific activities of the pools of nucleotides used for RNA synthesis, a correction necessary for the conversion of comparative rates of precursor incorporation into comparative rates of RNA synthesis during lymphocyte stimulation.

The prospects for using ^{32}P -phosphate incorporation into RNA to measure RNA synthesis also appear discouraging, as it has been shown that the rate of uptake of ^{32}P -phosphate by lymphocytes fluctuates rapidly after the addition of PHA (§ 12.3). Alternative approaches to this problem are discussed in the next section.

13.1.4. Qualitative and quantitative changes in RNA synthesis

The types of RNA found in animal cells can be divided broadly into the structural and the informational. The structural RNAs include the ribosomal and transfer RNAs (rRNA and tRNA), which are part of the cells' machinery for protein synthesis, and also a number of low molecular weight species of unknown function found predominantly in the nucleus (Darnell, 1968). The informational RNAs comprise messenger RNA (mRNA) and the high molecular weight heterogeneous nuclear RNA (HnRNA). The function of HnRNA has been argued over for a decade. It has many of the properties expected for mRNA, but much of it is degraded rapidly without ever leaving the nucleus. The rRNA and tRNA are transcribed from DNA as molecules significantly longer than the final product, and are then trimmed to the

appropriate size. At the time of writing it seems probable that at least some of the HnRNA will prove to be an extended precursor of mRNA.

The structural RNAs comprise more than 90% of the RNA in most animal cells. The informational RNAs, because of their high turnover rate, usually account for most of the RNA synthesized. These types of RNA can be distinguished by their labelling properties, by the ease with which they can be extracted from the cell, and by their behaviour during sucrose gradient centrifugation.

Most of the RNA synthesized by lymphocytes at all stages of stimulation by PHA is of the informational type (Rubin and Cooper, 1965; Cooper, 1969a). The proportion of RNA synthesized given over to rRNA and tRNA synthesis is unusually low in resting lymphocytes, and increases markedly after stimulation. Cooper (1969a) calculated that in the 24 hr following the addition of PHA the proportion of RNA synthesized that gave rise to mature cytoplasmic rRNA rose from 3 to 15% of the total.

Cooper (1972) has attempted to calculate the absolute increase in the rate of rRNA synthesis by determining its rate of methylation by methyl-³H-methionine. Unlike the nucleotide pools, the amino acid pools of lymphocytes saturate rapidly, and Cooper has found that PHA does not affect the rate of saturation of the S-adenosyl methionine pool. After 20 hr incubation with PHA he finds the rate of cytoplasmic rRNA methylation to have increased about 10-fold. This, together with the increase in the proportion of rRNA synthesis gives an overall increase in the rate of RNA synthesis of about 2-fold. This calculation rests on two as yet untested assumptions; that all the RNA in each cell is synthesized from the same pool of uridine nucleotides and that PHA does not affect the frequency of methylation of rRNA. These assumptions seem reasonable, and the answer obtained, an overall two-fold increase in the rate of RNA synthesis 24 hr after the addition of PHA, correlates interestingly with the increase in RNA polymerase activity of lymphocyte nuclei (see § 13.1.8).

The main drawbacks to Cooper's method are that it requires a great deal of accurate measurement from sucrose gradients, and it needs determinations to be made over a period of several hours, during which the rate of RNA synthesis is assumed to be constant. It is thus not applicable to periods when the rate of RNA synthesis is changing rapidly.

The increases in rRNA and tRNA synthesis begin very soon after the addition of PHA. Such increases have now been found to be an almost invariable accompaniment of the growth stimulation of animal cells. The

changes in rRNA synthesis are examined in greater detail in § 13.1.5 and the changes in tRNA synthesis in § 13.1.6.

The types of informational RNA synthesized have also been studied but as yet no qualitative changes have been detected following stimulation. This probably indicates that the methods used have not been sufficiently sensitive, rather than that there are no new types of informational RNA synthesized. This is discussed further in § 13.1.7.

Most of the detailed studies on changes in RNA metabolism following lymphocyte stimulation have used PHA as the stimulant, but it is highly probable that the changes brought about by other stimulants will prove to be very similar.

13.1.5. Ribosomal RNA synthesis

Ribosomal RNA is transcribed from DNA in the nucleoli of animal cells as a 45S precursor. This 45S RNA is very rapidly methylated and then more slowly processed within the nucleolus, giving rise to one molecule of each of the mature 28S and 18S forms of rRNA. About half the nucleotides of the 45S precursor are lost during the maturation process, but all the methylated nucleotides are conserved. These RNAs are not found free in the nucleoli, but all are complexed with protein. The details of this process were first established for HeLa cells (reviewed by Darnell, 1968) but Cooper (1968) has shown that rRNA synthesis in lymphocytes is very similar.

The increase in rRNA synthesis following lymphocyte stimulation by PHA involves not only an increase in the rate of synthesis of the 45S precursor, but also an increase in both the rate and the efficiency of the processing of 45S RNA to 28S and 18S RNA (Cooper, 1969a, b; Rubin, 1970).

The rate at which 45S RNA is processed in unstimulated lymphocytes is unusually slow. In HeLa cells it is the major species of labelled rRNA only following a pulse with a radioactive precursor for 20 min or less (Penman, 1966). Comparable results have been obtained for non-dividing cells such as rat hepatocytes (Muramatsu et al., 1966) and density inhibited chick fibroblasts (Emerson, 1971). However, if unstimulated lymphocytes are labelled for 1 hr with ^3H -methionine almost all the radioactivity in ribosomal RNA sediments at 45S (Cooper, 1970). After addition of PHA the rate of 45S processing is accelerated rapidly, and after 6 hr reaches a minimum time similar to that found for HeLa cells (Cooper, 1969a; Rubin, 1970). This accelerated rate of processing is maintained for at least 48 hr.

The maturation of 45S RNA is not only slow in unstimulated lymphocytes, it is also inefficient. About half the precursor molecules are completely degraded, and never give rise to mature rRNA. The existence of this wastage was first detected because the degradation of the 18S component is more rapid than that of the 28S rRNA, so that under appropriate labelling conditions the labelling of 28S and 18S rRNA deviates from the expected 1:1 molar ratio (Cooper, 1969b; Rubin, 1970). This has since been confirmed by more direct methods (Cooper and Gibson, 1971; Cooper, 1972). This wastage of precursors is rapidly reduced by PHA and virtually eliminated after 6 hr. It remains very low for the next 18 hr, but then rises again rapidly. Two days after the addition of PHA the rate of 45S precursor synthesis is at its peak and the rate of 45S maturation is still very rapid, but the efficiency of maturation has returned to the initial value (Cooper, 1969b). There is some indication that wastage may also occur in other types of cells, but this has not yet been systematically investigated.

The maturation of 45S RNA seems to be dependent on continued protein synthesis, as formation of mature 18S rRNA is almost completely prevented by inhibitors of protein synthesis such as cycloheximide. Cooper and Gibson (1971) determined the effect on the efficiency of maturation of treatments which varied independently the rates of rRNA and protein synthesis, and they concluded that the survival of the 18S rRNA depended on the rate of synthesis of a continuously produced protein which protected the 18S rRNA from degradative enzymes. PHA must increase the availability of this protein before it causes any detectable increase in the overall rate of protein synthesis (see § 13.2.1).

The effects of PHA on the rate of 45S RNA synthesis and on the efficiency of its maturation seem to be independent. Cooper (1970) has shown that PHA can prevent the wastage of 45S RNA synthesized prior to the addition of the stimulant, even when further RNA synthesis after the addition of PHA is prevented by actinomycin. In the reciprocal experiment PHA could still stimulate 45S RNA synthesis when maturation was almost totally prevented by the inhibition of protein synthesis.

PHA thus has multiple effects on the synthesis of rRNA. The synthesis of the protein required for maturation seems to be rate limiting in unstimulated lymphocytes, but this rises more rapidly than the rate of 45S RNA transcription during the first few hours after the addition of PHA, such that after about 6 hr the rate of transcription becomes limiting. Both these parameters continue to rise, but the reappearance of wastage during the second

day of culture indicates that the rate of synthesis of the protein has again become the critical factor.

The changes in rRNA synthesis are accompanied by marked changes in the morphology of the nucleoli. The ring-shaped nucleoli characteristic of cells synthesizing little rRNA are found in 95% of blood lymphocytes. Six hr after the addition of PHA 30% of the cells develop the dense nucleoli with nucleolonemas characteristic of cells active in rRNA synthesis, and this proportion rises steadily to a maximum of over 70% at 48 hr (Potmesil and Smetana, 1969).

While this increase in rRNA synthesis is one of the most marked and universal accompaniments of growth stimulation in animal cells, its significance is not entirely clear. Protein synthesis in lymphocytes is not limited by the availability of ribosomes, and when rRNA synthesis is selectively inhibited the rise in the rate of protein synthesis is essentially normal during the first 24 hr after the addition of PHA (Kay et al., 1969). However, inhibition of rRNA synthesis does prevent the initiation of DNA synthesis.

13.1.6. Synthesis of low molecular weight RNA

The synthesis of tRNA by animal cells resembles the synthesis of rRNA in that the product of transcription is longer than the mature tRNA. It differs from rRNA synthesis in that it occurs in the nucleoplasmic region of the nucleus, while the maturation and methylation of the precursors seem to be cytoplasmic events (Burdon et al., 1967).

As with rRNA, the synthesis of tRNA is increased relative to RNA synthesis as a whole after the addition of PHA. Both the rate of synthesis and the rate of maturation of tRNA precursors are low in unstimulated lymphocytes, and both are accelerated within the first few hours of PHA stimulation (Kay, 1968; Kay and Cooper, 1969). It is not known whether these changes reflect increases in the synthesis of specific types of tRNA, or whether they merely represent an increase in the overall rate of tRNA synthesis. Models have been developed in which both the rate of protein synthesis and the types of protein synthesized can be controlled by the availability of key tRNA species (Ames and Hartman, 1963; Stent, 1964), but while it has been shown that changes in growth rates or differentiated states of cells are frequently accompanied by modifications of individual tRNA species (Sueoka and Kano-Sueoka, 1970), the relative importance of such mechanisms for growth control is not yet established.

Studies on the ability of lymphocyte extracts to catalyse the methylation of undermethylated bacterial tRNA, showed that the tRNA methylase activity increased 5-fold by 48 hr after the addition of PHA (Riddick and Gallo, 1971). This was accompanied by a similar increase in the number of methyl groups that could be transferred to each tRNA, indicating that tRNA methylases with additional specificities had been synthesized. The increases in the activities of these enzymes were not early events, as they occurred at approximately the same period as the initiation of DNA synthesis. The distribution of radioactivity from methyl-³H-methionine between different modified bases in tRNA was not greatly affected by incubation with PHA, although there was a 50–100% increase in the proportion of labelled N²-methyl guanine and a 50% decrease in the proportion of labelled 1-methyladenine (Sharma and Loeb, 1973). These changes became apparent fairly rapidly after the addition of PHA, and were complete within 12 hr.

Animal cells also contain a number of types of low molecular weight RNA other than tRNA. Most are methylated, and largely confined to the nucleus, but their functions are not yet known (Weinberg and Penman, 1968). Low molecular weight nuclear RNAs of this type are found in both unstimulated and PHA stimulated lymphocytes (Howard and Stubblefield, 1972; Hellung-Larsen et al., 1973). Howard and Stubblefield (1972) found one component to be synthesized 48 hr after the addition of PHA, but not by unstimulated lymphocytes or immediately after the addition of PHA. However, Hellung-Larsen et al. (1973) were unable to confirm this. They found that the relative amounts of the different components did not change after stimulation and concluded that they were not influenced by the rate of cell division.

13.1.7. *Informational RNA synthesis*

The majority of RNA synthesized by lymphocytes at all stages of stimulation is informational RNA (Rubin and Cooper, 1965; Cooper, 1969a), but this has been the most difficult class to study. Even calculations of its rate of synthesis are indirect and rather tentative. If the assumptions discussed above are correct, the rate of synthesis of this class of RNA also increases during stimulation, but less, proportionally, than the synthesis of rRNA and tRNA.

Almost all the HnRNA molecules that are destined to become mRNAs have a poly(A) sequence added to the 3'-hydroxyl terminal end. The poly(A) sequence is thought to be added after the completion of transcription, but

while the HnRNA is still in the nucleus. The function of the poly(A) is not known, but it has provided a most valuable method of recognition of mRNAs and their precursors.

Poly(A)-containing RNA has been found in lymphocytes and the amount present increases after stimulation by PHA (Rosenfeld et al., 1972; Cooper, 1974). The rate of transport of the poly(A)-containing RNA to the cytoplasm is also increased. Not all the poly(A)-containing RNA found in the cytoplasm of unstimulated lymphocytes is attached to ribosomes, which implies that the low rate of protein synthesis in these cells is not due simply to a lack of mRNA.

In theory qualitative changes in the synthesis of informational RNA could be detected by competitive DNA-RNA hybridization. This technique has been applied most successfully to bacterial and viral systems, but the complexity of mammalian DNA is such that highly specialized conditions are required for the hybridization reaction to go to completion (Gelderman et al., 1971; Bishop, 1972). Such conditions have not yet been used in any of the studies on informational RNA of lymphocytes. These studies have all used less sophisticated conditions, in which only the hybridization of those classes of RNA complementary to highly or moderately repetitive DNA is achieved. The exact types of RNA involved vary with the DNA and RNA concentrations and the conditions of hybridization.

Using these techniques no changes in the types of RNA synthesized have been detected following lymphocyte stimulation by PHA (Torelli et al., 1968; Berke et al., 1971; Neiman and MacDonnell, 1971) or after sensitization of rat lymphocytes to mouse fibroblasts *in vitro* (Clark et al., 1971). Neiman and MacDonnell also failed to find any differences in the types of RNA present in the cytoplasm after stimulation. These results do not, of course, show that new RNA species are not synthesized during lymphocyte stimulation. They are, however, rather surprising, as similar methods have proved sufficiently sensitive to demonstrate differences between the RNA synthesized at different stages during liver regeneration (Church and McCarthy, 1967) and by normal lymphocytes and the lymphocytes of chronic lymphocytic leukaemia (Neiman and Henry, 1969).

Torelli et al. (1968) found that at the highest DNA/RNA ratios they studied up to 22% of the pulse labelled RNA of unstimulated lymphocytes would hybridize to DNA, compared to less than 4% of such RNA from 24 hr PHA lymphocytes. This difference seems too large to be accounted for by the relative increase in rRNA and tRNA synthesis, and may indicate that

after stimulation a lower proportion of the informational RNA is complementary to repetitive DNA. Berke et al. (1971) studied lower DNA/RNA ratios. They found the RNA synthesized between 19 and 24 hr after PHA to hybridize with no more than 3% of the DNA, but that higher degrees of hybridization, with up to a maximum of 15% of the DNA, could be obtained with RNA synthesized either before or after this period. This puzzling result is difficult to reconcile with the failure of competitive hybridization to detect differences between the RNAs synthesized at these different periods.

Given the alterations in protein synthesis that occur during lymphocyte stimulation, it seems unlikely that there will prove to be no differences in the types of informational RNA present. However, such differences could affect only a small part of the total RNA synthesized, or could affect only its distribution within the cell. Changes of this type could be very difficult to detect and almost impossible to eliminate experimentally.

13.1.8. RNA polymerase activity

In an attempt to understand more about the factors controlling RNA synthesis during lymphocyte stimulation, several groups of workers have studied the RNA synthesizing capacity of lymphocyte extracts. Animal cells contain two major classes of RNA polymerases, and probably also a number of minor polymerases which may be important in certain types of cells (Roeder and Rutter, 1969). Polymerase I is thought to be responsible for the synthesis of rRNA, polymerase II for the synthesis of informational RNA. Polymerase II is specifically sensitive to inhibition by the drug α -amanitin (Kedinger et al., 1970).

The activities of these polymerases can be determined under a number of conditions. Isolated nuclei incubated in media of low ionic strength can synthesize RNA *in vitro*, but this RNA is almost entirely the product of polymerase I activity. If the ionic strength is raised, normally by the addition of about 0.25 M $(\text{NH}_4)_2\text{SO}_4$, the nuclei are disrupted, and the amount of RNA synthesized greatly increased (Widnell and Tata, 1966). The increase in RNA synthesis is due to the activation of polymerase II, as it can be inhibited by α -amanitin. The RNA synthesis by these preparations is directed by endogenous DNA, but the factors limiting synthesis are clearly rather uncertain.

Under certain conditions the RNA polymerases can be separated from the DNA, purified, and their ability to catalyse transcription of added DNA

determined. Various methods of solubilization have been attempted, but perhaps the most successful has been to sonicate nuclei in media of high ionic strength. After purification, polymerase I and II can be separated from each other and from minor polymerases by chromatography on DEAE-cellulose or DEAE-Sephadex (Roeder and Rutter, 1969). The relative amounts of different polymerases can be determined in this way, although it is clearly hazardous to relate such determinations to polymerase activity in whole nuclei or cells until much more is known about the factors affecting the solubilization of the polymerases and their subsequent template activity.

Chromatin, of varying degrees of purity and physical state, can also be prepared from nuclei, and its ability to direct RNA synthesis by soluble polymerases determined. Under some circumstances the chromatin may direct the synthesis of informational RNA characteristic of the tissue from which it was derived (Paul and Gilmour, 1968), indicating that its natural template capacity may be conserved during isolation.

Conditions suitable for determination of RNA polymerase activity in lymphocyte nuclei were established by Wolff et al. (1967), and Handmaker and Graef (1970) determined the changes in polymerase activities of such preparations at both high and low ionic strength after the addition of PHA. They found that the activity at low ionic strength rose more rapidly and to a greater extent than that at high ionic strength. The low ionic strength activity was increased significantly 2 to 4 hr after PHA addition and rose to a maximum of twice the initial level after 20 hr. The high ionic strength activity did not increase in the first 4 hr, and increased only about 1.5-fold after 20 hr. Similar results were obtained by Kay and Cooke (1971) when they determined the effect of PHA on similar preparations assayed at high ionic strength in the presence and absence of α -amanitin. The amanitin-resistant polymerase increased more rapidly and to a greater extent than the amanitin-sensitive enzyme, although in their experiments the overall stimulation by PHA was somewhat higher.

The pattern of increases of these two activities in isolated nuclei correlates remarkably well with the changes in the synthesis of rRNA and informational RNA deduced by other methods (Cooke and Kay, 1973). This suggests that the factors limiting RNA polymerase activity in the isolated nuclei and the intact cell may be the same. If this proves to be generally true, such determinations of RNA polymerase activity may prove to be the simplest and most widely applicable method of assessing changes in RNA synthesis. Lymphocyte nuclear preparations assayed at high ionic strength probably

do not initiate the synthesis of new RNA chains as they are not inhibited by rifamycin derivatives which prevent initiation by soluble mammalian polymerases (Cooke and Kay, 1973). The RNA synthesis of isolated nuclei thus probably represents the completion of preexisting RNA chains, and would be proportional to the rate of RNA synthesis in the intact cell if the latter was limited by the rate of initiation of new chains.

Hausen et al. (1969) disrupted lymphocytes by homogenization at very low ionic strength, and then assayed both solubilized RNA polymerases and those which remained attached to deoxyribonucleoprotein. Both activities began to increase about 6 hr after PHA addition, and reached about twice the initial value after 24 hr. This probably indicates that the absolute amounts of RNA polymerase increase after PHA, along with its activity in nuclei. When the polymerases were solubilized from nuclei by the method of Roeder and Rutter (1969) and then assayed with or without α -amanitin both polymerase I and polymerase II activities were increased after incubation with PHA for 20 hr (Cooke and Brown, 1973). The increase in polymerase I activity was greater than that of polymerase II, and the changes in both activities were similar to the changes in the corresponding enzyme activities assayed in intact nuclei.

The priming activity of lymphocyte deoxyribonucleoprotein, as measured by its ability to direct RNA synthesis by added *Escherichia coli* RNA polymerase, has been reported to increase rapidly after the addition of PHA (Hirschhorn et al., 1969), but others have failed to confirm this result (Ono et al., 1970; Panijel et al., 1972). Final establishment of the factors controlling RNA synthesis in lymphocyte nuclei must await further characterization of lymphocyte polymerases, the factors which affect their activity, and those which affect the template capacity of the chromatin.

Panijel et al. (1972) have shown that immunisation *in vivo* with ovalbumin leads to increases in the RNA polymerase activities of lymph node cell nuclei comparable in magnitude to those found after PHA stimulation. They found that polymerase I and II were both increased to about the same extent. Detailed comparison with PHA stimulation is not possible, as the paper does not make clear the interval between the relevant antigenic stimulation and the performance of the assay.

13.1.9. Ribonuclease

Many animal cells contain an alkaline ribonuclease found throughout the

cytoplasmic fraction, and also a specific inhibitor of the enzyme. Kraft and Shortman (1970) have noted that inhibitor:alkaline ribonuclease levels are high in tissues characterized by high RNA levels and rates of RNA synthesis, and that these ratios drop when the rate of protein synthesis decreases. They have found that the inhibitor is extremely labile and suggested that this system may have a physiological role in the control of the level of cytoplasmic RNA or the stability of mRNA. Determination of the changes in levels of alkaline ribonuclease and the inhibitor during lymphocyte stimulation by PHA showed that adult human peripheral blood lymphocytes had high free ribonuclease activities but low levels of inhibitor (Kraft and Shortman, 1970). The amount of the inhibitor began to increase within the first 12 hr after PHA and continued to rise for the next 48 hr. From about 24 hr after PHA addition, the level of inhibitor was sufficient to neutralise all the alkaline ribonuclease. The increase in the inhibitor correlates well with the increase in total RNA levels.

13.2. Protein synthesis

That lymphocyte stimulation results in an increase in cell protein content has been shown at both the individual cell level (Darzynkiewicz et al., 1967; Steffen and Sören, 1968) and the culture level (Hausen et al., 1969; Fillingham and Morris, 1973). The mean dry mass of unstimulated lymphocytes has been variously estimated at between 36 and 60 pg/cell. This value increases between two- and three-fold in the 48–72 hr after the addition of PHA. The majority of cells in stimulated cultures have cell masses greater than those of unstimulated lymphocytes, but the response is heterogeneous with individual cells reaching up to six times the initial value. At later times after stimulation some enormous cells are found, and the mean dry mass of mitotic cells on the fourth day of culture is five times the initial level. Sören (1970) showed that protein accumulates in both nucleus and cytoplasm, but that the cytoplasmic accumulation is relatively greater. Much of the increase in nuclear mass occurs prior to the initiation of DNA synthesis.

While many workers have measured the changes in rates of incorporation of amino acids into protein during lymphocyte stimulation, comparatively little attention has been paid to the ways in which the increase in protein synthesis is brought about. It seems frequently to have been assumed that the increase results simply from increased synthesis of the relevant mRNAs, an assumption that is supported by little firm evidence (§ 12.5). Changes in

the activities of a number of lymphocyte enzymes have been documented, although these have not usually been studied in sufficient detail to establish whether the increased activity results from increased enzyme synthesis. Much more attention has been paid to the possibility that lymphocyte stimulation might result in the synthesis of immunological mediators, in particular immunoglobulins. This question was once highly controversial, but now a general agreement seems to have been reached.

13.2.1. *The rate of protein synthesis*

The ability of lymphocytes in culture to incorporate labelled amino acids into protein can readily be demonstrated by autoradiography. Nearly all small lymphocytes become labelled, and the labelling is fairly uniform (Cooper, 1961). The rate at which labelled amino acids are incorporated into protein is greatly increased after lymphocyte stimulation by all mitogens tested. While the effects of many stimulants on amino acid incorporation have not been studied, there seems no reason to doubt that this increase is a universal feature of lymphocyte stimulation.

The effect of PHA on amino acid incorporation depends on a number of factors including the culture conditions, the PHA concentration, the labelling protocol and the labelled amino acid chosen. PHA has little effect on the incorporation of amino acids which enter the cell by the L group transport system, such as leucine, phenylalanine and valine, within the first 2 hr, but thereafter the rate of incorporation rises continually to 5–10 times the initial rate after 24 hr and about 20 times the initial rate after 48 hr (Kay, 1966, 1968; Hausen et al., 1969). Changes at later times are more variable, probably reflecting variation in culture conditions at these times. PHA increases incorporation into protein of the A group amino acid alanine much more rapidly (Pogo et al., 1966; Rabinowitz et al., 1968), but this probably represents an effect on the uptake of amino acids of this group (§ 12.3). PHA also increases the rate of incorporation of ^{14}C -glucosamine into membrane glycoprotein very rapidly (Hayden et al., 1970). The rate of incorporation doubles within 3 hr, and rises to 10–20 times the initial rate after 4 days.

The rate of amino acid incorporation seems to be a reasonably accurate index of the rate of lymphocyte protein synthesis, at least when amino acids such as leucine and phenylalanine are used (Kay et al., 1971). In theory most of the complexities affecting the relationship between nucleoside incorpora-

tion and RNA synthesis could also apply to protein synthesis, but in practice most seem to be unimportant. The main difference is that, unlike the pool of nucleotide triphosphates, the pool of amino acids used for protein synthesis seems to equilibrate rapidly with exogenous precursor. Few direct studies have been done on this point, but the observation that the rates of incorporation of amino acids into protein are approximately linear for several hours, with any lag period very short relative to the radioactive pulses used, and that the proteins labelled are very largely metabolically stable, implies that there is no serious delay in pool saturation for amino acids such as leucine.

Extended culture of lymphocytes might be expected to deplete the amino acid content of the medium, and thus increase the effective specific activity of an added pulse of radioactive amino acid. In practice this effect proves to be significant for leucine only after culture with PHA for more than 48 hr (Kay et al., 1971), although it might become relevant sooner for amino acids present in smaller quantities or more readily metabolised. Such effects can be avoided by replacing the exhausted culture medium shortly before the pulse. With this precaution, and with a radioactive pulse sufficiently long to render insignificant the brief initial lag period, but short enough to avoid significant losses resulting from secretion or metabolic degradation of the protein product, the rate of incorporation of an amino acid such as leucine should provide an accurate comparison of the rates of protein synthesis of lymphocytes in different metabolic states.

13.2.2. Control of protein synthesis

Protein synthesis in animal cells is a complex process. In theory any of the steps involved could be rate-limiting for the overall process, and the rate-limiting step could differ in different cells or vary with metabolic state. It is important to realise that the limiting factor for overall protein synthesis may differ from that limiting the synthesis of an individual protein. The synthesis of a particular protein could be controlled by the rate of synthesis, transport to the cytoplasm or degradation of its mRNA, or by the availability of specific factors required for the initiation of termination of the translation of this mRNA, or of any rare amino-acyl tRNAs it required. The overall rate of protein synthesis could simply be the sum of the rates of synthesis of individual proteins, or could be limited by the availability of a component of the protein synthesizing machinery, such as ribosomes or a common amino-acyl tRNA.

In growing bacteria the rate of protein synthesis is limited by the availability of ribosomes, and most of the ribosomes at any instant seem to be actively engaged in protein synthesis. In animal cells too there is a general correlation between the cellular ribosome content and the rate of protein synthesis. The synthesis of the first enzyme to be studied in detail, the β -galactosidase of *Escherichia coli*, has proved to be controlled very largely by the rate of synthesis of its mRNA, and this method seems to be very widely employed in bacteria, at least for inducible enzymes. There is however some evidence that bacteria do contain factors which enable their ribosomes to discriminate between mRNAs.

The situation in animal cells has proved to be much more complex. The induction kinetics of many enzymes are compatible with the limiting factor being the synthesis of mRNA, but there are also a number of systems in which the synthesis of protein can be increased in the absence of RNA synthesis, in which some other limitation must be removed. Perhaps the most striking example of the control of protein synthesis at the translational level is the observation that sea urchin eggs can be fertilized and develop to the gastrula stage in the absence of RNA synthesis, or even of a nucleus (Gross and Cousineau, 1963).

First reports suggested that the early PHA-dependent increase in amino acid incorporation into lymphocyte protein was abolished when RNA synthesis was inhibited by actinomycin (Hirschhorn et al., 1963; Pogo et al., 1966). However, these studies were not properly controlled. They showed that actinomycin reduced amino acid incorporation in the presence of PHA to the control rate, but did not study the effect of actinomycin on the unstimulated cultures. Kay (1967) showed that actinomycin significantly reduced the rate of incorporation by control cultures within 1–2 hr and nearly abolished protein synthesis within 3 hr, the time at which a significant PHA-dependent increase was first detectable. Neiman and MacDonnell (1971) have reported a residual early stimulation of leucine incorporation into protein by PHA in the presence of actinomycin, implying that the early effects of PHA are at the translational level. Their conclusion is supported by the observation that PHA could still increase the survival of 18S rRNA, a process thought to reflect increased synthesis of a protective protein (§ 13.1.5), in the presence of actinomycin. These conclusions apply only to the very early period of the PHA response. The general requirement of protein synthesis for continued RNA synthesis means that the later stages of stimulation cannot be studied in this way. The lability of protein syn-

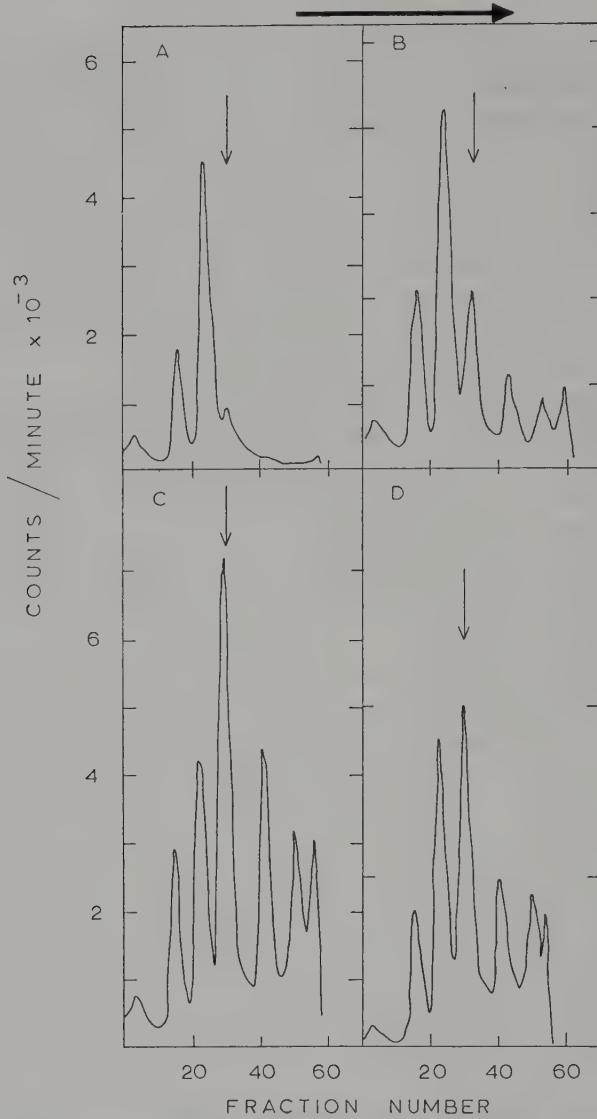


Fig. 13.5. Proportion of lymphocyte ribosomes active in protein synthesis before and after stimulation by PHA. ^3H -labelled ribosomes from lymphocytes incubated without PHA (A) or with PHA for 12 hr (B), 24 hr (C) or 48 hr (D) were sedimented in sucrose gradients containing 0.5 M NaCl. In these conditions ribosomes not carrying a nascent polypeptide chain are dissociated to 60S and 40S subunits, but active ribosomes and polyribosomes remain intact. The arrows indicate the position of monomeric 80S ribosomes on each gradient. Redrawn from Kay et al. (1971).

thesis in the presence of actinomycin does not imply that all mRNA in lymphocytes is metabolically unstable. RNA synthesis could easily be required for the continued synthesis of an unstable protein essential for protein synthesis, or the inhibition might be due to a side effect of actinomycin.

PHA stimulation results in increased synthesis of both tRNA and rRNA. The requirement for tRNA has not been investigated. However, the synthesis of rRNA can be inhibited selectively by low concentrations of actinomycin, and under these conditions much of the normal increase in protein synthesis during the first 24 hr after PHA addition still occurs (Kay et al., 1969). Further increase in the rate of protein synthesis is prevented, but this could as easily be secondary to inhibition of DNA synthesis as to a dependence of this further increase on the availability of new ribosomes.

Electron microscope studies have shown that unstimulated lymphocytes contain mainly single ribosomes, while stimulated cells contain many polyribosomes (Sören and Biberfeld, 1973). The percentage of ribosomes active in protein synthesis at different stages of PHA stimulation has also been determined by taking advantage of the selective resistance of ribosomes actively synthesizing protein to dissociation to subunits at high ionic strength

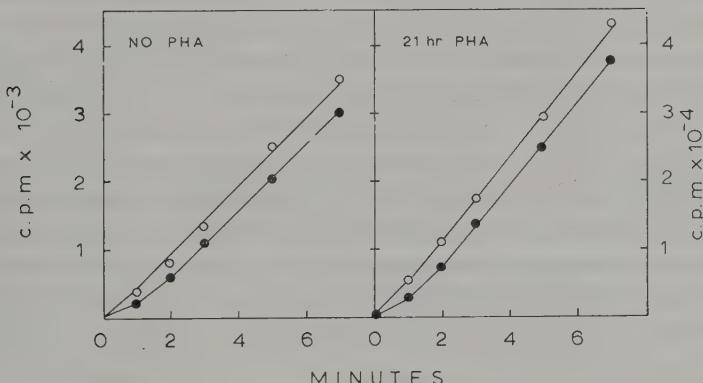


Fig. 13.6. Translation time in lymphocytes incubated with or without PHA. Incorporation of ^{14}C -leucine into the cytoplasmic fraction of lymphocytes (\circ) or into cytoplasm from which ribosomes had been removed by sedimentation (\bullet) was determined. The difference between the two parallel rates of incorporation represents the time required to synthesize one half of an average protein molecule. The average translation time is approximately 1.4 min in each case.

(Kay et al., 1971). They found that less than 30% of the ribosomes of unstimulated lymphocytes were active, but that this figure increased rapidly after the addition of PHA, rose to as high as 85% after 24 hr, and then slowly declined (fig. 13.5). Ribosomes synthesized before PHA addition were as active as those synthesized after stimulation. Parallel measurements showed that the rate of protein synthesis per ribosome by the intact cells continued to rise for 48 hr after PHA addition. The time taken for a ribosome to translate a single mRNA does not change significantly (fig. 13.6), so the accumulation of polyribosomes after activation must be due to an increase in the rate at which ribosomes initiate new rounds of mRNA translation. The ribosomes of unstimulated lymphocytes were as efficient as those of stimulated cells at attaching to the synthetic mRNA poly(U) under conditions in which the normal initiation factors are not required. There is a small reduction in the rate at which they synthesize polyphenylalanine, but enough to account for only a small part of the increase in protein synthesis after stimulation (Ahern and Kay, 1973). The reason for the large number of inactive ribosomes in unstimulated lymphocytes must be a lack of some factor required for the initiation of protein synthesis, either mRNA or an initiation factor.

To discriminate between these possibilities, Ahern et al. (1974) compared the abilities of cell-free protein synthesizing systems from PHA-stimulated and unstimulated lymphocytes to carry out the partial reactions of the initiation process with ^{35}S -methionyl tRNA_F^{met}, the initiator tRNA. Extracts of PHA-stimulated cells were found to be much more active than unstimulated extracts. This difference was not much affected by the addition of exogenous mRNA to the systems, but was overcome by the addition of partially purified initiation factors derived from rabbit reticulocytes. Extrapolating conclusions drawn from cell-free protein synthesizing systems to intact cells is not without risk but these results lend support to the hypothesis that protein synthesis in unstimulated lymphocytes is limited by the availability of one or more of the initiation factors, and that the amount of mRNA present is not rate-limiting.

13.2.3. Types of protein synthesized: cellular proteins

Most of the protein synthesized after lymphocyte stimulation remains associated with the cells. Only a small proportion is secreted to the culture medium, except when cell death is occurring (Smith et al., 1967). Analysis by

Table 13.3
Changes in enzyme activities during lymphocyte stimulation.

A. Enzymes showing substantial increases in activity which begin well before the initiation of DNA synthesis		
Adenyl cyclase	Smith et al. (1971)	12.4
Acyl CoA:lysolecithin acyl transferase	Ferber et al. (1974)	13.5
Phosphatidyl-choline cytidyl transferase	Nelson and Sribney (1972)	13.5
Phosphatidyl-choline glyceride transferase	Nelson and Sribney (1972)	13.5
Histone kinase	Cross and Ord (1971)	12.5
Histone phosphatase	Cross and Ord (1971)	12.5
Uridine kinase	Lucas (1967)	13.1.3
Cytidine kinase	Lucas (1967)	13.1.3
RNA polymerase I	Handmaker and Graef (1970)	13.1.8
RNA-directed DNA polymerase	Penner et al. (1971b)	14.3.3
Ribonuclease inhibitor	Kraft and Shortman (1970)	13.1.9
Ornithine decarboxylase	Kay and Cooke (1971)	13.3
S-adenosyl methionine decarboxylase	Kay and Lindsay (1973)	13.3
B. Enzymes showing substantial increases in activity which begin together with or following the initiation of DNA synthesis		
DNA polymerase	Loeb et al. (1968)	14.3.2
DNase	Loeb et al. (1970)	14.3.2
Polynucleotide ligase	Pedrini et al. (1972)	14.3.2
Thymidine kinase	Wilms and Wilmanns (1969)	14.3.1
TMP kinase	Loeb et al. (1970)	14.3.1
Deoxycytidine kinase	Pegoraro and Bernengo (1971)	14.3.1
Deoxycytidylate deaminase	Pegoraro and Bernengo (1971)	14.3.1
PolyADP-ribose polymerase	Lehmann and Shall (1972)	14.3.2
Carbamyl phosphate synthetase	Ito and Uchino (1971)	13.1.3
Aspartate transcarbamylase	Ito and Uchino (1971)	13.1.3
tRNA methylases	Riddick and Gallo (1971)	13.1.6
Cystathione synthetase	Goldstein et al. (1972)	13.2.3
C. Enzymes which show about a two-fold rise in activity, beginning together with or following the initiation of DNA synthesis		
RNA polymerase II	Handmaker and Graef (1970)	13.1.8
Acid phosphatase	Hirschhorn et al. (1967)	12.6
Aryl sulphatase	Hirschhorn et al. (1967)	12.6
β -glucuronidase	Nadler et al. (1969)	12.6
α -glucosidase	Nadler et al. (1969)	12.6
Lactate dehydrogenase	Rabinowitz and Dietz (1967)	13.2.3
Malate dehydrogenase	Rabinowitz and Dietz (1967)	13.2.3
Glucose-6-phosphate dehydrogenase	Nadler et al. (1969)	12.6
Fumarase	Loeb et al. (1970)	14.3.1
D. Enzymes which show little or no change in activity		
UMP kinase	Lucas (1967)	13.1.3
AMP kinase	Lucas (1967)	13.1.3
Uridine phosphorylase	Lucas (1971)	13.2.3
GMP kinase	Loeb et al. (1970)	14.3.1
dGMP kinase	Loeb et al. (1970)	14.3.1

Note: More detailed information and references may be found in the section referred to in the right hand column.

polyacrylamide gel electrophoresis of cellular proteins of stimulated and unstimulated lymphocytes did not lead to the detection of major new proteins, and Neiman and MacDonnell (1971) concluded that a large proportion of the increased protein synthesis after stimulation represented more rapid production of protein molecules already being synthesized.

Such methods are applicable only to the study of major structural proteins. Increased synthesis of individual enzymes would not be detected and changes in the activities of a great many enzymes have been documented (see table 13.3). However, an increase in the activity of an enzyme does not necessarily indicate an increase in its rate of synthesis. It could equally be due to a decrease in its rate of degradation, activation from an inactive apoenzyme, accumulation of an activator or co-factor, or loss of an inhibitor. Distinguishing between these possibilities is frequently difficult in mammalian systems, and even the most preliminary attempts to do so have not been made for most of the lymphocyte enzymes studied. Demonstrations that normal rises in enzyme activity do not occur when stimulation is accompanied by gross inhibition of RNA and protein synthesis by agents such as actinomycin, puromycin and cycloheximide are not usually helpful in this respect. Such experiments are of value only when the increase in activity is a reasonably rapid and direct effect of the stimulant. Most of the enzymic changes seen during lymphocyte stimulation become apparent only after several hours or days, and are probably not directly induced by the stimulant but are in response to the changing internal environment of the cell. Inhibitors are as likely to affect these environmental changes, and thus inhibit the development of the stimulus for the enzymic changes, as they are to have direct effects on the synthesis of enzyme mRNA or protein. In addition, any drug is liable to have direct but unrecognised side effects, while in the many published experiments in which cells have been incubated with inhibitors of RNA or protein synthesis for a day or more it is difficult to believe that they are still alive when studied. Experiments in which enzymic changes are demonstrated to occur even when RNA or protein synthesis are inhibited are much more convincing.

A problem to which no universal solution has been adopted is the way in which enzyme activity of lymphocytes should be expressed. Activities are conventionally expressed relative to protein content of extracts analysed, but unless unusual precautions are taken to remove the erythrocytes that generally contaminate cultures of blood lymphocytes, much of this protein will be haemoglobin. Unlike the lymphocyte protein, the amount of haemoglobin

present during culture will not rise. It may well fall, if some erythrocyte lysis occurs. This problem is clearly not serious when large rises in enzyme activities are studied, but may well explain some of the smaller changes reported to occur during the later stages of stimulation. This problem can be avoided by the removal of erythrocytes from cultures, or by expression of results relative to the number of cells, or the amount of DNA. It should be recognised that if the latter two procedures are adopted, rises in enzyme activity might be seen when the specific activity of the enzyme did not alter.

The most rapid change in an enzyme activity that has been reported is the rather controversial three-fold increase in adenyl cyclase found 1–2 min after the addition of PHA to lymphocytes (§ 12.4). The increased uptake of many different types of small molecules (§ 12.3) presumably indicates similar rapid increases in the activities of the relevant permeases. These changes must result from the activation of existing enzymes, as they still occur when protein synthesis is inhibited by cycloheximide.

The activities of several other enzymes show large increases which, while not so rapid as those above, begin well before the initiation of DNA synthesis, although most do not reach their maximum levels until 24–72 hr. The most prominent of these are uridine and cytidine kinases (§ 13.1.3), RNA polymerase I (§ 13.1.8), and the enzymes involved in synthesis of polyamines (§ 13.3). At least some of these increases are probably due to increased enzyme synthesis.

The increase in uridine kinase activity following lymphocyte stimulation is described in § 13.1.3. A significant increase in activity first becomes apparent 1–3 hr after the addition of PHA. Inhibition of protein synthesis prior to PHA addition does not prevent the increased rate of uridine phosphorylation and incorporation into RNA, but does prevent this early increase in uridine kinase activity. As discussed above, this kind of experiment can never be entirely convincing, but in this case the time period is sufficiently short and the evidence of continuation of the probable stimulus adequate to suggest that the increase in activity does represent increased enzyme synthesis.

The activities of many other lymphocyte enzymes begin to increase together with or following the initiation of DNA synthesis. In some cases the rises are substantial, in others less so. Many of the largest rises are in enzymes associated with DNA synthesis, such as DNA polymerase and thymidine kinase (§ 14.3). Increased synthesis of histones also occurs at this time (Pogo et al., 1966) and there are marked increases in enzymes as varied as

tRNA methylases (§ 13.1.6), carbamyl phosphate synthetase (§ 13.1.2) and cystathione synthetase (Goldstein et al., 1972). The significance of the smaller increases in the activities of a number of other enzymes is often difficult to assess, for reasons discussed above. This category includes many enzymes of the catabolic pathways of the cell. Although these changes are relatively small, some are of interest. The total increase in lactic dehydrogenase activity is small, but is accompanied by a change from the heart type isoenzyme to the muscle type. This isoenzyme change is probably due to the change in pO_2 in the stimulated cultures (Hellung-Larsen and Andersen, 1968). It is likely that changes in several other enzymes are of this consequential type, although this case is the best documented. The activities of several lysosomal enzymes, notably acid phosphatase, have also been shown to change relatively little after stimulation, but there are changes in the distribution of these enzymes between the different cell fractions (§ 12.6). Several other enzymes which have been studied do not show significant changes in activity.

13.2.4. Types of protein synthesized: immunological mediators

The early reports of Elves et al. (1963) and Hirschhorn et al. (1963) claimed that stimulation of human peripheral blood lymphocytes by PHA or antigens resulted in the initiation of immunoglobulin synthesis in the responding cells. It is now clear that, at least as far as PHA is concerned, this idea in its simple form is mistaken. These early papers have, however, been extremely influential, and the source of a controversy that has only recently been resolved.

Hirschhorn et al. (1963) reported that nearly all stimulated lymphocytes synthesized immunoglobulins, and that this synthesis accounted for a major part of the protein synthesized and secreted into the culture medium. Attempts to follow up this work soon established that most of the proteins synthesized were cellular proteins and not immunoglobulins (Sell et al., 1965; Turner and Forbes, 1966; Smith et al., 1967). It would indeed be surprising if human peripheral blood lymphocytes responded to PHA by large-scale immunoglobulin synthesis, as this cell population contains a high proportion of T lymphocytes and PHA seems to be primarily a T cell mitogen, while immunoglobulin synthesis is thought to be a B lymphocyte function.

The most convincing evidence for mitogen-stimulated immunoglobulin

synthesis has come from studies of the effects on mouse spleen cell populations enriched with B cells of mitogens known to activate mouse B lymphocytes such as pokeweed mitogen (Parkhouse et al., 1972), LPS (Andersson et al., 1972) and Sepharose-bound con A (Andersson and Melchers, 1973). PHA did not increase immunoglobulin synthesis in these cultures, and reduced the proportion of the protein synthesized that was immunoglobulin in normal mouse spleen cell cultures. The immunoglobulin was identified by specific precipitation with anti-immunoglobulin, and in some experiments antibody synthesizing cells were detected by the Jerne plaque technique. At least 95% of the immunoglobulin synthesized seems to be IgM. Immunoglobulin synthesis can be detected 10–15 hr after the addition of mitogen and its secretion into the culture medium begins after 24–30 hr. After incubation of B lymphocytes from mouse spleen with Sepharose-bound con A for 3 days, nearly 20% of the protein synthesized was immunoglobulin, a proportion comparable to that found for myeloma cells (Andersson and Melchers, 1973). Some cells resembling plasma cells, presumably derived from B lymphocytes, are seen in cultures of human lymphocytes stimulated by pokeweed mitogen but not in cultures stimulated by PHA (Douglas et al., 1967).

Much effort has been expended on the development of techniques sufficiently sensitive to detect such small quantities of immunoglobulins as might realistically be expected to be synthesized by human blood lymphocytes stimulated by PHA. The problem is further complicated in that even peripheral blood may contain a minority of cells actively synthesizing immunoglobulins (reviewed by Van Furth, 1969), and that the proportion of B lymphocytes present, even if not otherwise synthesizing antibodies, will contain and presumably synthesize immunoglobulin surface receptors.

The main techniques used to study this problem have been analysis of the protein synthesized by lymphocytes by immunological precipitation and immunoelectrophoresis, and the identification of immunoglobulin synthesizing cells with fluorescein-conjugated anti-immunoglobulin antisera. The results and main conclusions of these studies have been reviewed by Greaves and Roitt (1968).

Several workers have determined the proportion of the protein synthesized or secreted by lymphocytes incubated with or without PHA that is precipitated by antisera to immunoglobulins. All have found 1–10% of the labelled protein to precipitate. While PHA increases the amount of radioactive protein precipitated along with the overall rate of protein synthesis, the proportion of the total labelled protein precipitated does not usually increase.

The chief difficulty of this method is in guarding against the danger of non-specific precipitation of labelled protein. Most workers have controlled their studies by carrying out precipitations with other antigen-antibody combinations either prior to the precipitation with anti-immunoglobulin or on parallel samples. Greaves and Roitt (1968) preceded their specific precipitation by two non-specific ovalbumin-anti-ovalbumin precipitations, and found no more radioactivity in the specific precipitate than in the second non-specific precipitate. Other workers have reported finding somewhat more radioactivity in the specific precipitate than in the non-specific precipitate, but the danger is that all immune precipitates may not have the same capacity for non-specific precipitation, and the method has clearly been used at near to the limit of its sensitivity.

Immunoelectrophoresis and autoradiography have been used by many workers to study the types of protein synthesized during stimulation. Hirschhorn et al. (1963) reported that most of the protein secreted to the culture medium was immunoglobulin, but subsequent studies have found the main serum proteins labelled to be α - and β -globulins and lipoproteins. Some workers have found minor labelling of immunoglobulins, while others have failed to detect any. Turner and Forbes (1966) found labelling of immunoglobulins only in the first 12 hr of culture, which could be attributed to the few antibody synthesizing cells initially present. The main danger with this method is again the possibility of non-specific precipitation. Epstein (1966) found that the labelling of the lipoprotein precipitate was due to the adherence of radioactive peptides, while Thiele and Stark (1970) found that radioactive proteins synthesized by HeLa cells and monkey kidney cells showed similar labelling patterns to those made by lymphocytes, with even the immunoglobulin precipitates being labelled. Marinkovich and Baluda (1966) found that a variety of cell types, including cloned chick embryonic muscle cells, synthesized protein which appeared to include 5–20% immunoglobulin, as judged by either precipitation with anti-immunoglobulin after three non-specific precipitates, or immunoelectrophoresis and autoradiography.

Perhaps the most sensitive and specific method used to detect immunoglobulin synthesis is the labelling of cells by fluorescein-conjugated anti-immunoglobulin. Hirschhorn et al. (1963) found that nearly 100% of lymphocytes stimulated by PHA and 5–35% of those stimulated by antigens showed marked fluorescence, but that unstimulated lymphocytes, or stimulated cells of agammaglobulinaemic patients did not. This result has proved

to be repeatable, provided that lymphocytes are incubated with fluorescent antisera prior to fixation (Greaves and Roitt, 1968), although usually only about half the PHA stimulated cells are found to fluoresce. Greaves and Roitt suggest that the fluorescein-conjugated anti-immunoglobulin enters the cytoplasm by endocytosis, perhaps after combination with immunoglobulin attached to the lymphocyte surface, and that the increased fluorescence seen in activated lymphocytes may be due to their more rapid rate of endocytosis.

When lymphocytes were fixed before incubation with the fluorescein-conjugated anti-immunoglobulin, the procedure normally employed to demonstrate antibody synthesis by plasma cells, little or no fluorescence was found in lymphocytes stimulated by PHA, staphylococcal filtrate or anti-lymphocyte serum (for references see Greaves and Roitt, 1968) except in a few cells present only at the beginning of culture. As originally reported by Elves et al. (1963), fluorescence was seen in a few cells of cultures stimulated by antigens and also in some of the cells stimulated by pokeweed (Greaves and Roitt, 1968). A variety of other methods have confirmed that synthesis of specific antibodies occurs in antigen stimulated cultures (Girard, 1967) and some cells resembling plasma cells are seen in these cultures (Michalowski et al., 1966), in addition to the lymphoblasts of the type seen in cultures stimulated by PHA.

There is one limited sense in which human lymphocytes may synthesize immunoglobulins after stimulation by mitogens such as PHA. It is probable that at least a proportion of them have immunoglobulin receptors on their surface, and Hellstrom et al. (1971) have shown that the density of these increases after stimulation with PHA, con A, antigens, or in the mixed lymphocyte reaction. Such receptors are rapidly internalised after combination with either antigen or anti-immunoglobulin (§ 12.2.3) and such internalization is one possible explanation for the uptake of fluorescein-conjugated anti-immunoglobulin seen when PHA stimulated lymphocytes are incubated with this reagent prior to fixation.

A variety of other immunological mediators are secreted by lymphocytes in response to stimulation (ch. 10). Most of these are not well characterized and their synthesis has not been studied in any detail. It is frequently not clear whether the effects seen represent the synthesis of these agents or merely the release of preformed material.

13.3. Polyamine metabolism

The polyamines are a family of basic molecules, with three main members of biological importance, putrescine, spermidine and spermine. Putrescine is synthesized by the decarboxylation of the amino acid ornithine, and spermidine is formed from putrescine by the addition of a propylamine group obtained by the decarboxylation of S-adenosyl methionine. Spermine is formed by the addition of a further propylamine group to spermidine. The concentrations of these polyamines in mammalian cells have been found to correlate well with their RNA content, and the activities of the rate-limiting enzymes for polyamine synthesis, ornithine decarboxylase and S-adenosyl methionine decarboxylase, are generally found to be low in non-growing cells and high in cells with a rapid growth rate (Cohen, 1971). While a variety of effects of polyamines on protein and nucleic acid synthesis in mammalian cells, and cell-free systems derived from them, have been reported, their biological functions are still far from clear.

The activities of both ornithine decarboxylase and S-adenosyl methionine decarboxylase are very low in resting lymphocytes, but increase markedly after activation with PHA, con A or other mitogens (Kay and Cooke, 1971; Kay and Lindsay, 1973). The increases are biphasic, with the first phase beginning 5–8 hr after the addition of PHA, and the second occurring shortly before the initiation of DNA synthesis. These changes lead to marked intracellular accumulation of all three polyamines (Fillingame and Morris, 1973).

Both these decarboxylases are unstable enzymes, and their activity declines with a half life of less than 1 hr in the presence of inhibitors of protein synthesis (Kay and Lindsay, 1973). In the case of ornithine decarboxylase the half life may be as little as 15 min. The activities of such enzymes could be increased either by increasing their rate of synthesis or their stability, but while there are situations in which physiological agents can increase enzyme activity via an effect on the rate of enzyme degradation (Kay et al., 1972), the increases brought about by mitogens seem to be due to an increased rate of enzyme synthesis (Kay and Lindsay, 1973).

Work in several experimental systems has shown a correlation between increased polyamine production and increased synthesis of ribosomal RNA, and it has often been suggested that these may be causally related. Studies during lymphocyte activation have shown that neither increase is dependent on the other. Increases in the activities of both decarboxylases still occur when lymphocytes are activated in the presence of low concentrations of

actinomycin, which completely suppress ribosomal RNA synthesis (Kay and Lindsay, 1973). Spermidine synthesis can be completely suppressed and putrescine accumulation greatly enhanced by the drug methyl glyoxal bis-(guanyl hydrazone), but this drug has no effect on the normal increases in the rate of ribosomal RNA synthesis and maturation that occur after activation by PHA or con A (Kay and Pegg, 1973; Fillingame and Morris, 1973). The increase in protein synthesis after activation is also not prevented by the presence of this drug, although the initiation of DNA synthesis is inhibited.

13.4. Carbohydrate metabolism

The increases in membrane transport and protein and nucleic acid synthesis that accompany lymphocyte stimulation would be expected to require increased energy production, and there have been many studies of the changes in carbohydrate metabolism which accompany stimulation. The earlier studies relied mainly on histochemical methods, but more recently biochemical techniques have been used. Biochemical methods are in some respects more difficult to apply to the study of energy metabolism than to protein and nucleic acid synthesis, as erythrocytes, which do not show the two latter activities, do consume glucose, and granulocytes have very active carbohydrate metabolism. Both must therefore be removed from the cultures.

The rate of glucose uptake more than doubles within 15–30 min of PHA addition (Peters and Hausen, 1971) and the rate of lactate production shows a similar increase in the same period (Roos and Loos, 1970). Lactate production rises to about 4 times the initial rate after 24 hr, and the majority of the glucose utilized is converted to pyruvate or lactic acid (Pachman, 1967; Hedeskov, 1968). Some oxidation of pyruvate to CO_2 by the tricarboxylic acid cycle does occur and as much as 85% of the ATP synthesized may be formed as a result of oxidative phosphorylation (Roos and Loos, 1973). The importance of aerobic oxidation may have been underestimated in some studies, as oxygen consumption declines dramatically as the cell concentration increases (Hedeskov and Esmann, 1966) and most studies on carbohydrate metabolism have, for technical reasons, been performed at unusually high cell concentrations. The initial stimulation of glycolysis by PHA precedes the earliest stimulation of tricarboxylic acid cycle activity (Roos and Loos, 1970) but either pathway can provide the energy needed during the first 4 hr of PHA stimulation. Several groups have attempted to determine

the relative importance of glycolysis and oxidative phosphorylation in the longer term by the use of selective inhibitors, and most have found that suppression of either pathway severely inhibits DNA synthesis (Roos and Loos, 1973).

A small proportion of glucose is metabolised by other pathways. About 2% of the glucose taken up is converted to glycogen, and the rate of conversion is stimulated by PHA (Hedeskov, 1968). Glycogen accumulates in lymphocytes during the early stages of stimulation (Quaglino et al., 1962) although it is not found in the large lymphoblasts present later in culture. 5% of glucose taken up is oxidised by the pentose phosphate pathway, and this too is increased after PHA (MacHaffie and Wang, 1967; Hedeskov, 1968). About 2% is converted to amino acids, mainly serine and glutamate, but less than 0.1% to fats (Hedeskov, 1968).

Increased glucose uptake begins very early after PHA stimulation. When this is prevented by omission of glucose from the culture medium the changes in RNA and protein metabolism proceed normally for the first 24 hr although DNA synthesis is inhibited (§ 12.3). Sufficient alternative substrates must be available for these early metabolic changes. Roos and Loos (1970) have shown that PHA normally causes an early but transient fall in lymphocyte ATP levels, and increases the rate at which the ATP level falls when glycolysis is inhibited. It would seem logical that an increased demand for ATP synthesis might cause increased glucose uptake and more rapid glycolysis, but this may not be the important control in the intact cell. The rise in glucose uptake seems to occur even before the ATP level falls, and the increase in glycogen accumulation seen during the early stages of stimulation also would not accord with such a simple control system. The rise in glucose uptake may be part of the primary response to growth stimulation and, like the synthesis of ribosomes, anticipate a future increase in demand rather than occur in response to it.

13.5. Lipid metabolism

The interaction of stimulants with the lymphocyte membrane, the early changes in membrane function and the later changes in lymphocyte morphology might all be expected to result in alterations in phospholipid metabolism. However, Huber et al. (1968) found that incubation of lymphocytes with PHA for 60 hr did not greatly affect the relative amounts of the different types of phospholipids, although Fisher and Mueller (1969 b) found that the

increase in phosphatidyl-ethanolamine and -serine preceded the increases in phosphatidyl-choline and -inositol.

There have been a number of studies on the effects of stimulation on the incorporation of isotopes into phospholipids. PHA has been shown to increase the rate of incorporation into this fraction of glucose, glycerol and choline (Kay, 1968), phosphate (Fisher and Mueller, 1968), acetate (Huber et al., 1968), the methyl group of methionine (Fisher and Mueller, 1969b), inositol (Pasternak and Friedrichs, 1970) and oleate (Resch and Ferber, 1972). The incorporation of glucosamine into membrane glycolipids is also increased (Hayden et al., 1970). Con A and anti-immunoglobulins also stimulate the incorporation of choline, acetate and oleate (Resch and Ferber, 1972). In several cases the increased incorporation begins within 1 hr of the addition of stimulant, and these early increases do not depend on the synthesis of new RNA or protein (Kay, 1968; Fisher and Mueller, 1969b). Maximum rates of incorporation, several times the initial levels, are usually found after 48–72 hr. The increase in choline incorporation appears to be biphasic. The initial stimulation begins almost immediately, and the second phase after about 24 hr (Fisher and Mueller, 1969b).

The most interesting of these changes is the increase in the incorporation of ^{32}P -phosphate. Fisher and Mueller (1968) showed that the rate of incorporation of phosphate into phosphatidyl-inositol increased ten-fold within 10 min and twenty-fold within 30 min of PHA addition, while the incorporation into phosphatidyl-choline, -serine and -ethanolamine were very much less affected. This incorporation into phosphatidyl-inositol reached a maximum level within 24 hr, and seems to be due to increased turnover rather than synthesis. The effect of PHA was abolished by N-acetyl-galactosamine, although the sugar itself caused a marked stimulation of incorporation (Fisher and Mueller, 1969a). N-acetyl glucosamine was much less effective. Lucas et al. (1971) found a similar increase when lymphocytes were stimulated with streptokinase-streptodornase, but when tetanus toxoid was the stimulant the labelling of phosphatidyl-inositol was inhibited while incorporation into phosphatidyl-choline and -ethanolamine showed a rapid increase.

Increased uptake of ^{14}C -oleate also can be detected within minutes of the addition of PHA or con A. Incorporation into phospholipids is stimulated more than incorporation into neutral lipids, and incorporation into the plasma membrane fraction is stimulated more than incorporation into other membrane fractions (Resch et al., 1972). Not only is the activity of the micro-

somal acyl CoA:lyssolecithin acyl transferase increased maximally within 30 min of the addition of con A to calf thymocytes, but also its substrate specificity is altered to favour unsaturated fatty acids (Ferber et al., 1974). As a result the proportion of unsaturated fatty acid residues at position 2 of the phospholipids is almost doubled within 4 hr, a change which would be expected to lead to increased fluidity of the lymphocyte membrane (Ferber et al., 1974).

While the interpretation of these changes is far from clear, they seem too complex to be simply explained by alterations in the rate of isotope uptake caused by the stimulants. The same cannot be said for many of the studies with the other isotopes. In several cases changes in rates of precursor uptake could account entirely for the changes observed, while in others they could have major quantitative effects. No direct studies on rates of uptake have been performed, and the precursors used are often not the obligatory or even usual ones. This is probably most obvious in the case of choline only because it has been the most intensively studied. Choline does not seem to be an obligatory component of the culture medium for lymphocyte stimulation, although it may normally be released slowly by the degradation of serum phospholipids. Fisher and Mueller (1969 b) have shown that it can be synthesized by lymphocytes, but the relative importance of synthesis and uptake from the medium are not known. The isotope normally used, ^{14}C -choline, is available only at low specific activity, and its addition to the cultures has usually grossly increased the medium concentration. The intracellular pool of choline seems to saturate only very slowly, and may well be inflated by the addition of the isotope. Fisher and Mueller (1969 b) have shown that after a short pulse with ^{14}C -choline, incorporation into phospholipids continues unabated for several hours after the removal of isotope, even when a hundred-fold excess of unlabelled choline was added to the chase medium. Under these circumstances changes in the rate of precursor uptake could clearly affect the rate of incorporation into phospholipids, and Fisher and Mueller (1969 b) found different effects of PHA on phosphatidyl-choline labelling when ^{14}C -choline and ^{14}C -methionine were used as precursors. Perhaps the best indication that the change in the rate of choline uptake does in fact represent an alteration in phospholipid metabolism is the observation of Nelson and Sribney (1972) that there are parallel changes in the activities of the enzymes responsible for phosphatidyl-choline synthesis.

That important changes in lymphocyte membranes occur during stimulation is beyond doubt, although it is less certain that the key changes will

involve the lipid fraction. Biochemical studies could contribute more to the elucidation of these changes if we understood more about the complex relationships between membrane structure and membrane function.

13.6. Viral replication

Nahmias et al. (1964) found that herpes simplex virus, which would not replicate in normal human leucocytes, would replicate if the leucocytes were stimulated by PHA. Subsequent studies, summarised in table 13.4, have revealed a number of other viruses which will only replicate, or which will replicate to much higher titres, in stimulated lymphocytes. Although many of these studies have been carried out in unpurified leucocyte cultures, it has been clearly shown that the increased viral replication occurs in the stimulated lymphocytes. At least in the case of vesicular stomatitis virus, the replication occurs mainly in T lymphocytes (Kano et al., 1973). The lower levels of replication of vesicular stomatitis virus (Edelman and Wheelock, 1967), yellow fever virus (Wheelock and Edelman, 1969) and poliovirus (Willems et al., 1969) seen in unstimulated cultures occur mainly in the

Table 13.4

Viruses whose replication only occurs or is greatly enhanced after lymphocyte stimulation.

Virus	Lymphocytes	Stimulant	Reference
Herpes simplex	human blood	PHA	Nahmias et al. (1964)
Mumps	human blood	PHA	Duc-Nguyen and Henle (1966)
Vesicular stomatitis	human blood	PHA	Edelman and Wheelock (1966)
Vesicular stomatitis	human blood	ALS	Edelman and Wheelock (1968)
Vesicular stomatitis	mouse lymph node	PHA	Eustatia and Van der Veen (1971)
Vesicular stomatitis	mouse spleen	PHA, con A, pokeweed	Kano et al. (1973)
Vaccinia	human blood	PHA	Miller and Enders (1968)
Yellow fever	human blood	PHA	Wheelock and Edelman (1969)
Polio	human blood	PHA	Willems et al. (1969)
Echo 9	human blood	PHA	Berkovich et al. (1969)
Mengovirus	mouse lymph node	PHA	Eustatia and Van der Veen (1971)
Murine leukaemia	mouse spleen	MLR	Hirsch et al. (1972)
Canine distemper	dog blood	ALS	Poste (1970)

monocytes. Viral replication is usually accompanied by at least partial inhibition of the PHA response, and similar inhibition is also caused by a number of other viruses which either do not replicate successfully in stimulated lymphocytes, or whose replication is not improved by lymphocyte stimulation (reviewed by Notkins et al., 1970).

Two possible explanations for the increased ability of stimulated lymphocytes to support viral replication have been proposed. The first is that the unstimulated lymphocytes might not possess the metabolic systems to support the DNA, RNA and protein synthesis required for replication, and these systems might be developed during stimulation. The deoxyribonucleotide and DNA synthesizing systems in particular are minimal in unstimulated lymphocytes (§ 14.3), and may explain the failure of the DNA viruses, herpes simplex and vaccinia, to replicate. Both these viruses seem to enter unstimulated lymphocytes (Bouroncle et al., 1970; Miller and Enders, 1968). The second explanation is that lymphocyte stimulation may increase viral absorption either by causing the production of viral receptors or stimulating endocytosis. This may be important for those RNA viruses which can replicate in non-dividing monocytes. However, Wheelock and Edelman (1969) found that addition of PHA even after yellow fever virus stimulated viral replication, and also that even unstimulated lymphocytes produced interferon in response to viral addition, suggesting that absorption had occurred. Furthermore, the activation of leukaemia virus from mouse spleen cells during the mixed lymphocyte reaction (Hirsch et al., 1972) is unlikely to be due to any effect on viral absorption. This activation also appears to be not solely the result of lymphocyte stimulation, as no virus was produced when PHA was used as the stimulant.

13.7. General considerations

Two conclusions can be drawn immediately from these studies on the metabolic consequences of lymphocyte stimulation. The first is that these changes are of great diversity and seem to affect practically every metabolic activity of the lymphocytes. This is perhaps not surprising in view of the major structural changes that occur and the different requirement of growing and non-growing cells. The second conclusion is that the changes which accompany growth stimulation in lymphocytes are very similar to those which occur during a switch to a more rapid rate of growth in other types of cells as, for example, in the remaining hepatocytes after partial hepatectomy, in

kidney cells after removal of the opposite kidney or after folic acid injection, in salivary glands after administration of isoproterenol, in uteri after exposure to oestrogens or in cultured fibroblasts after subculture from a confluent monolayer.

Examples of the biochemical changes which occur in many or all of these systems prior to the stimulation of DNA synthesis are a rapid increase in uptake of many metabolites; marked increases in the rates of synthesis of ribosomal RNA and transfer RNA; increased utilization of pre-existing ribosomes for protein synthesis, mediated mainly by an increase in the rate of initiation of new protein chains; and increased synthesis of polyamines. There are some differences in the responses of these different cells. The precise range of metabolites whose uptake is increased, and the magnitude of these increases, may show some variation (§ 12.3 and § 13.1.3). Differences in the types of informational RNA synthesized by hepatocytes can be detected rapidly after partial hepatectomy by DNA-RNA hybridization techniques, even when the hybridization is carried out at quite low DNA:RNA ratios, but it has not proved possible to demonstrate such differences after lymphocyte stimulation (§ 13.1.7). Nevertheless, the similarities between these systems are so much more pronounced than the differences as to lend considerable support to the concept of a common growth programme for all mammalian cells, which can be activated in individual types of cells in response to a cell-type specific growth signal (Hershko et al., 1971).

Much effort has been expended in attempts to explore the inter-relationship of the different elements of the growth programme. The usual procedure has been to try to establish the sequence of metabolic events, and then to study the effect of inhibition of early changes on later ones. Such experiments are usually convincing only when they give the negative answer that a late change still occurs when an earlier one is prevented. Positive answers are more suspect, as it is difficult to eliminate the possibility that even supposedly selective inhibitors may have unsuspected side effects. In any case, such experiments normally show only that the later increase is dependent on the continuation of the activity inhibited, not that the later increase is caused by the earlier.

Such topics as have been studied in detail have shown that control mechanisms may be extremely complex. Cooper's studies on the control of ribosomal RNA metabolism have shown that the rate of synthesis of the 45S rRNA precursor, the rate of processing of the precursor and the efficiency with which the precursor molecules are converted to mature 28S and 18S

rRNA are all increased within a few hours of the addition of PHA to lymphocytes, and that these changes are not interdependent (§ 13.1.5). Less detailed studies suggest that the changes in mRNA and tRNA synthesis may be equally complicated. Similarly the increase in the rate of initiation of protein synthesis is accompanied by an increase in availability of both mRNA and initiation factors (§ 13.1.7 and § 13.2.2), although the increase in initiation factor activity seems to be the more important. All the precedents suggest that as other control mechanisms are studied in more detail they will prove to be equally complex.

The metabolic changes after activation seem to form a sequence beginning immediately after the addition of mitogen and becoming progressively more complicated as DNA synthesis approaches. As the activities of many metabolic pathways are controlled by the concentration of their end product or their initial substrate, the concept that all the changes could be part of a consequential cascade dependent on a single initial change is obviously an attractive one. Some of the events observed are most probably of this type. For example, the increased ATP utilization required for increases in rates of ion transport, protein synthesis and RNA synthesis would be expected to lead to increased ATP synthesis, and thus increased oxidation of substrates such as glucose.

There is, however, evidence that at least some of the metabolic alterations occur in anticipation of an increased demand for their product rather than in response to such a demand. Thus, while the increase in ATP synthesis might be expected to provide a stimulus to the rate of glucose uptake, in fact glucose uptake increases more rapidly than the demand for intracellular glucose for energy production. As a result there is a transient intracellular accumulation of a store of glycogen early after activation (§ 13.4). Similarly, the function of ribosomes is to synthesize protein and it would be logical to imagine that the increase in rRNA synthesis after stimulation was connected with the increase in protein synthesis. In fact the increase in rRNA synthesis begins while the cell still has a high proportion of inactive ribosomes, and most of the normal increase in protein synthesis in the first 24 hr after the addition of PHA can still occur when rRNA synthesis is selectively inhibited (§ 13.2.2).

This ability of cells to anticipate future increases in demand implies the existence of a sophisticated growth control programme, in addition to the simple feedback control systems geared to existing demands. Such a programme could operate via intracellular mediators such as the pleiotypic

effector postulated by Hershko et al. (1971). It is rather difficult to imagine that the complete sequence of changes, spread over 48 hr or more after lymphocyte stimulation, are due to changes in the concentration of a single mediator of this type. It could be accomplished by a hierarchy of such mediators, if the concentration of the first were affected by the interaction of the mitogen at the cell membrane, and the concentration changes of subsequent mediators were effected by the satisfactory completion of earlier parts of the response.

References

Section 13.1.

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DNA synthesis and the cell cycle

DNA synthesis is not continuous even in rapidly dividing cells, but is confined to one period of the cell cycle, the S phase. In a normal cell cycle mitosis is followed first by a growth phase, G_1 , in which there is no DNA synthesis, then by the S phase, and then by G_2 phase in which DNA synthesis has been completed and the cells prepare for another mitosis. Non-dividing cells, such as unstimulated lymphocytes, are usually found to be arrested early in G_1 . The selective ability of cells in S phase to incorporate DNA precursors can be used to identify those cells and, indirectly, to determine the duration of the cell cycle and its individual stages. Several such studies have been carried out on stimulated lymphocytes.

Lymphocyte stimulation has also been used as a model system in which to study the factors controlling the initiation of DNA synthesis in mammalian cells. The main emphasis has been on the induction of the enzyme systems needed, most of which are absent from unstimulated lymphocytes. Some attention has also been paid to the low rate of incorporation of DNA precursors, not associated with replication, that may occur outside the S phase. Such incorporation may represent either the repair of damaged DNA, or synthesis using an RNA template.

As DNA synthesizing cells are rare in many populations of lymphocytes, the initiation of DNA synthesis has provided a sensitive low background assay that has become the standard method for determining whether or not lymphocyte transformation has occurred. This assay can readily be made at least semi-quantitative, provided that a few simple precautions are observed.

14.1. The incorporation of precursors into DNA

The most frequently used radioactive precursors for the study of DNA synthesis are the ^3H - and ^{14}C -isotopes of thymidine. The main differences between these are that ^3H -thymidine is available at much higher specific activities, and is significantly the cheaper. Isotopes of deoxycytidine, deoxyuridine and iododeoxyuridine have also been used.

The incorporation of thymidine into DNA by lymphocytes stimulated with PHA is virtually confined to those cells which have DNA contents between the diploid (G_1) and tetraploid (G_2) values, and which were thus presumably in S phase (Cooper et al., 1963). DNA contents of individual cells were measured in Feulgen stained smears, and isotope incorporation was determined by autoradiography. Unlabelled cells were almost exclusively diploid or tetraploid showing that DNA synthesis was continuous throughout S phase. The rate of thymidine incorporation was found to be fairly constant throughout S phase by Cooper et al. (1963), but Zank et al. (1971) have suggested that there may be a temporary decrease in the rate in mid-S phase.

The location of the newly synthesized DNA has been studied by Ribas-Mundo (1966). He found that DNA synthesis began diffusely throughout the nucleus, but that the nucleolus-associated chromatin and some other localized regions replicated at the end of S phase. According to Bader et al. (1963), who analysed the ^3H -thymidine labelling of human metaphase figures, a chromosome in group 16–18 and another in group 19–20 are the first to complete DNA synthesis. Some larger chromosomes completed DNA synthesis when small chromosomes were still replicating, indicating that conditions other than size determined the way in which chromosomes terminated DNA synthesis.

The incorporation of thymidine has proved an extremely satisfactory way of determining whether or not lymphocytes have initiated DNA synthesis, but a number of problems have arisen in the quantitation of lymphocyte stimulation by this method. One difficulty is that thymidine breaks down slowly on storage, and rapidly on incubation with leucocytes at 37°C , to thymine and dihydrothymine, which are not utilised as precursors for DNA synthesis (Cooper and Milton, 1964; Milton et al., 1965). Degradation products are found within 5 min of addition of isotope to leucocytes, and are formed at a rate proportional to the thymidine concentration. Granulocytes are much more active than lymphocytes in this respect, but the

degradative activity of lymphocytes is increased after stimulation. Such degradation probably accounts for the very low thymidine incorporation found by some workers who have added isotope at the beginning of culture, particularly if the cultures contain granulocytes. It is probably of only minor importance when the isotope is added for short pulses late in culture.

The second difficulty is that thymidine is not a normal precursor for lymphocyte DNA synthesis. Any present in the medium at the beginning of culture would be destroyed, and thymidine nucleotides will normally be synthesized at the nucleotide level from ribonucleotides. This clearly raises the possibility that the rate limiting step in the incorporation of thymidine into DNA could be the uptake or phosphorylation of the thymidine rather than the rate of DNA synthesis. The rate of thymidine incorporation by lymphocytes stimulated in a variety of ways increases with the concentration of thymidine added, and saturation occurs only at thymidine concentrations of about 20 µg/ml (Hartog et al., 1967; Schellekens and Eijssvoogel, 1968; Bain, 1970; Sample and Chretien, 1971). At such concentrations endogenous synthesis of thymidine nucleotides is probably completely suppressed but saturation can be fully maintained for only about 4 hr (Sample and Chretien, 1971). Very high thymidine concentrations inhibit DNA synthesis (Hartog et al., 1967) by interfering with the synthesis of other deoxyribonucleotides.

Most workers have used very much lower thymidine concentrations. Addition, for example, of 1 µCi/ml of ^3H -thymidine at a specific activity of 5 Ci/mmol gives a thymidine concentration of less than 0.1 µg/ml. Under these conditions most of the thymidine nucleotides entering DNA are derived from the endogenous pathway, and the relative rates of ^3H -thymidine incorporation into DNA by different cultures will only indicate their relative rates of DNA synthesis if the proportion of nucleotides entering DNA derived from the labelled thymidine is constant. While this would seem a dangerous assumption, there is considerable empirical evidence that the rate of thymidine incorporation does correlate quite well with other parameters of lymphocyte stimulation. At these low thymidine concentrations incorporation is linear for only a few hours (Bain, 1970). The subsequent decrease is probably due in part to degradation of the isotope, and in part to radiation damage to the cells. Both these effects are likely to be more serious for more highly stimulated cells, and thus mask rather than exaggerate differences between cultures. At very low thymidine concentrations, below 0.02 µg/ml, incorporation into DNA is disproportionately

reduced, indicating a threshold concentration needed for the utilization of exogenous thymidine.

In summary, DNA synthesis can be determined most reliably from the theoretical viewpoint by measurement of the incorporation of high concentrations of thymidine during short pulses. Raising the specific activity of the isotope or increasing the labelling time with low specific activity isotope increases the sensitivity of determination, and seems to be justified empirically. Experiments in which the incorporation of high specific activity isotope is determined over long periods, in which very low thymidine concentrations are used, or in which the isotope is added at the beginning of culture, should be regarded with more suspicion.

14.2. The cell cycle in activated lymphocytes

A great many factors can affect the onset, extent and duration of DNA synthesis during stimulation of lymphocyte cultures. The main factors involved are the nature, concentration and metabolism of the stimulant used, the source and prior history of the responding cells, and the culture conditions. With this number of variables, some of them difficult to control, it is scarcely surprising that there is only a general similarity between the changes in rates of DNA synthesis during culture seen by different workers. This section is not intended to review the results obtained by different groups, but to establish the main factors responsible for the variation.

14.2.1. The effect of nature and dose of stimulant on the onset of DNA synthesis

Maximal DNA synthesis occurs more rapidly in response to stimulants affecting a high proportion of the lymphocytes than to those initially affecting only a small proportion. Thus, after the addition of PHA DNA synthesis begins after about 24 hr, and is usually found to reach a maximum at about 72 hr, dying away at later times. Other stimulants affecting a high proportion of the lymphocytes such as staphylococcal filtrate, con A or pokeweed produce maximum DNA synthesis after 3–5 days, while weak stimulants such as antigens and allogeneic lymphocytes, produce maxima after 5–7 days or later. When the effects of different concentrations of strong stimulants are studied, suboptimal concentrations are generally found to give a delayed response, as judged by peak DNA synthesis (Ling and Holt,

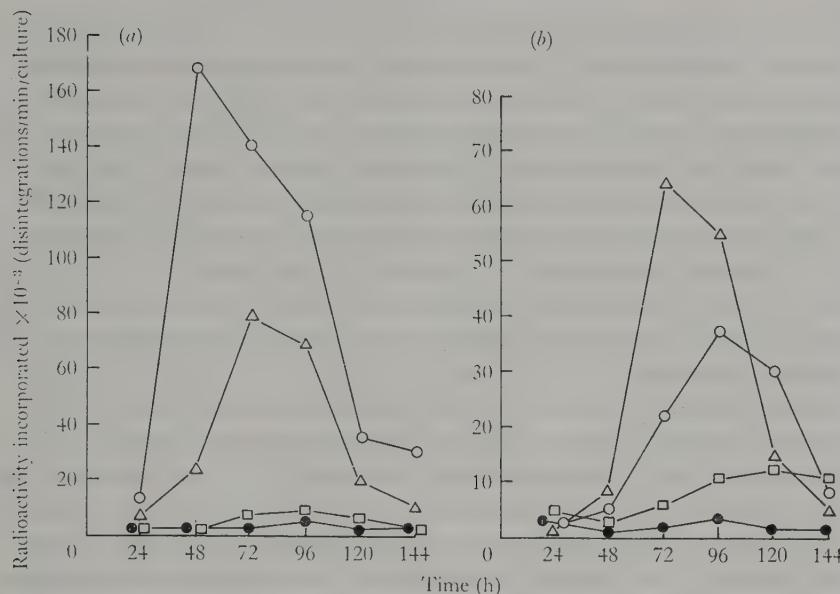


Fig. 14.1. Time-dose response curves for pig lymphocytes stimulated with PHA (a) or staphylococcal filtrate (b). Stimulant was added at the beginning of culture and ^3H -thymidine ($0.5 \mu\text{Ci}/\text{ml}$; $150 \text{ mCi}/\text{mmole}$) 24 hr before harvesting. Cultures were incubated without stimulant (●), with crude Burroughs Wellcome PHA at $100 \mu\text{g}/\text{ml}$ (○), $10 \mu\text{g}/\text{ml}$ (△) or $1 \mu\text{g}/\text{ml}$ (□) or staphylococcal filtrate at a dilution of 1:10 (△), 1:100 (○) or 1:1000 (□). Taken from Hardy and Ling (1973).

1967; Robbins and Levis, 1970). Typical results are shown in fig. 14.1. Delayed responses have also been found when lymphocytes were incubated with PHA at very low cell concentrations (Robbins and Levis, 1970).

A number of explanations can be advanced to account for these different time courses of DNA synthesis. The simplest is that the time taken for a lymphocyte to begin to synthesize DNA depends on the strength of the stimulus it receives which in turn will depend on the number of molecules of mitogen bound to its surface receptors and on the nature of the mitogen. Other possibilities are that the stronger stimulants, and higher stimulant concentrations may be more toxic to lymphocytes, or that they may lead to more rapid depletion of the culture medium. There have been a number of reports of beneficial effects of changing all or part of the culture medium, and it has also been claimed that the first mitoses in antigen-stimulated cultures occur as early as in cultures stimulated by PHA, but that subsequent

replication in the antigen-stimulated cultures was much more rapid and extensive (Marshall et al., 1969). However, it has been shown that under the conditions of the experiment shown in fig. 14.1 there were no significant differences in viability in cultures incubated with different concentrations of PHA or staphylococcal filtrate, and that the differences were not reduced if the medium was replaced at intervals during culture (Hardy and Ling, 1973). The conclusion that the time taken for lymphocytes to enter DNA synthesis is a function of the stimulant concentration is supported by direct determinations of the time taken for individual lymphocytes to accumulate at the G₁/S boundary in cultures stimulated by different PHA concentrations (Jasinska et al., 1970).

The mechanism of this dose-dependent response to mitogens is as yet unknown. The interaction of weaker stimulants, or lower concentrations of stimulants with lymphocytes may be slower (§ 12.2.2), and may be dependent on lymphocyte-macrophage interactions (§ 12.1.2), either of which could delay the response. It is conceivable that under these conditions only a population of lymphocytes capable of a slow response to mitogens would be activated, while another population, capable of a more rapid response, would not be affected. However, the most likely explanation is that the response of individual cells to activation is not an all-or-none phenomenon, but that weaker stimulation will result in a more extended G₁ period.

One practical conclusion is that when comparing the responses to different stimulants or even to different concentrations of the same stimulant, it is important to be aware of the stage of the response being studied. For example, after 7 days in culture an antigen stimulated culture near its peak might synthesize more DNA than a declining PHA stimulated culture. It would, however, be rash to conclude that the antigen was the stronger stimulant.

14.2.2. Heterogeneity of the response

Even with the strongest lymphocyte stimulants there are always a small number of non-responding cells, and there are clear morphological differences amongst those that do respond. A high proportion of lymphocytes respond to PHA by increased RNA and protein synthesis, but only about half initiate DNA synthesis.

The rate at which cells initiate DNA synthesis is also heterogeneous.

Some lymphocytes can be found in their first mitosis at times when others have progressed to their second or even third division (Bender and Prescott, 1962). More quantitative studies on the rates at which lymphocytes initiate DNA synthesis have been performed by Jasinska et al. (1970) who stimulated lymphocytes with PHA in media containing amethopterine and adenosine. In these conditions the synthesis of thymidine nucleotides, and thus DNA synthesis, is inhibited and the cells accumulate at the G₁/S boundary. When thymidine is added to these cultures the block is bypassed, and DNA synthesis resumes within minutes. The percentage of cells accumulated at the block can be determined by autoradiography, after a brief pulse with ³H-thymidine. Jasinska et al. (1970) found that some cells were ready to initiate DNA synthesis after 36 hr, but that the G₁ period was very heterogeneous and cells continued to reach the G₁/S boundary until at least 72 hr. In some experiments the stimulated cells reached this point in a biphasic pattern. Very similar results were obtained by Soren (1973a) using a different technique. He incubated cells with PHA in medium containing ¹⁴C-thymidine, and then shifted them to medium containing ³H-thymidine for the final 8 hr of culture. Cells initiating DNA synthesis during the 8 hr period could be identified autoradiographically as they were labelled with ³H-thymidine only. Most cells entered their first period of DNA synthesis between 48 and 72 hr after the addition of PHA, but some cells entered DNA synthesis after as little as 24 hr and others took more than 100 hr.

Studies on the effect of adding anti-PHA to lymphocytes at various times after PHA have shown that the cells become committed to DNA synthesis irrespective of the continued presence of PHA about 24 hr before DNA synthesis actually starts (Younkin, 1972). The period between the commitment and the initiation of DNA synthesis seemed to be relatively constant from cell to cell, while the time required for the commitment to occur was much more variable.

The rate at which cells reach mitosis after reversal of DNA synthesis inhibition is also rather variable, implying further heterogeneity in the time required to complete the S and G₂ phases (Steffen and Stolzmann, 1969).

14.2.3. Cell cycle time for second and subsequent rounds of DNA synthesis and cell division

Stimulated lymphocytes may pass through a number of sequential divisions. This was first shown by Bender and Prescott (1962) who gave a pulse of

^3H -thymidine to PHA stimulated cultures after 48 hr, and studied the subsequent frequency of labelled chromatids in metaphases. After 72 hr most dividing cells were in their first division since the pulse, but at 84 hr two thirds of the labelled metaphases were in their second division, as only one chromatid of each pair was labelled. At later times metaphases were found in which only a half or a quarter of the chromosomes were labelled, showing that some cells pass through at least four divisions.

The cell cycle times for second and subsequent divisions are generally much shorter than that of the first division. Few mitoses are found in PHA stimulated cells before 48 hr, but by 72 hr the majority of cells in metaphase are in their second division (Buckton and Pike, 1964; Sasaki and Norman, 1966). The cell cycle time for second and subsequent divisions in PHA stimulated cultures is generally found to be about 18–24 hr. Most workers have calculated that S phase occupies half such a cycle and that the remainder is split equally between G_1 and $G_2 + \text{mitosis}$ (Marshall and Roberts, 1965; Sasaki and Norman, 1966), but some have suggested that the post-mitotic G_1 may be very short (Soren, 1973b). Detailed reference to the durations of the different stages of the cell cycle reported by various workers may be found in Hardy and Ling (1973).

When cultures are stimulated by weaker stimulants, such as antigens, allogeneic lymphocytes or pokeweed, the cell cycle time after the first division, determined by time-lapse cinemicrography, has been found to be even lower, about 8–12 hr, and large clones of cells may be produced (Marshall et al., 1969). Similar results have been found in the rat mixed lymphocyte reaction (Wilson et al., 1968). The reduced cell cycle time compared to PHA stimulated cells may reflect the more favourable culture conditions when the proportion of responding cells is low. When the cell cycle is as short as this the response seen after several days in culture could be accounted for by the repeated division of a very small proportion of the initial cell population.

The decrease in cell cycle time seen in second and subsequent divisions must be due very largely to a decrease in the G_1 period, although the very short cell cycle times seen after antigenic stimulation must also involve the reduction of other phases. The 8–24 hr division cycle is comparable to the cycles found in rapidly dividing cell lines of human origin, while the extended G_1 periods for the first division are generally found when cell division is activated in non-dividing animal cells.

14.2.4. Restimulation of lymphocytes which have recovered from a first stimulus

When lymphocytes are exposed to PHA or staphylococcal filtrate for 16 hr, and then cultured in medium free of stimulant, a peak of DNA synthesis occurs after 3–4 days. The rate of DNA synthesis then falls to near resting levels. Parallel morphological changes also occur, so that after 6–7 days many of the cells in culture resemble small lymphocytes, although they have a higher dry mass than unstimulated lymphocytes (Steffen and Sören, 1968) and higher levels of at least one enzyme, acid phosphatase (Polgar et al., 1972). When such cultures were exposed to a second stimulant, a further increase in DNA synthesis occurred (Ling and Holt, 1967). This second increase occurred much more rapidly, and was more marked than in cells not previously exposed to stimulant (fig. 14.2). It appeared to be immaterial

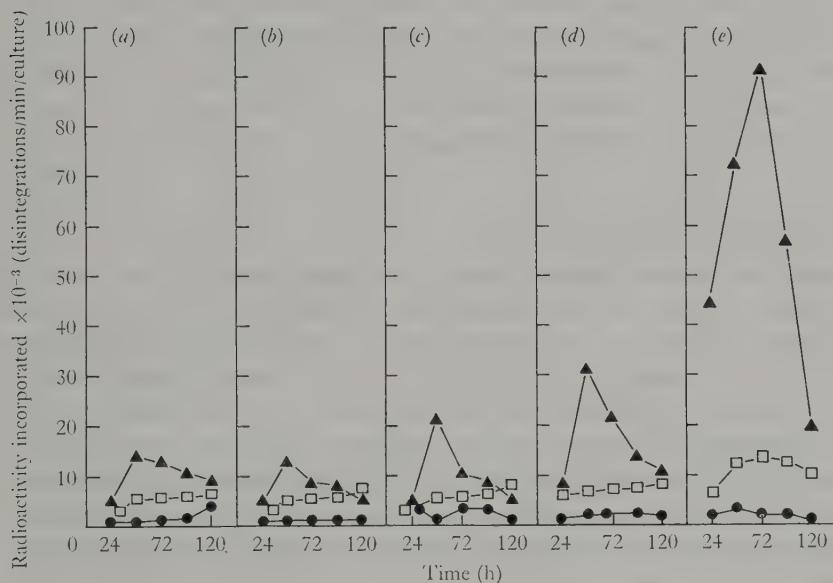


Fig. 14.2. Restimulation of pig lymphocytes pulsed without PHA (a), or with semi-purified Burroughs Wellcome PHA at 0.02 µg/ml (b); 0.1 µg/ml (c); 0.5 µg/ml (d) or 2.0 µg/ml (e). PHA was removed after 16 hr, and cultures incubated for a total of 5 days. Incubation was then continued for the time shown either without additional stimulant (●) or with optimal stimulating doses of PHA (▲) or staphylococcal filtrate (□). ^3H -thymidine (0.5 µCi/ml; 150 mCi/mmol) was added 24 hr before harvesting. Taken from Hardy and Ling (1973).

whether or not the first and second stimulants were identical, and even responses to antigens or allogeneic lymphocytes were accelerated by prior non-specific stimulation. Significant augmentation of the second response could occur even if the concentration of the primary stimulant used was too low to cause much DNA synthesis itself. The increase in the rate of rRNA synthesis after activation (§ 13.1.5) also occurred more rapidly than usual after restimulation (Cooper, 1969). There thus appear to be clear functional differences between lymphocytes which have been stimulated recently and those which have not, even though, morphologically, they may appear quite similar.

It has also been shown that those rat lymphocytes which have divided most recently *in vivo* respond most readily to PHA or pokeweed *in vitro* (Stayner and Schwarz, 1969), while rabbit spleen and guinea pig lymph node cells from animals killed after a booster injection of antigen give a greater response to PHA than those of unimmunized animals (Harris and Littleton, 1966; Eurenus and McIntyre, 1970). The previous history of the lymphocytes is clearly of considerable importance in determining the rate at which they respond to stimulation, and may be the main explanation for the heterogeneity of the initial G₁ period (§ 14.2.2).

14.3. DNA synthesis

The nature of the signal for the initiation of DNA synthesis in mammalian cells is quite unknown, but several lines of evidence suggest that it may be a cytoplasmic function. Transplantation of normally non-dividing frog brain nuclei into fertilized eggs results in their initiating DNA synthesis just as the egg nucleus would (Gurdon and Brown, 1965) while fusion of lymphocytes with dividing HeLa cells results in the initiation of DNA synthesis in the lymphocyte nucleus in the resulting heterokaryon (Harris et al., 1966). The slime mould *Physarum* has many nuclei in the same cytoplasm, and all initiate DNA synthesis in a highly synchronous manner (Rusch, 1969).

The initiation of DNA synthesis in non-dividing mammalian cells is generally accompanied by the induction of several of the enzymes involved in DNA synthesis and deoxyribonucleotide metabolism. While such induction may well be necessary for the initiation of DNA synthesis, and DNA synthesis can be limited experimentally by nucleotide deprivation, it is probable that this is not the normal limitation. DNA polymerase activity

remains constant throughout the cell cycle of synchronized mammalian cells, and while the activities of several of the enzymes for deoxyribonucleotide synthesis are increased during S phase, their correlation with DNA synthesis is frequently not precise, and they are often also present during G₁ and G₂ phases (for references see Turner et al., 1968).

There does seem to be a close relationship between the synthesis of ribosomes and the initiation of DNA synthesis. The initiation of DNA synthesis in nearly all non-dividing mammalian cells is preceded by an increase in the rate of ribosome synthesis. The only major exception is the very rapid cell division that follows fertilization of eggs, and here large numbers of ribosomes, synthesized and stored during oocyte maturation, are already available. The induction of DNA synthesis in several mammalian systems is prevented by low concentrations of actinomycin which are thought to inhibit rRNA synthesis quite selectively. Such low actinomycin concentrations also inhibit DNA synthesis in stimulated lymphocytes, although they have comparatively little effect on protein synthesis or morphological changes (Kay et al., 1969). The possibility that low actinomycin concentrations may have some as yet unrecognised additional effects cannot, of course, be ruled out. Whatever the cause of the inhibition it does not seem to be due to a direct effect on DNA synthesis, as DNA synthesis is inhibited only if the actinomycin is added at least 8 hr before DNA synthesis begins. DNA synthesis in animal cells is also closely linked to the synthesis of proteins, particularly the synthesis of histones, and the initiation of DNA synthesis in lymphocytes, like other mammalian cells, seems to have a requirement for Zn²⁺, which may be essential for one of the enzymes involved (Chesters, 1972).

Many compounds have been reported to inhibit DNA synthesis in stimulated lymphocytes, but most of these probably act by preventing RNA, protein or nucleotide synthesis, or by killing the cells, and give little additional information. Several studies on the induction of DNA polymerase and other enzymes associated with DNA synthesis, and on the nature of the DNA synthesized, have been carried out, and are discussed below.

14.3.1. Enzymes concerned with deoxyribonucleotide metabolism

The pools of the deoxyribonucleotides dATP and TTP have been found to be 0.2 and 0.05 pmoles/10⁶ unstimulated lymphocytes respectively but increase 20–200-fold after stimulation with PHA (Munch-Petersen et al.,

1973). This increase begins after about 20 hr and reaches a maximum at the same time as DNA synthesis. The activities of both thymidine and TMP kinases and dCMP deaminase increase, although the magnitude of the increases observed have varied from 2-fold to more than 20-fold (Wilms and Wilmanns, 1969; Loeb et al., 1970; Pegoraro and Bernengo, 1971). The activities of enzymes concerned with synthesis of other nucleotides have been studied in less detail. An increase in the activity of deoxycytidine kinase has been reported (Pegoraro and Bernengo, 1971), while the activity of dGMP kinase is much higher in unstimulated lymphocytes and does not change after stimulation (Loeb et al., 1970). In general the increases in these enzyme activities parallel the increase in DNA synthesis. Pegoraro and Bernengo (1971) found that while the activity of dCMP deaminase followed the increase in DNA synthesis closely, the increases in thymidine and deoxycytidine kinases were delayed for a further 24 hr. Similar delays can be seen in the results of some but not all other workers. Very little information is available on either the factors controlling the activities of these enzymes, or their role, if any, in the control of DNA synthesis.

14.3.2. DNA polymerase

The exact mechanism of DNA synthesis in animal cells has not yet been elucidated, but there seems little doubt that the enzyme DNA polymerase is involved in this process. Extracts of stimulated or unstimulated lymphocytes synthesize little DNA on their own, but after stimulation with PHA contain a soluble DNA polymerase that can synthesize DNA using denatured calf thymus or salmon sperm DNA as a primer (Loeb et al., 1968; Rabinowitz et al., 1969). Native calf thymus DNA is not active as a primer, but the double stranded synthetic copolymer poly dAT can be copied accurately (Loeb and Agarwal, 1971). DNA synthesis during the mixed lymphocyte reaction is also accompanied by the induction of DNA polymerase (Agarwal et al., 1970).

The increase in DNA polymerase after PHA stimulation was very closely linked to the initiation of DNA synthesis as judged by ^3H -thymidine incorporation, although the most detailed studies showed that the rise in DNA polymerase began about 2 hr earlier (Loeb et al., 1968; Loeb and Agarwal, 1971). Co-incubation of stimulated and unstimulated extracts provided no evidence for the inactivity of the unstimulated extracts being due to an inhibitor or lack of an activator. Inhibition of protein synthesis at

any time completely prevented any further increase in DNA polymerase, and the enzyme decayed with a half life of about 6 hr when protein synthesis was inhibited after induction had begun (Agarwal and Loeb, 1972). It thus seems likely that the increased activity of the enzyme is due to increased synthesis.

The increase in DNA polymerase activity, like the increase in DNA synthesis, is sensitive to even very low concentrations of actinomycin added together with or soon after PHA, but becomes resistant to such doses prior to the initiation of DNA synthesis (Agarwal and Loeb, 1972). Enzyme levels are maintained for at least 12 hr in the presence of much higher concentrations of actinomycin added after enzyme induction has occurred.

If extracts are prepared from PHA-stimulated lymphocytes at high salt concentrations they are found to contain a second DNA polymerase, DNA polymerase II, in addition to that normally extracted (Smith and Gallo, 1972). DNA polymerase II has a much lower molecular weight than DNA polymerase I, is more strongly adsorbed to phosphocellulose, is less sensitive to sulphhydryl blocking reagents, and prefers different synthetic DNA primers. Most of the DNA polymerase II is found in the nuclear fraction after homogenisation, while DNA polymerase I is found mainly in the cytoplasm. Several other types of mammalian cell have been found to contain two DNA polymerases with analogous properties. The relationship between them is uncertain, but they are antigenically cross-reactive, and may thus contain amino sequences in common in spite of their different properties. A third distinct DNA polymerase has been found in a particulate cytoplasmic fraction of PHA-stimulated, but not unstimulated lymphocytes (Bobrow et al., 1972).

The DNA polymerase activity of isolated lymphocyte nuclei using endogenous DNA as template is very much lower than the soluble DNA polymerase activity. However, this low level of nuclear DNA polymerase is also increased after lymphocyte stimulation by PHA (Lehmann and Shall, 1972). The significance of the endogenous DNA polymerase is currently unclear. It is not known which enzyme is responsible, but the low activity associated with nuclei seems much too low to account for the DNA synthesis of the intact cell. Lehmann and Shall have also shown that the activity of another enzyme which may be associated with DNA synthesis, the DNA-dependent polyADP-ribose polymerase, is increased after incubation of lymphocytes with PHA for 48 hr. While the role of this enzyme is currently obscure, there are a number of indications that the metabolism of its sub-

strate, NAD, may be related to the control of DNA synthesis (Shall, 1972).

The activities of DNase (Loeb et al., 1970) and polynucleotide ligase (Pedrini et al., 1972), two other enzymes which are probably involved in DNA replication, are also very low in unstimulated lymphocytes, but increase after stimulation with PHA. The increase in DNase occurs together with, or perhaps slightly before, the increase in DNA synthesis, but the rise in polynucleotide ligase is delayed for a further 24 hr. The exact functions of both these enzymes in replication are rather uncertain, but the delayed rise of the ligase activity would seem to limit the roles that could be ascribed to it.

14.3.3. RNA-directed DNA polymerase

Enzymes which can use RNA templates to synthesize complementary DNA were first discovered as components of RNA tumor viruses (Temin and Mizutani, 1970). Subsequent studies found such enzymes in animal cells infected with RNA tumour viruses, and Gallo et al. (1970) found RNA-directed DNA polymerase in leukaemic lymphocytes but not normal lymphocytes, even after their stimulation by PHA.

RNA-directed DNA polymerases have since been reported in both normal and PHA stimulated lymphocytes (Penner et al., 1971a, b). These authors confirmed the finding of Gallo et al. (1970) that no activity dependent on an RNA template could be detected in crude extracts of lymphocytes, but found that activity could be detected after fractionation of the extracts by electrofocussing. This enzyme was completely separated from the DNA-directed DNA polymerase, and was active with either yeast RNA or the synthetic co-polymer poly rA.rU as primer but not with calf thymus DNA. The RNA strand of the mixed DNA-RNA copolymer poly rA.dT can be copied about 20 times more effectively, and the changes in this activity in crude extracts during the course of stimulation by PHA have been determined (Penner et al., 1971b). The activity began to increase well before the initiation of DNA synthesis, and reached a maximum of 4–10 times the unstimulated level. It did not appear to change in parallel with the changes in DNA polymerase activity, and even at the peak of activity did not exceed 3% of the DNA-directed activity. However, it has since become apparent that normal cellular DNA polymerases are able to copy the RNA strand of poly rA.dT at low efficiency, and under certain conditions they may also use poly rA.rU or the poly A regions of single stranded RNA as a template

(Smith and Gallo, 1973). No normal lymphocyte RNA-directed DNA polymerase has yet been shown to satisfy the most stringent criterion, the ability to copy the heteropolymeric regions of single stranded RNA.

14.3.4. Nature of the DNA synthesized

It is generally assumed that the DNA synthesis observed after activation of lymphocytes by mitogens is due simply to the normal replication of the chromosomal DNA required for mitosis. There is a great deal of evidence to support this view, in that the incorporation of DNA precursors is restricted to those cells with DNA levels between the $2n$ and $4n$ values (§ 14.1), and that the labelled cells pass through mitosis soon afterwards. However, there have been a few observations which have suggested that DNA synthesis might not be quite so straightforward as is generally assumed.

Newly synthesized DNA can be separated from other DNA if cells are grown in the presence of bromodeoxyuridine. This nucleoside analogue is incorporated into DNA in place of thymidine, and DNA containing it has a higher density than normal DNA and can be separated from it by centrifugation to equilibrium in caesium chloride density gradients. Analysis of the DNA synthesized by activated lymph node cells showed that the DNA synthesized after addition of PHA behaved as if it were the product of normal semi-conservative replication, containing one strand of old DNA and one strand of newly synthesized DNA, but that the DNA synthesized after antigenic stimulation also contained another, less dense, component (Souleil and Panijel, 1970). This lighter component was also observed by Gottlieb et al. (1970), but their finding that such a component was also present in PHA-stimulated cells has been shown to be due to contamination of their cultures by antigen used to prime the lymph node cells *in vivo* (Souleil and Panijel, 1973).

The nature of this peak of bromodeoxyuridine-substituted DNA of low density has been the subject of some discussion. In some experiments it appeared to have a density even lower than that of unsubstituted DNA. This could be due to the attachment of a low density substance, such as protein or lipid, and Gottlieb et al. (1970) proposed that it was DNA complexed with lipid on the basis of its conversion to the normal hybrid form by phospholipase A. However, this hypothesis was rejected by Souleil and Panijel (1972) who found that the low-density DNA containing bromodeoxyuridine was not affected by incubation with pronase or phospholipase, or by

detergents. They found that the low-density DNA was of higher than average molecular weight, and that it could be converted to the normal hybrid form by sonication, and suggested that it was composed of end-to-end joining of labelled and unlabelled segments of DNA. They suggest that the apparent reduction in density below the value for unsubstituted DNA is an artefact of the collection procedure used to sample the gradients, which would appear to reduce the density of high molecular weight DNA. The significance of this DNA is not clear. The labelling periods employed in these experiments are too long and the proportion of the low density DNA too high, for it to be likely to be a normal intermediate in DNA replication. The possibility that this DNA synthesis may be associated with differentiation induced by antigen has been suggested.

Another unexpected observation was that a high proportion of the DNA labelled with ^3H -thymidine after lymphocyte activation by specific or non-specific mitogens appears free in the culture medium within the next two or three days (Rogers et al., 1972; Sarma and Rutman, 1972). The release of the labelled DNA is not accompanied by a comparable release of unlabelled DNA. The DNA released has a molecular weight of about 10^7 , and a low density which suggests that it is complexed with protein or lipid. This could indicate a surprisingly high mortality, affecting selectively the progeny of those cells that have incorporated ^3H -thymidine, but Rogers et al. (1972) have raised the possibility that this DNA might have been synthesized for some purpose other than mitosis, and then secreted when it is no longer required.

There have also been suggestions that there may be a very low rate of DNA synthesis during the G_1 phase of lymphocyte activation (Akifjev and Aingorn, 1972). This could be detected either by autoradiography with long exposure times of cells labelled with ^3H -thymidine, or by the ability of inhibitors of DNA synthesis such as 5-aminouracil and fluorodeoxyuridine present during G_1 to induce chromosome breaks. Both of these properties were found in lymphocytes incubated with PHA but not in unstimulated lymphocytes. The sensitivity to inhibitors of this G_1 phase DNA synthesis showed that it was not due to DNA repair (§ 14.4).

14.4. DNA repair

Exposure of cells to ultraviolet or ionizing radiations causes damage to the cellular DNA, the type of damage caused depending on the type of ir-

radiation used. Ultra-violet light causes mainly the formation of cyclobutane dimers between adjacent thymidine residues, which are then excised and replaced. Excision occurs efficiently in human cells, but very much less rapidly in rodents. Ionizing radiations cause single-strand, and with a lower frequency double-strand, breaks in DNA chains, together with some base damage. Repair of single-strand breaks in DNA of many animals has been demonstrated by an increase in the molecular weight of the DNA or incubation after irradiation.

Both bacteria and animal cells are capable of incorporating nucleotides into DNA after exposure to either ultra-violet light or ionizing radiations. Such incorporation can occur outside the S phase in animal cells, and is referred to as 'unscheduled' DNA synthesis. It is thought to indicate repair or DNA damaged by irradiation, and to be the result of insertion of nucleotides into gaps in DNA left after excision of damaged regions.

At the cellular level, repair or recovery can be demonstrated in some types of animal cells by a variety of tests. The most usual method is to compare the effects of a given dose of irradiation when given as a single dose or split into two separate parts. The total number of primary molecular lesions will increase linearly with the total dose of irradiation, and be the same in each case. However, secondary effects, such as cell death or the number of chromosomal breaks, may increase more than linearly with the dose of irradiation. Repair processes occurring between the two parts of a split dose may be shown by a decreased effect of the split dose relative to the single dose. Although in some cases a correlation has been shown between repair of DNA at the molecular level and cellular recovery, the relationship between the processes at the molecular, chromosomal and cellular levels is often not straightforward. The analysis of mitotic figures in PHA stimulated lymphocyte cultures from donors irradiated months or years previously has shown that these cells may survive *in vivo* for extended periods containing irradiation-induced chromosome abnormalities which would have been lethal if the cells had previously attempted to divide (Buckton and Pike, 1964).

Unscheduled incorporation of ^3H -thymidine into DNA occurs in lymphocytes after irradiation with either ultra-violet light (Evans and Norman, 1968a) or ionizing radiations (Spiegler and Norman, 1969). In both cases incorporation of ^3H -thymidine begins immediately and is complete in about 6 hr. As in other animal cells, this incorporation is not inhibited by hydroxyurea, a potent inhibitor of normal S phase DNA synthesis, but

has the kinetics of an enzymic process. Spiegler and Norman (1970) have calculated that the limiting enzyme for unscheduled DNA synthesis after irradiation with ionizing radiations is present at a concentration of about 10^4 units/cell and that only 1-2 nucleotides are incorporated at each lesion.

After irradiation with ultra-violet light about 90% of lymphocytes show unscheduled ^3H -thymidine incorporation. Such cultures respond normally to PHA, and all mitotic cells and 98% of the lymphoblasts are derived from those cells labelled with ^3H -thymidine (Evans and Norman, 1968b). In contrast 30% of the untransformed lymphocytes were derived from those cells not labelled after irradiation. Other cells which have lost the capacity to divide, such as granulocytes, muscle cells and chick erythrocytes, also have little or no capacity for unscheduled DNA synthesis (Darzynkiewicz, 1971). The significant correlation is with the potential of the cell for DNA synthesis rather than the actual rate, as lymphocyte stimulation by PHA results in a less than 2-fold stimulation of the rate of unscheduled DNA synthesis (Spiegler and Norman, 1969; Darzynkiewicz, 1971). If unscheduled DNA synthesis requires the enzymes involved in DNA replication, the low levels present in unstimulated lymphocytes (§ 14.3) must be adequate.

Ionizing radiations, at doses much lower than those used to provoke unscheduled DNA synthesis, give rise to chromosome aberrations which can be seen in the mitotic figures of lymphocytes subsequently exposed to PHA. Prempee and Merz (1969) concluded from split dose experiments that unstimulated lymphocytes could repair some of these aberrations. They found that such repair took 60-90 min in lymphocytes early in G_1 , but was complete in 30-60 min in cells in late S or G_2 phases. Rather similar results were found by Wolff (1972) except that he found that maximum repair required an interval of 4-5 hr between the two doses of irradiation, with either stimulated or unstimulated lymphocytes. Such results fit quite well with the observations on unscheduled DNA synthesis. However, in a very similar series of experiments Stefanescu et al. (1972) were not able to find any evidence for repair of chromosome aberrations caused by ionizing radiations in the first 20 hr after PHA addition. Repair began only shortly before the beginning of S phase. Repair of such chromosome breaks has also been reported to occur in regenerating, but not normal, mouse hepatocytes (Coggle, 1968).

Lymphocytes are unusually susceptible to irradiation or treatment with radiomimetic agents such as nitrogen mustard, but their survival is very greatly increased after stimulation with PHA or antigens (§ 10.5). Only 5%

of unstimulated lymphocytes survived for 6 days after treatment with 150 rads, while after PHA 30% survived 1200 rads for the same period. Similarly, no unstimulated lymphocytes survived treatment with 5 µg/ml nitrogen mustard, but 50% did so in PHA treated cultures. These very marked changes do not seem to correlate well with the small changes in DNA repair seen by most workers after lymphocyte stimulation.

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