

The Regulatory Role of Macrophages in Antigenic Stimulation

E. R. UNANUE

Department of Pathology, Harvard Medical School, Boston, Massachusetts

I. Introduction	95
II. Association of Macrophage-Bound Antigen with Immunogenicity	97
A. <i>In Vivo</i> Experiments	97
B. <i>In Vitro</i> Experiments	120
III. Handling of Antigen by Macrophages	128
A. Uptake of Antigen	128
B. The Immunogenic Moiety	137
IV. Macrophage-Lymphocyte Contact	147
V. Macrophages and Adjuvants	149
VI. Summary	151
References	157

I. Introduction

An optimal immune response to most antigens usually needs interaction or collaboration among three distinct cell types as is evidenced by the results of extensive research in cellular immunology during the past five years. These three cells are the B and T types of lymphocytes (i.e., B lymphocytes are those derived from the bone marrow in mammals or the bursa of Fabricius in chickens; T lymphocytes are those derived from the thymus) and the macrophages. The B and T lymphocytes have specific recognition units on their surfaces and express humoral or cell-mediated immunity, respectively. Macrophages do not elaborate antibody; they do interact with a multiplicity of antigens, catabolize, and destroy antigen and also play a helper role in the inductive process, in part, by presentation of some of the antigen. This kind of collaboration between macrophages (or their products) and lymphocytes was actually the first immunological cell-to-cell interaction recognized, preceding by a few years the discovery of the interaction between T and B cells. The present review considers the role played by macrophages in handling antigen and in regulating the response of B and T lymphocytes.

The history of the macrophage is complex. The immunological importance given to this cell has varied throughout the years. In the 1930s when our understanding of lymphocytes and antibody formation was poor, it was thought that macrophages themselves were responsible for both uptake of antigen and synthesis of antibody (Sabin, 1923). As a

consequence, a great part of research in immunology employed techniques which evaluated uptake of antigen or physiology of the macrophage system. Following recognition that lymphocytes and plasma cells were the cells involved in antibody responses, the interest in macrophages dwindled for a time only to be revived by Fishman and Adler's provocative observations in the early 1960s (Fishman, 1961; Fishman and Adler, 1963a,b). These authors demonstrated that under certain conditions antibody responses by lymphocytes did not occur upon exposure to antigen alone but rather upon interaction with extracts of macrophages which had previously phagocytized the antigen. These early experiments were interpreted as denoting that a special processing of antigen by macrophages was an essential step in immune recognition. The macrophage again became the center of attention but only for a short time. Later, as more experimental data became available, it was clear to many that this early interpretation might not be entirely correct. Subsequent experiments have shown that lymphocytes do interact with antigens bound to macrophages but that this interaction on occasion may not be absolutely necessary and, moreover, may not involve processing of antigen molecules. During recent years the interactions between T and B lymphocytes and antigen have concerned immunologists. We now better understand how lymphocytes interact with antigens and the nature and the state of immunogenic molecules. Although all the facts about macrophages and lymphocytes are not available and will not be for some time, our knowledge of the physiology of these cells is, nevertheless, growing, and the role of the macrophage in the inductive state of immunity may now be placed in a better perspective. Apart from its role in the induction of immunity, the macrophage has held the interest of those studying delayed hypersensitivities (or cell-mediated immunities) (Benacerraf and Green, 1969). We now recognize the macrophage as one of the cells involved in the nonspecific components of these reactions, playing an essential role in resistance to some bacterial and viral infections (Mackaness and Blanden, 1967).

This review will focus on the role of the macrophage in removing antigen from extracellular fluids, degrading the larger part of this antigen while presenting a small part of it in persisting immunogenic form. This handling of antigen is done without contributing to the specificity of the immune response which is determined by the antigen-reactive T and B lymphocytes. During the process of uptake of antigen, macrophages appear to retain a few molecules of antigen, undegraded or with few chemical changes. Macrophage-associated antigen becomes an effective immunogenic stimulus mainly in conditions that require that two types of lymphocytes meet with antigen molecules. These lympho-

cytes are specifically antigen committed and few in number. (Perhaps with some antigens, the molecules need to be partially degraded in order to "allow" them to trigger lymphocytes.) The positive immunogenic role of macrophages is related to their capacity to (1) remove extracellular antigen which might be capable of interacting with and eliminating isolated T or B lymphocytes and (2) retain antigen in lymphoid tissues and promote its necessary meeting with both T and B cells. The importance of the regulatory role of macrophages depends upon the need for concentration of antigen molecules, a need which is conditioned to number of T and B cells, requirements for T cells, physiochemistry of the antigen, etc. Other interpretations of the role of macrophages have been published (Fishman and Adler, 1970; R. S. Schwartz *et al.*, 1970; Perkins, 1970; Cohn, 1968).

II. Association of Macrophage-Bound Antigen with Immunogenicity

The role of macrophages in the induction of an immune response has been evaluated *in vivo* and *in vitro* in a wide variety of experiments. Among the *in vivo* procedures are included those experiments that involve either modifying the antigen so as to make it more-or-less phagocytizable by the macrophage system or, alternatively, modifying or altering the macrophage or reticuloendothelial system (RES) in order to reduce the amount of antigen taken up. Also analyzed in this section are those experiments that involve isolation and transfer of macrophages with bound antigen as well as attempts to sensitize lymphocytes *in vitro* upon contact with macrophages. Tissue culture experimentation involves mainly the study of the proliferative or antibody response of lymphocytes to antigen in the presence or absence of macrophages.

A. *In Vivo* EXPERIMENTS

1. *Fate of Antigen in Vivo*

For several years immunologists have attempted to follow antigen in experimental animals. One approach used was to label the antigen with isotopes, such as ^{125}I , ^{35}S , or ^{32}P , and, subsequently to study the tissue extracts for the presence or absence of labeled material and/or immunogenic moieties. The latter was investigated by immunizing nonimmune or boosting immune animals with the tissue extracts. These early approaches, of the 1950s and early 1960s, have been reviewed (Humphrey, 1960; Campbell and Garvey, 1961, 1963). The main information from these extensive studies is that small immunogenic moieties could be extracted from tissues many weeks and months after the initial injection of antigen. Campbell and Garvey identified small fragments of antigen

which they believed were associated with ribonucleic acid (RNA). Yet, information on the localization of the antigen and its role *in vivo* was not elucidated by these studies. A better means to follow antigen *in vivo* utilized fluorescent antibody (Kaplan *et al.*, 1950) or radioautography. The fluorescent method, although giving precise histological localization, was not sensitive enough for the detection of only a few molecules of antigen. The method of choice then has been to use proteins tagged with radioiodine ^{125}I at a high specific activity. This method has been used extensively and successfully by Nossal, Ada, and collaborators at both the light and electron microscopic levels. The work of the Australian group has been recently summarized in a book to which the reader is referred for a detailed analysis of their results (Nossal and Ada, 1971). The usual method is to inject antigen labeled with carrier-free ^{125}I at high specific activity (Greenwood *et al.*, 1963; McConahey and Dixon, 1966) and to trace it in the lymph node or spleen, hours or days after local or intravenous injection, respectively. The tissues can be processed for radioautography and/or immunofluorescence (in order to investigate for antibody) at the same time. The latter procedure enables us to locate the labeled antigen and indicates the presence of immunoglobulin (Ig) either extracellularly or associated with cells (McDevitt *et al.*, 1966). Fates of a synthetic polypeptide labeled externally with ^{125}I and internally with tritium were compared and gave similar results, indicating the validity of the external label (Humphrey *et al.*, 1967). It is clear that radioautography, although showing precise histological localization, does not yield quantitative information. Some quantitative aspects of radioautography are discussed in a paper by Ada *et al.* (1966). Still, identification of antigen in a tissue does not necessarily imply that it participates in immune induction.

With antigen tracing studies, two main areas or sites in lymph nodes where antigen becomes trapped have been identified: one is associated with typical macrophages situated in medullary areas or perifollicular zones of lymph nodes, or perifollicular or marginal zones of spleen; the second is associated with the lymphoid follicles. The characteristics of antigen trapping in these two areas appear to differ (Ada *et al.*, 1964; Nossal *et al.*, 1964; Nossal, 1967; Humphrey and Frank, 1967; Humphrey, 1969; Nossal and Ada, 1971; Hanna and Hunter, 1971).

At the first site, antigens enter into a node via afferent lymphatics and are readily taken up by the macrophages of the medulla and perifollicular areas. The experimental antigens most frequently used include albumins, hemocyanins, *Salmonella* flagellin, Ig, ferritin, and synthetic polypeptides. Ultrastructurally, flagellin- ^{125}I was readily found in association with macrophages and usually in intracellular vesicles or in

phagolysosomes (Nossal *et al.*, 1968a). The macrophages responsible for this uptake showed morphological characteristics in general similar to macrophages isolated from bone marrow or the peritoneal cavity. Of importance was the observation that injection of antigen into tolerant, unresponsive recipients led to its uptake by these macrophages to an extent comparable to that exhibited by macrophages from normal animals (Humphrey and Frank, 1967; Humphrey, 1969). Injection of autologous proteins, such as Ig, also led to their trapping by extrafollicular macrophages, although no attempts were made to exclude aggregates from the preparation of autologous Ig. The amount of antigen associated with these macrophages rapidly declined and became difficult to demonstrate after a few days (Nossal, 1967). Most likely, a great part of the antigen had been lost as a result of catabolism by the macrophage. To this effect, synthetic polypeptides made up of D-amino acids, which are degraded poorly by the enzymes of macrophages, persisted in the sinus and peri-follicular macrophages for prolonged periods of time and probably throughout the life of the experimental animal (Medlin *et al.*, 1970; Janeway and Humphrey, 1969). Uptake of antigen by macrophages was not reduced by X-irradiation (G. M. Williams, 1966b; Jaroslow and Nossal, 1966), but was greatly enhanced if the antigen was bound to antibody as an immune complex (Nossal *et al.*, 1965).

The second site of antigen uptake is the lymphoid follicles. White (1963), by using fluorescent antibody, was the first to call attention to the presence of Ig in a weblike pattern among cells of lymphoid follicles (1963). Later, Nossal, Ada, and collaborators reported on the appearance and persistence of flagellin-¹²⁵I in these structures and on the marked enhancement of the trapping mechanism in immune animals (Nossal *et al.*, 1965; Jaroslow and Nossal, 1966; Lang and Ada, 1967). Humphrey and Frank's work (1967) on follicular localization of albumins or hemocyanins in normal and tolerant rabbits has clarified our understanding of the mechanisms of localization of antigen. They found, on the one hand, that adult rabbits made tolerant by injections of antigen at birth were unable to localize antigen in the lymphoid follicles (although, as mentioned before, the medullary macrophages were perfectly capable of antigen uptake); normal rabbits, on the other hand, localized the antigen in the follicle but not until several days after its injection, at a time when antibody synthesis had started. Further studies in which radioautography was combined with fluorescent antibody methods indicated that both antigen and Ig were present on the same sites in the follicles (Balfour and Humphrey, 1966). It should be noted that early studies showed localization of flagellin-¹²⁵I in the follicles of tolerant rats (Mitchell and Nossal, 1966), but this trapping was most likely asso-

ciated with the presence of natural antibodies in the serum (G. M. Williams, 1966a). Hence, antigen must be bound with antibody in order to be localized in the lymphoid follicles. As would be expected, Ig-¹²⁵I (either autologous or foreign) injected subcutaneously could be found localized in the follicles of draining nodes (Hanna *et al.*, 1968; Herd and Ada, 1969a,b). Herd and Ada indicated that this localization appeared to be associated with the presence of the Fc portion of the Ig molecules (1969a,b). The third component of complement (C), C3, was also identified in the follicles together with antigen and Ig; furthermore, there was good evidence that C could fix *in vitro* to these areas (Gajl-Peczalska *et al.*, 1969). The localization of C in the follicles adds further proof that antigen is found at this site as part of an immune complex.

The fate of antigen in the medullary and perifollicular macrophages and in follicles is strikingly different. By ultrastructural examination it was possible to detect most of the antigen in the follicles in between closely packed cells on what appeared to be membranes of specialized dendritic-type cells (Mitchell and Abbott, 1965; Szakal and Hanna, 1967; Nossal *et al.*, 1968b; Cottier and Sordat, 1971). Another characteristic of antigen localized in the follicle was its persistence for prolonged periods of time, whereas most of the antigen associated with the extrafollicular macrophages was eliminated. Finally, in contrast to perifollicular and medullary macrophages, the antigen-trapping mechanism appeared to be sensitive to X-irradiation (G. M. Williams, 1966b; Hanna and Hunter, 1971; Nettesheim and Hammons, 1971). Table I is a summary of the antigen-trapping systems.

Two points are not yet clarified with respect to the follicular trapping system. They are (1) the kinds of cells that trap antigen and (2) the manner in which antigen or trapping cells reach the follicles.

Lymphoid follicles appear to have a complicated web of interdigitating cell processes, from lymphocytes in various stages of differentiation and nonlymphocytic cells characterized by large pale nuclei. The non-lymphoid cell has received several descriptive names: dendritic macrophage, reticulum cell, and dendritic cell. The term macrophage has been abandoned since small amount of antigen is inside the vesicles of this cell in contrast to typical macrophages. The evidence that this cell is a reticulum cell is not clear and, hence, the descriptive, noncommittal term of dendritic cell appears most appropriate. Based on observations of fluorescent antibody, White postulated that the antibody present in follicles was localized on these special "dendritic"-type cells. As previously mentioned, by combined radioautography and immunofluorescence antigen and antibody were identified at the same loci. Ultrastructural studies showed that the antigen (or the antibody) was localized in between

TABLE I
ANTIGEN-TRAPPING SYSTEMS IN LYMPH NODES AND SPLEEN

Extrafollicular	Follicular
Associated with typical macrophages	Associated with dendritic cells and perhaps with some type of lymphocytes
Many antigens are taken up without apparent need of antibody; antibody increases trapping	Antigen must be complexed to antibody (and C?) in order to bind to the follicles (foreign Ig can bind directly)
Tolerance does not affect binding of protein antigen	Tolerant animals do not trap antigen unless natural antibody is present in serum
Not affected by X-irradiation	Affected by X-irradiation
Most of the antigen is rapidly degraded—persistence of antigen is short	Antigen persists for long periods
May participate in early immune induction	May participate in immune memory
Trapping does not necessarily correlate with immunogenicity	Trapping follows early immune induction and does not insure immunogenicity

tightly packed cells. In many instances the antigen was clearly localized on the membrane of the dendritic cell in close contact to the surrounding lymphocytes. The problem in this study is the proper identification of the site of localization of the immune complex. As Nossal *et al.* carefully stated in their ultrastructural study in 1968(b), "it could not be claimed that every thin process seen in the follicles came from the reticular cell, nor was every antigen depot necessarily associated with a reticular cell process. . . . In fact in many cases where a complexity of processes was encountered, it was not possible to determine from which type of cell a particular process was derived." We now know that follicles contain B-type lymphocytes which have a membrane receptor for C3 (Bianco *et al.*, 1970) and can bind antigen-antibody complexes. Therefore, an important new problem is to determine the extent to which these lymphocytes contribute to the binding of antigen-antibody complexes in the follicles. Notwithstanding this reservation and assuming that, as expected, the dendritic cell bears most of the antigen-antibody complexes in the follicles, we must still question the nature of this cell. Is this a new type of cell differing completely from the monocyte-macrophage line while also having a membrane receptor for Ig? Or, is it possible that this cell may represent a stage of maturation or functional differentiation of monocyte-macrophages? I raise this latter possibility because of indications that monocytes and macrophages do exhibit some degree of functional changes which might make them express one function or

another to a variable extent during their differentiative process. The physiological behavior of the brain macrophage may be pertinent to keep in mind—in inflammation the microglia appears as a round cell, whereas under normal conditions it is a small cell with interdigitating processes. Similar morphological changes are seen in macrophages of the omentum (Fischer *et al.*, 1970).

The manner in which antigen (with antibody) becomes trapped in the follicles is not determined. The two obvious considerations are that (a) the antigen-antibody complexes enter as free complexes to become trapped by the cells or (b) the complexes enter already bound to the membranes of cells. White and collaborators (1967) have done a very detailed analysis of this process in the chicken spleen and are of the impression that the dendritic cells outside the arteriolar sheath trap the antigen, move along the sheath, and trap the lymphocyte to form the reactive follicle. In the mammalian spleen there is also some suggestion that antigen is taken up by the cells of the marginal zone and is carried into the follicles (Nossal *et al.*, 1966).

The association between uptake of antigen by extrafollicular macrophages or follicular cells and the immune process is not known. It is understandable that this problem must depend for its solution upon a series of indirect pieces of evidence. For example, we know that extrafollicular macrophages handle antigen in a way very similar to that of peritoneal macrophages (Ada and Lang, 1966; J. M. Williams and Ada, 1967); we also know that some antigen bound to transplanted peritoneal macrophages is immunogenic. (Some of these macrophages even localize to perifollicular areas after injection.) The logical assumption, then, is that antigen bound to extrafollicular macrophages of the spleen and lymph node should be available for immune recognition at least for some time. Unfortunately little is known about the turnover and fate of extrafollicular macrophages. Insofar as the follicular antigen-trapping system is concerned, we know that it is not necessary for immune induction. Induction of antibody precedes localization of antigen in the follicle (Humphrey and Frank, 1967). Indeed, the first histological evidence of antibody formation usually involves the perifollicular areas (Oort and Turk, 1965; Leduc *et al.*, 1968). It is also apparent that follicular localization of antigen is not a necessary condition for induction of antibody (Humphrey, 1969). It has been reasonably speculated by many that the follicular trapping of antigen may be a mechanism for maintenance of immunological memory. In order to fully understand the meaning of this process of follicular localization of antigen, information must be sought on dynamics of cells in the follicles, effects of concentration of antibody, reaction of lymphocytes to antigen-antibody-C complexes, origin of dendritic cells, etc.

2. Persistence of Immunogenicity

Potentially immunogenic molecules can persist in tissues for prolonged periods of time. Whether some or all of these molecules are associated with macrophages and the extent to which they contribute to the maintenance of a state of immunity (i.e., of memory) have not been ascertained. The experimental approaches that have been used to study persistence of immunogens are diverse. Humphrey in 1960 reviewed an early technical procedure employed during the 1950s. It consisted of making a tissue extract days or weeks after injection of antigen into one experimental animal and testing for immunogenicity by injection into a second animal. Indeed, immunogenic material from pneumococcal polysaccharide, hemocyanin, Ig, or albumins was identified in tissues several days after its injection.

A different approach consisted of transferring immune cells into animals that previously had received antigen and which were immunoincompetent (by either tolerance induction or X-irradiation). Murine lymphocytes primed to human albumin made antibody when transferred into tolerant mice that had received the antigen 10 weeks previously (Mitchison, 1965). In this system, but with X-irradiated hosts as recipients, immunogens from sheep red blood cells (SRBC) and *Escherichia coli* lipopolysaccharide were found to persist in mice for as long as 14 and 45 days, respectively (Britton *et al.*, 1968). However, the immunogenicity of human albumin was short-lived, about 17½ hours (Britton and Celada, 1968). These experiments, therefore, indicated that potential immunogens persisted although their quantity varied from antigen to antigen.

Is the persisting immunogen necessary for the production of immune memory? There have not been many experiments designed to answer this important question. The evidence suggests that the initial interaction with antigen leads to a population of long-lived, memory lymphocytes that do not necessarily require continuous antigenic stimulation (Celada, 1967). Nevertheless, the retained immunogen may add to an optimal state of low active immunity or of immune memory. Celada (1967) transferred lymphocytes from immune mice into X-irradiated recipients and challenged these with antigen at periods of up to 6 months after transfer. Because his dose-response system was well-calibrated and sensitive, he estimated that the capacity of the transferred cells to mount a secondary response decayed in two phases: during the first month with a half-life of 15 days, and thereafter of 100 days. The reader should recall that the decline of immunogenicity of the same antigen *in vivo* was 17½ hours. Hence, the memory response to albumin did not appear to be dependent upon the presence of retained antigen.

The well-known fact that antibody can block the immune response at the level of the antigenic stimulus has been used to estimate whether persisting immunogen may be active *in vivo*. Britton and Möller (1968) observed an interesting cyclical fluctuation in the number of antibody-synthesizing cells to *E. coli* lipopolysaccharide in mice for as long as 50 days. The phenomenon was explained by feedback suppression of the response by antibody, implying that antigen persisted throughout the observed period of time and became periodically available for immune recognition. Graf and Uhr (1969) injected rabbits with bovine albumin. Two to 3 weeks later the rabbits were bled; the antialbumin antibody was removed from the plasma by a special method, and the absorbed plasma was reinfused into the rabbits. This resulted in exclusive removal of the specific antibody. Subsequently, the level of antialbumin antibody rose in the circulation. The interpretation was that the antibody in the serum was, in fact, playing a homeostatic, inhibitory role, and once removed, immunogenic material again became available for immune stimulation. The induction of optimal immune memory to hemocyanin required the persistence of antigen for at least 3 to 4 weeks as also judged by effects of passive antibody (Cerottini and Trnka, 1970).

The site where immunogenic antigen persists has not been determined. Perhaps this immunogen is on the dendritic cells of the follicles or on typical extrafollicular macrophages or even is associated with other cells. Immunogens could be shown to persist in macrophages for at least 3 weeks by the use of the macrophage transfer system (Unanue and Askonas, 1968b). Cells containing antigen could be isolated from spleens of mice as long as 7 months after antigen injection (Mitchison, 1969a).

In summary, there is strong evidence that a small amount of immunogen can persist for prolonged periods of time in tissues. Its availability to immunocompetent cells may depend upon the level of extracellular antibody and, perhaps, anatomical factors. The exact locus of the antigen is not known, although there is a possibility that it could be associated with RES cells. The persisting immunogen, although not entirely necessary, may contribute to the maintenance of memory; it persists in extremely small amounts, probably of the order of 0.001% or less of the injected dose (McConahey *et al.*, 1968).

3. Immune Response to Antigens Differing in Their Degree of Phagocytosis

There is an association between the degree of uptake of antigen by macrophages of the lymphoid organs and immunogenicity. Antigens, such as red cells and bacteria, trigger strong immune responses with

ease, while soluble proteins or polysaccharides trigger poor immune states. Experiments that show the clearest relationship between uptake of antigen by macrophages and immunogenicity are those that employ as antigens proteins differing in their state of polymerization (Dresser, 1962; Claman, 1963; Frei *et al.*, 1965; Biro and Garcia, 1965; Spiegelberg and Weigle, 1967; Golub and Weigle, 1969). The two proteins most commonly employed are albumins and Ig. Solutions of albumins or Ig are made up of monomers and a variable quantity of polymers of differing sizes. When injected into an animal, the monomers are poorly taken up by the RES and circulate for some time; the polymers are immediately taken up and disappear from the circulation. Monomers can be obtained free of polymers either by ultracentrifugation (Dresser, 1962; Claman, 1963) or by biological filtration (Frei *et al.*, 1965; Golub and Weigle, 1969). Biological filtration consists of injecting the solution containing a mixture of monomers and polymers into an experimental animal which is exsanguinated some hours later. The serum contains the monomers inasmuch as the polymers have been removed from the circulation by the RES. For experimental purposes, polymers are readily obtained by aggregation of the protein by either physical or chemical means. There is general agreement that removal of the polymers from a solution of foreign albumin or Ig by ultracentrifugation (Dresser, 1962; Claman, 1963) or biological filtration (Frei *et al.*, 1965; Golub and Weigle, 1969) results in a significant loss of the immunogenic potency of these proteins. Furthermore, one or several injections of albumins or Ig in their monomeric form may lead to a state of tolerance (Dresser, 1962; Frei *et al.*, 1965; Biro and Garcia, 1965; Dresser and Mitchison, 1968; Golub and Weigle, 1969). In contrast, the polymeric form of albumin or Ig is highly immunogenic. Some commercial preparations of albumins are contaminated with endotoxin (Dvorak and Bast, 1970) and are unsuitable for evaluating immunogenicity, inasmuch as endotoxins are powerful immunological adjuvants. Yet, the higher immunogenicity of polymers cannot be accounted for by contamination with endotoxin, since polymers free of endotoxin are themselves highly immunogenic (Fauci *et al.*, 1971; Schmidtke and Unanue, 1971a). These observations led to the "direct access" theory of tolerance, i.e., the interaction of lymphocytes "directly" with monomers produces immunological paralysis, whereas the interaction after macrophage uptake produces immunity (Dresser and Mitchison, 1968). Many events apart from uptake by the RES could explain the immunogenicity of polymers and monomers. It is possible that aggregated moieties trapped in lymphoid tissues not necessarily by the RES may be the best form of antigen and at the best site to trigger a lymphocyte to proliferate and differentiate. Perhaps the mere

fact that these aggregates are readily removed from the serum and extracellular tissues favors the immune response, since specific lymphocytes are then free to interact with antigen now concentrated in critical lymphoid organs. Nevertheless, there is direct evidence that part of the above effect can be explained by the uptake of polymeric molecules by the macrophages. This evidence derives from experiments in which macrophages containing antigens are isolated from experimental animals and transplanted into a syngeneic recipient which is then tested for its capacity to achieve an immune state. The recipient makes a strong response to some macrophage-associated antigens. The results of the experiments involving antigen bound to live macrophages are detailed in a later section.

In contrast to the above situation, there are other experiments that show an immunologically detrimental effect of macrophages. In these experiments a highly phagocytizable antigen, such as a red cell or an aggregated protein, is injected into the peritoneal cavity of normal mice or mice having a rich macrophage exudate. Such mice exhibit smaller immune responses than counterparts injected intravenously (i.v.) (where the antigen has not had contact with macrophages before its arrival in the spleen) (Perkins and Makinodan, 1964; Franzl and Morrello, 1966; Perkins, 1970). Moreover, if before intraperitoneal (i.p.) injection of antigen, a particle capable of blocking the RES is injected i.p., the immune response is restored. In these experiments there is a direct relationship between the amount of radiolabeled red cell or aggregated Ig trapped in the spleen after injection i.v. or i.p. and the degree of immunogenicity—those mice injected with antigen i.v. or i.p. into a cavity with blockade of the RES had about ten to twenty-fold more antigen in their spleens than mice injected i.p. without RES blockade. These experiments clearly indicate that (1) depriving a lymphoid organ of antigen results in poor immunogenicity, and (2) local peritoneal macrophages can degrade antigens and reduce the amount of effective immunogenic stimulus to reach lymphoid sites. The catabolic function (or negative immunogenic function) of macrophages is readily demonstrable under these conditions. These experiments do not rule out that in the spleen at least part of the response could be regulated or associated with the antigen taken up by the local RES. Still, since local lymphoid macrophages are in a better anatomical relationship with lymphocytes than are peritoneal macrophages, the former would be more suitable for helping in immune induction (aside from catabolizing antigen). In contrast, local peritoneal macrophages have little chance for interaction with lymphoid cells, since there are relatively few lymphocytes in a normal peritoneal cavity. An alternative and not mu-

tually exclusive explanation is that direct interaction with some antigens and splenic lymphocytes results in immune induction. The above experiments gave different results with antigens that were poorly taken up by macrophages. Injection of poorly phagocytizable antigens into the peritoneal cavity of mice with a rich macrophage exudate results in immune responses comparable or higher than those obtained in mice injected i.v. (Unanue, 1968a). In this case, free antigen flowing directly into the spleen is taken up very poorly, and the presumed arrival into the spleen of peripheral macrophages with antigens, although small in number, results in enhanced immunity. It is reasonable to conclude that many antigens taken up by macrophages at nonlymphoid sites, such as the peritoneal cavity or liver, may be lost from interacting with lymphocytes because the macrophages destroy the antigen, and the macrophage-associated antigen that is not destroyed has little opportunity at these sites to interact with immunocompetent elements. Hence, the helper role of macrophages (or positive immunogenic function) should be sought mainly among macrophages in intimate association with lymphoid elements such as in spleen or local lymph nodes.

4. RES Blockade

There have been numerous attempts to study immune responses to antigen administered at the time of an RES blockade (Gay and Clark, 1924; Cannon *et al.*, 1929; Stern *et al.*, 1955; Cruchaud, 1968, Sabet and Friedman, 1969). Reticuloendothelial blockade consists of saturation of the RES as a result of overloading with a given colloid and is measured by estimating the rate of elimination of a test colloidal suspension from the circulation. The extent and duration of blocking depend upon the dose of colloid administered. Colloidal carbon which has been used frequently produces RES blockade for up to 3 days. Other colloids, such as saccharated iron oxide depress for only a few hours (Benacerraf *et al.*, 1956) and are followed by a hyperphagocytic phase. Blockade of the RES is never complete. Also large doses of some of the materials injected may be "toxic" for experimental animals. For these reasons, it is obvious that many studies of the immune response during RES blockade are of dubious value and are difficult to interpret conclusively. In some instances, RES blockade has even resulted in increased antibody formation (Lewis, 1954; C. R. Jenkin *et al.*, 1965; Fisher, 1966). Recently, Friedman and collaborators have carefully examined the immune response to SRBC in mice that have received injections of colloidal carbon (Sabet and Friedman, 1969; Sabet *et al.*, 1969) or ethyl stereate (Melnick and Friedman, 1969). Their experiments show quite clearly that a significant depression in the number of antibody-forming cells

can be consistently obtained if the blocking agents are given usually a day or two before injection of antigen.

Injection of carageenan, a high molecular weight polygalactose specifically cytotoxic for macrophages, can result in depression of delayed hypersensitivity in sensitized guinea pigs (H. J. Schwartz and Leskowitz, 1969). More important in the context of our review is that injection of antigen and carageenan into unsensitized guinea pigs led to depressed cell-mediated reactions (H. J. Schwartz, 1971).

5. Use of Antimacrophage Serum

Attempts to use antibodies against a cell line in order to evaluate its role in a given phenomenon date as far back as 1899 when Metchnikoff proposed to use antimacrophage serum (AMS). However, it was not until the past two decades that the use of specific antibodies to cells became more popular, probably as a result of Humphrey's successful experiments (1955a,b) in which he showed that Arthus reactions in guinea pigs depleted of circulating leukocytes by antipolymorphonuclear leukocyte sera were markedly reduced. In this way it was established that the infiltration by polymorphonuclear leukocytes was essential in the production of the inflammatory reaction. Heterologous antibodies to polymorphonuclear leukocytes (Humphrey, 1955a,b; Cochrane *et al.*, 1965), mast cells (Valentine *et al.*, 1967), and lymphocytes (Lance, 1970) have been used quite extensively. Heterologous antibodies specific for macrophages have been obtained now and used to study different aspects of the function of these cells.

Macrophages have specific, surface antigenic determinants identifiable by their reaction with heterologous antibodies. Thus, for example, antibodies made in rabbits against mouse macrophages react only with macrophages and spare lymphocytes and hematopoietic cells (Cayeux *et al.*, 1966; Unanue, 1968b; Despont and Cruchaud, 1969). In order to obtain antibodies to macrophages, experimental animals have been immunized with purified or semipurified suspensions of macrophages (usually peritoneal or alveolar) either lightly, i.e., with relatively few cells and for a few times, or strongly (Boros and Warren, 1971), i.e., with large number of cells, several times, and usually associated with strong adjuvants. In the former cases, selected antisera can be shown to react with macrophages exclusively; in the latter cases, all antisera have to be absorbed first with lymphocytes in order to make them specific for macrophages. The specificity of AMS has been tested by agglutination, cytotoxic, or immunofluorescent tests (Fig. 1). By each of these methods, appropriate antibodies can be shown to bind to macrophage membranes and to kill the cell in the presence of C. Lymphocytes are

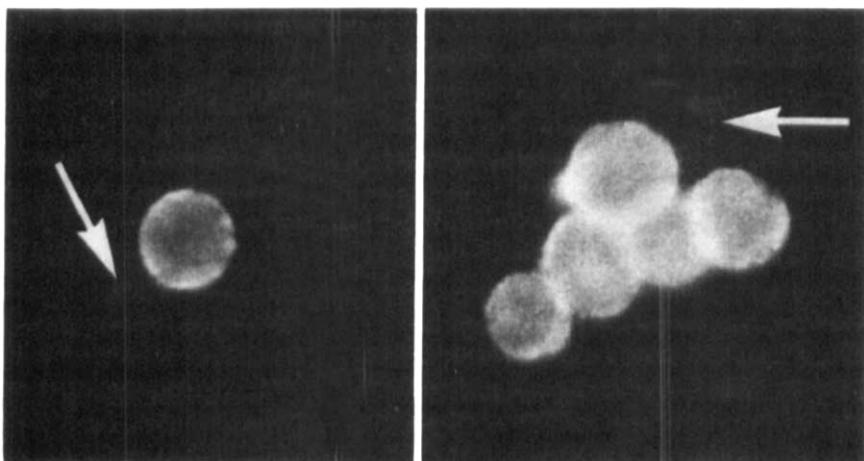


FIG. 1. Photomicrograph of peritoneal exudate cells after reaction with anti-macrophage serum (AMS). Guinea pig cells were incubated first with rabbit anti-guinea-pig macrophage serum and second with fluorescein-conjugated sheep anti-rabbit IgG. The macrophage reaction is strongly positive, whereas that of the lymphocytes (arrows) is negative. The photomicrograph has been overexposed in order to bring out the faint negative lymphocyte. (From J. D. Feldman and Unanue, 1971, p. 269.)

not killed by specific AMS indicating that either macrophages contain specific surface determinants or, alternatively, that the lymphocytes have the antigens but in amounts and/or spatial distributions below the level of detection or C fixation. Cohn and Parks (1967) reported the presence of natural macroglobulin antibodies in bovine sera directed against mouse macrophages and erythrocytes.

The effects of antimacrophage antibodies were studied both *in vitro* and *in vivo*. Macrophages treated *in vitro* with the antibodies (in the absence of C) adhered poorly to glass (Unanue, 1968b; Hirsch *et al.*, 1969) and showed reduced uptake of some particulate (Unanue, 1968b; Hirsch *et al.*, 1969; Jennings and Hughes, 1969; Argyris and Plotkin, 1969) and soluble antigens (Unanue, 1968b). This blocking of the uptake probably takes place by direct or indirect interference by AMS with the site on the cell surface to which the antigens bind. Macrophages cultured for several days in the presence of bovine sera containing AMS activity had increased pinocytic activity, increased levels of acid hydrolases, and larger numbers of lysosomes (Cohn and Parks, 1967).

Experimental animals were injected with AMS i.v., i.p., or subcutaneously. Injection of AMS into the peritoneal cavity produced rapid agglutination and/or death of the local macrophage population (Unanue,

1968b, Boros and Warren, 1971). This effect, as expected, was transitory, and by a day or two after injection of AMS, the peritoneal cavity contained newly formed macrophages. Macrophages with nuclear aberrations were frequently seen within hours after AMS injection (Unanue, 1968b); peritoneal lymphocytes were not affected by injection of AMS (Unanue, 1968b). The i.v. injection of specific AMS led to a transitory drop in monocyte counts (Boros and Warren, 1971) but not in the number of circulating lymphocytes (Hirsch *et al.*, 1969; Boros and Warren, 1971). The phagocytic index was depressed after several injections of AMS (Hirsch *et al.*, 1969). There were few histological studies of spleen or lymph nodes following AMS injection—some congestion of splenic red pulp and death of local macrophages were reported (Unanue, 1968b; Muller-Hermelink and Muller-Ruchholtz, 1971).

Antimacrophage serum has been used to study three types of phenomena: (1) immune responses to a variety of antigens; (2) delayed hypersensitivity responses in sensitized animals; and (3) infectious processes. Although only the first interests us in this review, a brief analysis of the other two will be made. The use of AMS in *in vitro* immune responses will be covered in a later section.

The use of AMS for evaluating the role of macrophages in *in vivo* immune reactions has not been very rewarding. In only some instances did AMS, given at a time close to the administration of antigen, produce some depression of immunity (Argyris and Plotkin, 1969; Isa, 1971); however, the specificity of an AMS was not altogether clear. Moreover, it has been reported that injection of some batches of normal foreign sera might depress immune responses (Despont and Cruchaud, 1969). The lack of consistent immunosuppressive effects by AMS (Unanue, 1968b; Despont and Cruchaud, 1969; Panigel and Cayeux, 1968) is difficult to interpret, since no clear answer can be given on whether or not macrophages play a role in immune induction. It may well be that AMS does not reach tissue macrophages at adequate concentrations or during a sufficient period of time to block macrophage function. Along these lines, it is known that antilymphocyte serum is an effective immunosuppressant mainly because it binds and opsonizes *circulating* lymphocytes (Lance, 1970). Immune reactions not dependent on circulating T cells are not affected by antilymphocyte sera.

Antimacrophage serum has been shown to be more effective in reducing delayed hypersensitivity elicited in sensitized animals. Thus, Heise and Weiser (1970) noted a small depression of skin tuberculin reactions in guinea pigs treated 2 days earlier with AMS. J. D. Feldman and Unanue (1971) resorted to an adoptive transfer system: irradiated guinea pigs that received immune lymphocytes plus bone marrow cells (as a

source of macrophages) showed depressed skin reactions if treated with AMS. Boros and Warren (1971) showed reduced formation of immune lung granulomas in mice treated with AMS. Most likely the effectiveness of AMS is due to its capacity to reduce levels of circulating monocytes, the cell line responsible for the nonspecific exudate of cell-mediated responses. Still, the results published so far are not impressive, and, perhaps, the use of stronger AMS or of continuous injection of the antisera might produce clearer answers. One point of importance in explaining the action of AMS *in vivo* is whether AMS can bind to macrophage precursors. J. D. Feldman and Unanue (1971) noted that AMS was not binding to cells in bone marrow, the tissue containing a rich pool of precursor cells. More recently, Virolainen and collaborators (1972) have shown directly that macrophage precursors did not react with AMS as did the mature monocytes and macrophages. These results indicate that the AMS-reacting antigen(s) in the macrophage develops during the process of differentiation and suggest that continuous production of this cell line can occur despite the presence of extracellular AMS.

Administration of AMS potentiates some viral infections (Hirsch *et al.*, 1969). In these infections the degree of viremia depends upon the capacity of the macrophage to take up viruses from the circulation. For example, AMS treatment hastened mortality of mice with vesicular stomatitis virus (Hirsch *et al.*, 1969), since depression of the macrophage system affects the degree of viremia and the consequent amount of virus seeded into the brain. Similar results were obtained with mice infected with yellow fever virus (Panigel and Cayeux, 1968). In contrast, treatment with AMS of mice infected with encephalomyocarditis virus lowered their mortality—apparently in this case the macrophage permitted the survival and multiplication of the virus (Panigel and Cayeux, 1968).

6. Transfer of Macrophages

A system employed to study the immunogenicity of macrophage-bound antigens consists of isolating live macrophages containing the antigen and transplanting them to syngeneic hosts which are then observed for their immune responses (Cohn, 1962; Gallily and Feldman, 1967; Argyris, 1967; Argyris and Askonas, 1968; Unanue and Askonas, 1968b; Unanue, 1969; Mitchison, 1969b; Spitznagel and Allison, 1970b; Cruchaud *et al.*, 1970). Hence, the immune response to an antigen initially carried exclusively by macrophages in the absence of free antigen is studied.

The macrophages in these experiments were obtained mainly from

the peritoneal cavities of mice or rats after injection of an irritating or inflammatory substance, such as glycogen, proteose peptone, or thioglycollate broth. These peritoneal macrophages are well-differentiated cells that have originated from a rapidly proliferating pool of cells in the bone marrow (Volkman, 1966; Volkman and Gowans, 1965a,b). In this experimental system there have been few comparisons of macrophages from different organs or of macrophages showing different degrees of maturation. However, when the immunogenicity of bacteriophage T4 bound to Kuppfer cells was compared to those bound to peritoneal macrophages, the former group showed reduced immunogenicity (Inchley, 1969). A critical analysis of the variables that are introduced in such a system in order to purify the Kuppfer cell was not done. Spitznagel and Allison compared the handling of antigen by normal macrophages and macrophages obtained after thioglycollate injection. The latter took up more antigen, although weight by weight the antigen in both cells as assayed in the transfer system had comparable immunogenicity (1970b). Other unpublished experiments (mentioned by Howard, 1970) claim that bovine albumin is of comparable immunogenicity when bound to isolated Kuppfer cells, alveolar macrophages, or peritoneal macrophages (cells were isolated and then transplanted into mice to assay for immunogenicity).

The technique of the macrophage transfer system is shown in Fig. 2. The macrophages are exposed to the antigen *in vivo*, i.e., after an i.p. injection, or *in vitro*; the macrophages are washed well after the uptake of antigen in order to eliminate extracellular antigen, and injected i.p. into histocompatible recipients. The recipient mice are subsequently studied either for their primary or secondary antibody responses and/or for a delayed hypersensitivity response. The antigens used in the system have included soluble proteins such as albumins, foreign Ig, hemocyanin, lysozyme or ovalbumins, or particulate antigens comprising *Escherichia coli*, red cells, or *Shigella*. In many instances the antigens are tagged with an isotope as in the cases of proteins with radioiodine, red cells with ⁵¹Cr or ¹²⁵I, or bacteria with ³²P. This allows an estimate of the amount of antigen associated with the live macrophage before transfer. The fates of the injected macrophages as well as the site where these cells meet with immunocompetent cells are not completely established. The problem with following macrophages after their injection is finding a suitable label which is sensitive, nontoxic, and not reutilized if released by the cell. Roser and collaborators (Roser, 1965, 1968; Russell and Roser, 1966), by using ¹⁹⁸Au as a marker, obtained evidence that a small number of injected macrophages end up in lymph nodes, liver, and spleen. In the spleen, macrophages were localized to the marginal zone

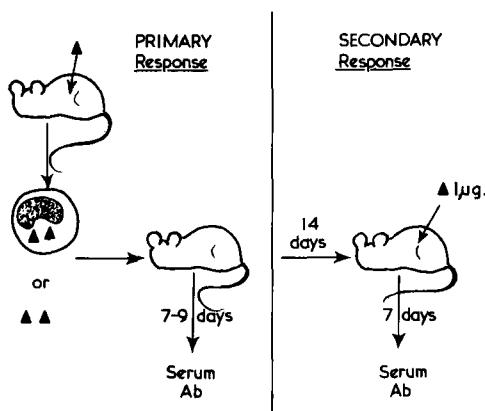


FIG. 2. Illustration of the technique of the macrophage transfer system. The antigen (in triangles) is injected i.p. and the peritoneal macrophages are isolated 30 minutes to 1 hour later. Alternatively, the macrophages can be isolated and incubated *in vitro* with the antigen. After uptake of antigen the cells are washed several times and then injected (i.p., i.v., or subcutaneously) into a normal recipient. If the antigen is tagged with an isotope, the radioactivity in the cells is measured before injection. The recipients are studied for their primary or secondary response to antigen.

and in lymph nodes to the medulla and perifollicular areas. Although all indications are that ^{198}Au is a good tag for following migration of macrophages, some released material may be reutilized. Gillette and Lance (1971) followed macrophages labeled with either ^{125}I -5'-iodo-2'-deoxyuridine (UDR) or ^{51}Cr . (Both labeling methods gave comparable results.) After i.v. or i.p. injection the label was found in liver and spleen, and, as regards the migration pattern of peritoneal macrophages after stimulation by different inflammatory substances, it was found that more of the "stimulated" tagged cells than controls left the peritoneal cavity and appeared in the spleen.

Several characteristics of the immune response to antigens transferred in live macrophages have been studied. It is clear that immunocompetent lymphocytes must be present in order to interact with antigen and initiate the immune process. Transfer of macrophages into X-irradiated recipients (Argyris, 1967; Unanue and Askonas, 1968b; Cruchaud and Unanue, 1971a,b) or into recipients immunologically tolerant (Mitchison, 1969b) to the antigen does not lead to immune responses. This type of experiment clearly emphasizes the well-known fact that macrophages are not involved in antibody synthesis. Antigen on dead macrophages (Mitchison, 1969b) or those from allogeneic (Mitchison, 1969b) or xenogenic (Unanue and Askonas, 1968b) donors (which are rejected by the host)

TABLE II
IMMUNOGENICITY OF ANTIGENS IN THE MACROPHAGE-TRANSFER SYSTEM^a

Antigen	Donor of macrophages	Recipients	Main findings (Refs.) ^b
Albumin (bovine)	Mice	Mice	Immunogenic (considerably more than free antigen when priming) (1) Macrophages catabolized most of the antigen taken up (1) Viable macrophages were required (1) Macrophages from tolerant mice were effective (1) Irradiation of donors of macrophages decreased immunogenicity of antigen in macrophages (1) Transfer into allogeneic recipients decreased immunogenicity (1)
Albumin (human)	Mice	Mice	Immunogenic (results comparable to those with bovine albumin) (1) Injection of free albumin decreased response to macrophage-bound albumin (2) Most of the antigen was catabolized except for a small fraction—some of the retained antigen was on surface of the membrane and accounted for a great part of the immunogenic moieties (i.e., trypsin-inhibited immunogenicity) (3)
<i>Escherichia coli</i>	Rabbits	Mice	Immunogenic (4)
γ -Globulin (bovine)	Rabbits	Rabbits	Immunogenicity persisted (4) Immunogenic (5) Irradiated recipients did not respond to transfer unless reconstituted with lymph node cells (5) Macrophages from irradiated (750 R) donors had decreased immunogenicity of antigen in macrophages (5)
γ -Globulin (human)	Mice	Mice	Immunogenic (6) Inhibition of RNA synthesis had no effect on the immunogenicity of antigen in macrophages (6)
Hemocyanin (from keyhole limpets, <i>Megathura crenulata</i>)	Mice	Mice	Immunogenic (less than free antigen) (7) Most of the antigen was catabolized except for a small fraction (7) Immunogenic molecules were retained on plasma membrane (8, 9) Immunogenicity was inhibited by incubating macrophages <i>in vitro</i> with antibody prior to transfer (9)

TABLE II (Continued)

Antigen	Donor of macrophages	Recipients	Main findings (Refs.) ^b
Hemocyanin (from <i>Maia squinado</i>)	Mice	Mice	Immunogenicity was increased in X-irradiated macrophages which retained more antigen on the membrane (10) Activated macrophages of cell-mediated immunities have more retained antigen on their membranes (11)
			Immunogenic (more than free antigen) (12) Antigen in live macrophages was more immunogenic than in dead macrophages (12)
			Antigen in macrophages placed in diffusion chambers into peritoneal cavity was less immunogenic than when injected i.p. (12)
			Immunogenicity persisted for long times and was associated with a retained fraction of antigen (13), some of which was on the plasma membrane (8)
			Immunogenicity was inhibited by incubating macrophages <i>in vitro</i> with antibody prior to transfer (14)
Lysozyme	Mice	Mice	Immunogenic (comparable to free antigen) (1)
Ovalbumin	Mice	Mice	Immunogenic (more than free antigen) (1)
Red blood cells (sheep)	Mice	Mice	Immunogenic (comparable to free antigen) (15) Some red cell antigen may be released from macrophages (16)
<i>Shigella</i>	Mice	Mice	Immunogenic and capable of eliciting antibody in 550-R irradiated mice (17) Antigen in macrophages from irradiated mice was not immunogenic (17)

^a This table was published in part in *Seminars in Hematology* 7, 225 (1970). In most of these experiments, a transfer system similar to that described in Fig. 2 was used.

^b Key to references:

- 1. Mitchison, 1969b
- 2. Spitznagel and Allison, 1970
- 3. Schmidtke and Unanue, 1971
- 4. Cohn, 1962
- 5. Pribrnow and Silverman, 1967
- 6. Cruchaud *et al.*, 1970
- 7. Unanue, 1969
- 8. Unanue *et al.*, 1969
- 9. Unanue and Cerottini, 1970
- 10. Schmidtke and Dixon, 1972
- 11. Lane and Unanue, 1972
- 12. Unanue and Askonas, 1968a
- 13. Unanue and Askonas, 1968b
- 14. Unanue, 1972
- 15. Argyris, 1967
- 16. Cruchaud and Unanue, 1971a,b
- 17. Gallily and Feldman, 1967

induces smaller responses than antigen bound to living syngeneic cells. Finally, antigen bound to other cells, such as fibroblasts, induces poor immune responses (Mitchison, 1969b). Intimate association between macrophages and lymphocytes is required, since macrophages isolated in impermeable diffusion chambers induce diminished immune responses (Unanue and Askonas, 1968b; Cruchaud and Unanue, 1971a).

Antigen bound to live macrophages is immunogenic and can induce primary immune responses, prime for a secondary challenge (i.e., induce a memory state), and induce a state of delayed hypersensitivity. Most of these studies are summarized in Table II. In all examples, the extent of the immune response depends upon the amount of antigen transferred in the cells (Mitchison, 1969b; Spitznagel and Allison, 1970b; Unanue and Askonas, 1968b). For example, small amounts of hemocyanin bound to macrophages primed mice for a small γM immune response, whereas larger amounts primed for strong γM (19 S) and γG (7 S) responses (Unanue and Askonas, 1968b). A partial inhibition of the immune response to macrophage-bound antigen occurs if normal macrophages are injected simultaneously. The reasons for this phenomenon are not known (Unanue and Askonas, 1968b). Delayed hypersensitivity has been induced in rats (Unanue and Feldman, 1971) and guinea pigs (Seeger and Oppenheim, 1970a). Such animals exhibited strong cutaneous delayed reactions if previously injected with live macrophages containing antigen.

Immune responses to a given amount of antigen injected either bound to live macrophages or in free form have been compared. Generally, those antigens that were taken up poorly by tissue macrophages, if transferred exclusively bound to macrophages, induced much higher immune responses than a comparable amount of free antigen (Mitchison, 1969b; Unanue and Askonas, 1968b). For example, albumin bound to live macrophages was about 1000-fold more immunogenic than free albumin (Mitchison, 1969b; Spitznagel and Allison, 1970b). (Such experiments support the contention that at least part of the strong immune responses to aggregated antigens may be explained by their uptake by tissue macrophages.) A different situation was observed when one compared the immune response of free or macrophage-bound antigen but using antigens which *in vivo* were taken up to a great extent by macrophages (Unanue, 1969). In this situation, the free antigen induced a higher immune response than the macrophage-bound antigen. For example, foreign red cells (Cruchaud and Unanue, 1971a) or keyhole limpet hemocyanin (Unanue, 1969) produced much greater responses (two to ten fold) when injected free rather than when bound to live macrophages. These results appear similar to those discussed in Section II,A,3. These large particulate antigens are readily trapped by lymph node and

spleen macrophages and, hence, are in suitable sites for triggering immune states. Thus, transfer of these latter antigens already bound to macrophages does not necessarily enhance the concentration of antigen in a lymphoid tissue; however, such enhancement does occur with antigens that are poorly taken up by tissue macrophages. In the latter case, transfer of antigen that is exclusively bound to live macrophages leads to a stronger immune state as a result of more antigen being concentrated in the lymphoid tissue (see Fig. 3).

Responses to macrophage-bound antigen in immune and nonimmune mice can differ. Mitchison (1969b) observed that the relative immunogenicity of albumin administered either macrophage-bound or free was much higher in nonimmune mice than in primed mice. With other antigens, such as hemocyanin, the immune response of primed or nonimmune mice was comparable (Unanue and Askonas, 1968b). The first results can be interpreted as denoting that immune mice with a large number of immunocompetent lymphocytes are less dependent on an antigen-concentrating cell; or, alternatively, that small amounts of antibody in the primed mice serve to concentrate the antigen.

The macrophage transfer system has been used to evaluate the role of macrophages in tolerance, in T and B lymphocyte collaboration, in some genetic disorders, and in immunoincompetence produced by X-irradiation. It is now very clear that immunological unresponsiveness is associated with a lack or an incapacity of T and/or B lymphocytes to respond to antigen (Chiller *et al.*, 1971). Macrophages from tolerant

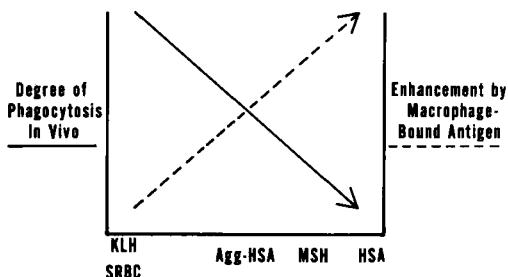


FIG. 3. Hypothetical graph illustrating the relationship between uptake of antigen by macrophages and the apparent enhancement (or decrease) of immunogenicity of antigen when injected macrophage-bound or free. Those antigens that are well taken up by macrophages show little improvement or even a decrease when administered macrophage-bound—the reverse is true for those antigens that are taken up poorly by macrophages where the response to macrophage-bound antigen is much higher than to a comparable amount of free antigen. (KLH) keyhole limpet hemocyanin; (SRBC) sheep red blood cells; (Agg-HSA) heat-aggregated human serum albumin; (MSH) hemocyanin from *Maia squinado*; (HSA) human serum albumin.

animals handle immunogens in the same way as do macrophages from normal animals. *In vivo* it was shown by radioautography that macrophages from tolerant animals took up as much antigen as macrophages from normal responsive animals (Humphrey and Frank, 1967). The association of antigen with cells in the follicles of normal and tolerant animals was analyzed in a previous section. By using the transfer system, Mitchison (1969b) showed directly that immunogenicity of antigens bound to macrophages of tolerant mice was not impaired and, thus, reemphasized that the state of tolerance was not associated with macrophage dysfunction. Similar results were obtained by Harris (1967) by using macrophages from tolerant rabbits and assaying for immunogenicity *in vitro*. It is also quite clear that the association of most catabolizable antigens with macrophages is a pathway for immunogenicity that does not lead to tolerance induction. In no instance has transfer of antigen bound to macrophages produced an unresponsive state (Table II). Furthermore, there appears to be competition between the tolerogenic forms of some antigens and macrophage-associated, immunogenic antigen for the immunocompetent cells. This very important observation was made by Spitznagel and Allison (1970b), who injected bovine albumin bound to live macrophages together with free albumin into syngeneic mice. The injection of albumin in live macrophages induced strong responses which were markedly reduced by concomitant injection of free antigen.

Macrophage-associated antigen can apparently interact with both T and B lymphocytes. This has been inferred from three sets of experimental observations. First, as mentioned above, recipient animals injected with antigen bound to live macrophages developed satisfactory cell-mediated immunity, presumably a manifestation of activity of T lymphocytes (Unanue and Feldman, 1971; Seeger and Oppenheim, 1970a). Along these same lines, if sensitized animals were injected with macrophages containing antigen, they readily developed strong delayed reactions (superimposed on a nonspecific inflammatory reaction) (Unanue and Feldman, 1971). A second line of evidence for the interaction of both T and B lymphocytes with macrophage-associated antigens derived from studies in thymectomized mice (Unanue, 1970) which did not respond to antigen bound to live macrophages. Also, the tissues of thymectomized mice were not impaired in their capacity to take up antigens (Unanue, 1970). These observations indicated that the response to antigen in macrophages required the presence of both types of lymphocytes and that the immunoincompetence of thymectomized mice was not associated with an impaired mechanism for concentrating antigen. Macrophages from thymectomized mice were normal *in vitro* (Mosier *et al.*, 1970). The third piece of evidence stems from the obser-

vation of Hamaoka *et al.*, who showed that hapten-primed cells and carrier-primed cells responded to hapten carriers bound to spleen macrophages (Hamaoka *et al.*, 1971). Presumably the carrier-primed lymphocytes are thymic derived, and the hapten-primed cells correspond to the B type. Similar results have been obtained by Katz and Unanue (1971), using peritoneal macrophages.

The macrophage transfer system has been used to evaluate the participation of macrophages in the genetically controlled immune response to hemocyanin among various strains of mice (Cerottini and Unanue, 1971). Macrophages from high and low responder strains handled antigen in the same way. Moreover, low responders were not restored to a high response by transferring macrophage-bound antigens from high responder strains. Clearly, the macrophage was not responsible for the differing responses among species in agreement with well-known observations in other systems (McDevitt and Benacerraf, 1969).

The macrophage transfer system was used to study the effects of X-irradiation on macrophages. Gallily and Feldman (1967) made the interesting observation that X-irradiated mice could mount an immune response to *Shigella* only if the organism was carried by macrophages and not free; such a response to *Shigella* bound to macrophages did not take place if the macrophages came from X-irradiated donors. Their conclusion was that part of the immunological defect of the X-irradiated mice could be attributed to defective antigen handling by macrophages. This study is difficult to interpret since there is at present no information on how *Shigella* immunogens are handled by macrophages. Pribnow and Silverman (1967) had some indications that macrophages from X-irradiated rabbits after uptake of bovine γ -globulin did not induce immune responses. Mitchison (1969b) also observed the effects of X-irradiation on the transfer of human albumin bound to murine macrophages. He subsequently showed with Kolsch that there were some differences in the intracellular distribution of antigens between macrophages from heavily X-irradiated mice and nonirradiated mice (Kolsch and Mitchison, 1968), although it was not clear whether these differences in handling correlated strictly with the impaired capacity to induce human albumin immunogenicity. Such effects of X-irradiation have not been observed with other antigens. Responses to hemocyanins (Unanue and Askonas, 1968b; Unanue and Cerottini, 1971) and SRBC bound to macrophages are not impaired by X-irradiation. Recently, Schmidtke and Dixon (1972) studied the handling of hemocyanin by X-irradiated macrophages and found that more antigen was retained on surface membranes of X-irradiated cells associated with increased immunogenicity. There was no effect by X-irradiation on the function of macrophages in *in vitro* (Roseman *et al.*, 1969). In summary, there is no

clear explanation for the impaired responses observed to only some antigens associated with macrophages from X-irradiated animals. Detailed studies on antigen handling by these cells should eventually explain the discrepancies.

7. Attempts to Sensitize Lymphocytes in Vitro

Ford, Gowans, and McCullagh attempted to sensitize lymphocytes by incubation on macrophage monolayers before adoptive transfer (Ford *et al.*, 1966; McCullagh, 1970). Rat thoracic duct lymphocytes were isolated and incubated with either solubilized SRBC or with monolayers of macrophages that had taken up SRBC. After 3 hours the lymphocytes were transplanted into syngeneic recipients. (The lymphocytes were separated from macrophages using a technique whereby iron particles were added to the cells which were then placed in a magnetic field—macrophages that took up the iron adhered, whereas the lymphocytes did not.) It was found that lymphocytes first incubated on macrophages made an anti-SRBC response, but those incubated in solubilized SRBC did not. This interesting experimental model, if developed further, could be used to study interactions of macrophage-bound antigen with lymphocytes.

B. In Vitro EXPERIMENTS

In 1967, Dutton reviewed the early literature concerning *in vitro* systems and included the work associated with macrophage extracts (Dutton, 1967, p. 295). The two *in vitro* systems most frequently used in recent years include the antibody-forming cell [plaque-forming cells (PFC)] response to foreign red cells (notably SRBC) or the proliferating cell response to soluble antigens or allogeneic cells. In this review we attempt to summarize the published results so far as they concern the role of the macrophage.

1. Response in the Mishell-Dutton or Marbrook System

An antibody-forming cell response can be readily obtained as outlined by Mishell and Dutton (1966) and Marbrook (1967). In both methods a suspension of spleen cells is used, most often from mice. In the Mishell-Dutton system the cells (usually $5-15 \times 10^6$ in 1 ml of media) are cultured in dishes that are rocked for several days; the medium which contains selected fetal calf sera is supplemented daily with culture nutrients. In the Marbrook method the cells ($\sim 10^7-1.6 \times 10^7$) are placed in a dialysis bag that is immersed in a flask with tissue culture medium; this method is stationary and does not require daily supplementation of the medium. In both methods the immune response

is measured by numbers of PFC present 4 or 5 days after the culture is initiated.

There is general agreement that the primary anti-RBC response requires the presence of at least three types of interacting cells. Two of the cells are lymphoid and correspond to the T and B lymphocytes identified *in vivo*; the remaining cell has all the characteristics of a macrophage and can best be identified by its property of adherence to glass surfaces. The evidence that this third type of cell (i.e., adherent cell) is a macrophage is based on the following: (1) the cell adheres to glass or plastic surfaces; (2) it has phagocytic properties; (3) it has classic morphological criteria of macrophages; (4) it is radioresistant; (5) it does not synthesize antibodies; (6) it is affected by treatments that block the RES *in vivo*; and, finally (7) it is affected by specific AMS (Shortman and Palmer, 1971). In general, other *in vitro* methods employed for separating cells identify the activity of the adherent cells with macrophages. In conclusion, it is rather unlikely that apart from macrophages there is another cell having similar properties of adherence, sedimentation, and reactivity with AMS. Therefore, I shall use the term adherent cell synonymously with macrophage.

The first demonstration that an adherent cell population was required in this system came from the studies of Mosier (1967). He cultured murine spleen cells on plastic dishes for 30 minutes and separated those that attached to the dish from those that did not attach. Neither cell type (i.e., nonadherent comprising about 90% of the original population and adherent comprising about 10%) by itself would respond *in vitro* to SRBC. However, when both were mixed together the anti-SRBC response was readily reconstituted. In other experiments the adherent cells were shown to interact with SRBC. The SRBC were added to the adherent cells for 0.5 hour after which most of the SRBC were removed. The adherent cells, presumably containing 5% phagocytized SRBC, were capable of interacting with the nonadherent lymphocytes. However, no exact estimate of the ratio of phagocytized to nonphagocytized SRBC was made, and it is most likely that some nonphagocytized SRBC must have remained along with the adherent cells. Subsequently, these early experiments were confirmed and expanded (Mosier, 1969; Pierce, 1969a,b; Pierce and Benacerraf, 1969; Talmage *et al.*, 1969; Dutton *et al.*, 1970; Hartman *et al.*, 1970; Haskill *et al.*, 1970; Shortman *et al.*, 1970; Shortman and Palmer, 1971). Thus, it was later shown conclusively that the nonadherent cells comprised the population of progenitors of PFC (Hartman *et al.*, 1970; Shortman and Palmer, 1971). This was best done by mixing adherent and nonadherent spleen cells, each from a different mouse strain. The source of resulting PFC was

easily determined by killing one or the other cell with appropriate antibodies to strain histocompatibility antigens prior to testing for PFC. Indeed, all PFC originated from the mouse strain that donated the nonadherent cells.

Adherent cells obtained 1-48 hours after *in vivo* X-irradiation of mice (with 250 to 1000 R) were capable of performing in culture as effectively as adherent cells from nonirradiated mice (Roseman, 1969; Roseman *et al.*, 1969; Dutton *et al.*, 1970; Shortman *et al.*, 1970). However, 3 days after X-irradiation the mouse spleens were depleted of adherent cells (Gorcynski *et al.*, 1971). As expected, the nonadherent population from X-irradiated mice were unable to respond.

The degree to which the adherent cells influence the response to SRBC may depend upon the number of progenitors of PFC and/or the cell density in culture. Thus, spleen cells from immune mice appeared, in the hands of some investigators, to be less sensitive to depletion of the adherent cell population than were cells from nonimmune mice (Pierce, 1969a). The influence of cell density in rabbits was observed by Theis and Thorbecke (1970); nonadherent cells were more responsive when cultured in stationary tubes where the total surface area was small than in dishes where the total area was large.

Although most investigators find that the macrophage is required for the *in vitro* response to SRBC, there is no general agreement on the mode of action or function of this cell. Is the macrophage favoring a response by handling the antigen in an appropriate way? Is the macrophage simply favoring cell-to-cell interactions regardless of its handling of antigen? Or, is the macrophage providing a source of nutrients or special factors required for the *in vitro* growth of lymphocytes? These three possible mechanisms are not mutually exclusive, and evidence in favor of one or two has been provided. Unfortunately, *in vitro* responses are not uniform, and results vary among different investigators. Each investigator has found subtle and sometimes ill-defined requirements for support of antibody formation in culture. We should be aware that *in vitro* systems introduce experimental variables which may apply only to the *in vitro* method and not to events occurring *in vivo*.

The fate of the SRBC (and its antigen) among the adherent cell population has not been fully studied. It is quite important to determine the form in which the SRBC immunogen is presented to the lymphocyte. Does such presentation occur before or after endocytosis of SRBC? Is modification or change of the SRBC by intra- or extra-cellular macrophage products a necessary step? Mosier's original method gave one the impression that most SRBC were phagocytized by the macrophages. Hence, immune recognition must have followed the in-

teriorization process. However, recently Leserman and Roseman (1971) have indicated that ammonium chloride treatment of adherent cells exposed to SRBC abolishes their capacity to support *in vitro* responses of lymphocytes. The ammonium chloride presumably is lysing extracellular SRBC. The treatment was not affecting macrophage function, since addition of SRBC restored their response. This last piece of evidence raises the possibility that the *in vitro* response may be directed to SRBC antigens not engulfed by macrophages but remaining extracellularly. Shortman and Palmer (1971) have recently presented some evidence for a soluble SRBC antigen resulting from interaction of macrophage culture fluid and SRBC. They incubated SRBC in the fluid from 24-hour culture of macrophages; after several hours the SRBC were centrifuged and discarded. The resulting supernatant stimulated nonadherent cells for an anti-SRBC response. (Sheep red blood cells by themselves were not stimulatory.) The conclusion from this experiment is that macrophages may generate some factors which "release" or "liberate" antigen from SRBC, and these are the antigenic moieties capable of triggering the response of the lymphocyte. Shortman and Palmer have cautioned against overinterpreting their data in that their supernatant-antigen fluid was about twenty-five-fold less immunogenic than SRBC associated with the adherent cell layer.

In favor of the macrophage serving as a cell that facilitates cellular interactions are the results of Mosier (1969) and Pierce and Benacerraf (1969). They found that after 24 or 48 hours of culture in the Mishell-Dutton system, clusters of cells appear, some of which contain PFC. A macrophage was required for the cluster in that nonadherent cells formed few or small aggregates of cells (Pierce and Benacerraf, 1969). All the PFC in a cluster (a mean in some cultures of about 6 per cluster) responded to a given foreign red cell, either sheep or burro, if both were added to the culture (Mosier, 1969). That these clusters may have a functional significance was gathered from results in which the cultures were interrupted at various times after their initiation and continued by keeping stationary instead of by rocking, i.e., the culture was initiated and interrupted at 6, 24, or 48 hours; the cells were then dispersed and recultured either by rocking or remaining stationary (Pierce and Benacerraf, 1969). Those cultures that were rocked developed clusters again, whereas those left stationary did not. The cultures left stationary after an interruption at 6 hours were found to have few PFC at day 4 or 5 as compared to similar cultures rocked after interruption at 6 hours. Interruption at 24 hours produced a lesser effect in that the responses of stationary cultures were one-third or one-half that of rocking cultures. At 48 hours there was no effect. On the basis

of these studies, Pierce and Benacerraf concluded that after a period of about 1 day the lymphocytes were already "activated" upon interaction with macrophage (and its antigen) and, hence, did not require close cell contacts and aggregates. It should be noted that not all culture systems require movement. The Marbrook system for studying SRBC response is a stationary one, although also sensitive to the depletion of adherent cells.

Evidence for macrophages supplying factors that improve the growth of lymphocytes comes from the experimental results of Dutton and collaborators (Dutton *et al.*, 1970; Hoffman and Dutton, 1971). They have found that culture fluids of adherent spleen cells or, better, of peritoneal macrophages will allow nonadherent spleen cells to respond to antigen. The chemical property of the factor in question has not been solved (it is stable to heating at 57°C for 30 minutes but sensitive to freezing and thawing). Hoffman and Dutton postulated that the factor could be an enzyme necessary for SRBC processing, an antibody-like molecule or a growth-conditioned media-type factor. The observation that SRBC treated *in vitro* with the culture fluid can stimulate non-adherent cells for antibody production favors an "antibody-like molecule" theory.

Recent findings of Shortman and Palmer (1971) are important in establishing a coherent picture among results in the literature. They have shown quite clearly that a given culture of nonadherent lymphocytes that does not respond to SRBC will respond to a different antigen—the polymerized form of flagellin from *Salmonella adelaide* (Table III shows one of their results). In some experiments both antigens were added to the same culture, yet the cells only responded to flagellin. This experiment tends to minimize the role of macrophages *in vitro* as cells simply providing a conditioned kind of media and implies a more active role of these cells in the process of antigen handling and presentation. It also emphasizes that the macrophage pathway may vary in importance depending upon the antigen in question. Along these lines, M. Feldman and Palmer (1971) claimed that macrophages were not required for the *in vitro* immune response to a soluble extract of SRBC. Their results imply that SRBC must be degraded to a small size in order to trigger lymphocytes for antibody synthesis. This important study needs confirmation.

Gorcynski *et al.* (1971) have correlated an adherent cell *in vitro* with spleen macrophages operational *in vivo*. They found that spleen cell suspensions devoid of adherent cells 3 days after X-irradiation of mice did not support *in vitro* responses by lymphocytes; at the same time suspensions of lymphocytes (depleted of macrophages) transplanted

TABLE III
EFFECT OF ANTIMACROPHAGE ANTISERUM ON IMMUNE
RESPONSES IN TISSUE CULTURE^{a,b}

Tissue cultures	Antibody-forming cells/culture	
	SRBC antigen	POL antigen
Unfractionated spleen cells		
Untreated	1660 (1020-2280)	1090 (568-1350)
+ Normal serum	893 (313-1500)	696 (278-1020)
+ Antimacrophage serum	36 (0-150)	446 (242-846)
Adherence column filtrate		
Untreated	92 (30-205)	1330 (468-2240)
+ Normal serum	6 (0-13)	405 (164-648)
+ Antimacrophage serum	0 (0-0)	298 (177-363)

^a From Shortman and Palmer (1971), p. 405. I thank the authors for allowing to reproduce the table.

^b This table shows the effect of macrophage depletion on the response to sheep red blood cells (SRBC) or polymerized flagellin (POL). Purified lymphocytes (by column filtration or as a result of antimacrophage serum treatment) respond to POL but not to SRBC. One milliliter of undiluted antiserum was added to each 8 ml. of tissue culture medium. The cells (12×10^6 viable nucleated cells in 1 ml. culture) were preincubated with this mixture for 90 minutes at 37°C, prior to initiation of the response by antigen addition. Results are the mean of three separate experiments, each involving three to four cultures per point. The range of individual experiments is given in parenthesis. Background values of untreated unfractionated spleen cells in the absence of added antigen averaged 65 antibody-forming cells per culture for POL, 99 per culture for SRBC.

into mice irradiated 3 days previously did not form antibody *in vivo*. A similar correlation was found when mice were injected with large doses of horse red cells; such mice did not make antibodies when immunized to SRBC *in vivo* (unless replenished with macrophages) nor did the spleens contain active adherent cells for *in vitro* responses (to SRBC). Furthermore, cells isolated from normal spleens were capable of restoring both *in vivo* and *in vitro* responses in both sets of experiments. These cells, presumably macrophages, had identical physical properties when separated in density gradients or velocity sedimentation. This last experiment strongly suggests that the role of macrophages *in vitro* may be akin to their role *in vivo*.

2. Proliferative Response of Immune Cells

Lymphoid cells from immunized experimental animals or humans proliferate in culture when exposed to antigens for which they are specific. This specific proliferative response could be determined by either pulsing the cells with a radiolabeled deoxyribonucleic acid (DNA)

precursor (such as tritiated thymidine) hours before harvesting or estimating the number of large blasts in cell smears. In humans the lymphoid cells were obtained from peripheral blood and the antigen most frequently used was purified protein derivative (PPD). In experimental animals (such as rabbits, guinea pigs, or mice) cells were obtained from the spleen, and the antigens included a variety of soluble proteins such as albumins, Ig, tetanus, and diphtheria toxoid. Cells from non-immune individuals responded poorly, if at all, in this *in vitro* system. The culture consisted of cells placed in stationary tubes with antigen and was examined 3 to 5 days later.

Several points seem to have been reasonably well established. For example, the presence of adherent cells or purified macrophages appears to be important in order for a purified population of lymphocytes to respond to antigen (Hersch and Harris, 1968; Gordon, 1968; Oppenheim *et al.*, 1966; Lamvik, 1969; Schechter and McFarland, 1970). This effect has been tested most often by separating the macrophages through their ability to adhere to glass. Customarily, human lymphocytes have been separated by passing them through a column of glass beads with appropriate media. These cultures of purified lymphocytes which responded poorly to antigen were restored by addition of purified macrophages. Other cell types, such as fibroblasts (Hersch and Harris, 1968) or neutrophiles (Cline and Sweet, 1968), could not be substituted for macrophages.

The extent of macrophage contribution to responses depended in part upon total cell density (Oppenheim *et al.*, 1968). In ordinary cultures purified lymphocytes responded poorly but, if the cells were concentrated 3 to 4 times, there was a partial restoration of the proliferative response. This effect could also be seen to a lesser extent on cultures of lymphocytes stimulated by phytohemagglutinin (PHA), where poor stimulation was noted at high dilutions of the mitogen unless the cell concentration was increased. This would imply that macrophages functioned as a means for concentrating lymphocytes with antigen or lymphocytes with lymphocytes. To this effect, many reports have emphasized and illustrated the presence of groups of lymphocytes, some of which are blasts around macrophages (Cline and Sweet, 1968; Schechter and McFarland, 1970). Depletion of macrophages from a suspension of spleen cells by AMS reduced the proliferative response to antigen but not to PHA (Jehn *et al.*, 1970). These last results agree with the previous series of experimental results.

Part of the effect of macrophages in these culture systems which utilize soluble antigens could be explained by the uptake of antigen. The evidence was reasonably convincing that the soluble antigen taken up by

the macrophages was capable of stimulating the DNA synthetic response. This was done by obtaining macrophages (often with a great degree of purity) from spleen (Harris, 1965), peripheral blood (Oppenheim *et al.*, 1966; Hersch and Harris, 1968; Schechter and McFarland, 1970; Cline and Sweet, 1968), peritoneal cavity (Seeger and Oppenheim, 1970b), or lung alveolar spaces (Seeger and Oppenheim, 1970b) and exposing them to antigen for various periods of time. The cells were washed well, mixed with the lymphocyte suspension, and then cultured for several days. [In one experiment the cells were separated from the glass and mixed with lymphocytes. This procedure insured that antigen adhering to glass was not operative in the system (Schechter and McFarland, 1970).] The lymphocytes, which in the usual culture conditions responded poorly to soluble antigen, now proliferated upon interaction with the macrophage-bound antigen. The response required close cell contact and was not associated with a soluble antigen product released from the macrophage. Thus, separation of the macrophages from the lymphocytes by a membrane (of $0.45\ \mu$ pore size) impaired the response (Hersch and Harris, 1968; Cline and Sweet, 1968). Also, culture fluid that harbored macrophages (with antigen) was not stimulatory (Cline and Sweet, 1968). Harris claimed that the antigen responsible for this response was on the membrane of the macrophage (1965). Macrophages containing antigen, if treated with the specific antibody, were inhibited in triggering the response of the lymphocyte. Live macrophages were also important here in that after antigen uptake, if killed by exposure to heat, they would not trigger lymphocyte proliferation (Cline and Sweet, 1968). Macrophages from immune individuals were as effective as those from normal unimmunized subjects (Cline and Sweet, 1968; Seeger and Oppenheim, 1970b). Unfortunately, no complete quantitative studies comparing the relative effectiveness of macrophage-bound antigen and soluble antigen are available. Harris had some indication of the uptake of radiolabeled antigen by the macrophage and claimed that macrophage-bound antigen must be more immunogenic than free (1965); others have made similar claims (Cline and Sweet, 1968).

Two further points are of importance and these concern the ratio of macrophage to lymphocyte in culture and the effects of X-irradiation. The ratio of macrophages to lymphocytes was critical in that high ratios depressed the response (as also observed in the previous section) (Harris, 1965; Parkhouse and Dutton, 1966). The best ratio at which to obtain a response was about 1 monocyte/10–50 lymphocytes. In all reports, macrophages exposed to antigen and irradiated were not impaired and responded as well as nonirradiated macrophages.

Whether the macrophage also contributed in producing a conditioned

media is under consideration. Bach and collaborators (1970) have shown that fluid from macrophage cultures can be substituted for these cells and allow lymphocytes to respond to allogeneic cells or soluble antigens. Hence the problem of distinguishing among several effects is still present in this system, and again technical differences probably account for non-uniformity of results.

III. Handling of Antigen by Macrophages

The handling of antigen by macrophages will be reviewed here with emphasis on identifying the immunogenic moiety of the macrophage bound antigens. Cohn (1968) described the physiology of macrophages in a previous volume of this series and presented details on the process of endocytosis. Also, excellent reviews on phagocytosis (Rabinovitch, 1968) and on lysosomes (Weissman and Dukor, 1970) have appeared.

Uptake of soluble and particulate antigens by macrophages consists of a two-stage process. The first is attachment of antigen to the surface of the cell, and the second is endocytosis of the bound antigen. Subsequently, some of the pinocytic or phagocytic vesicles with the endocytized antigen fuse with lysosomes; the lysosomal enzymes then degrade most of the antigen. We consider two main points: (1) the capacity of a macrophage to take up antigen and to discriminate between foreign and nonforeign elements and (2) the possible locus of the immunogenic moiety among the total antigen taken up by this cell.

A. UPTAKE OF ANTIGEN

In trying to understand the extent to which the macrophage plays a role in the immunological process, it is necessary to evaluate the capacity of these cells to discriminate among a variety of antigenic or nonantigenic materials. To do so one needs information on types and amounts of antigen taken up, on whether the macrophage has the capacity to discriminate among antigens or between foreign and nonforeign substances, on whether there are different populations of macrophages differing in their capacity to take up antigen, and finally on the biochemical nature of macrophage-antigen interaction.

Both *in vivo* and *in vitro* procedures have been employed to study uptake of antigen by macrophages. *In vivo* procedures include (1) the study of blood clearance of suitably labeled antigens and (2) radioautographic studies of the fate of antigen. (The techniques and main results of this last procedure were described in Section II,A,6.) The *in vitro* methods consist of studying the uptake by macrophages of a variety of suitably tagged antigens under a series of tissue culture conditions. It is obvious that the *in vitro* methods are the methods of choice because

they give direct quantitative results amenable to experimental variation without interference with *in vivo* variables. Macrophages are cells that are relatively easy to culture for hours or days without fastidious cultural requirements (Jacoby, 1965).

Knowledge of the mechanisms of antigen uptake and of the physiology of macrophages has been obtained by studying the rate of elimination of suitably tagged antigens from the circulation. This method gives an indication of the activity of the RES mainly of liver and spleen. Colloidal suspensions, such as carbon, saccharated iron oxide (Benacerraf *et al.*, 1954), chromic phosphate (Dobson and Jones, 1951; Dobson, 1957), or gold (Zilversmit *et al.*, 1952), as well as particulate antigens such as heat-aggregated proteins (Halpern *et al.*, 1957) have been employed. The ideal material must not be toxic, should be of a homogeneous size, and should be easy to trace and measure in the blood. The kinetics of clearance from the circulation of colloidal suspensions were studied extensively by Benacerraf, Biozzi, and collaborators (reviewed in Biozzi *et al.*, 1957; Benacerraf *et al.*, 1957a; Benacerraf, 1964). They found that the liver and spleen took up most of the injected material (80–90%) of which two-thirds was taken by the liver. Indeed there was a direct relationship between size of these organs and the rate of blood clearance. The distribution of material between liver and spleen varied depending upon the amount injected. The liver RES was probably the most active because of its large number of RES cells and great blood flow (Benacerraf *et al.*, 1955a,b, 1957b; Biozzi *et al.*, 1958). In one passage through the liver about 80 to 90% of material could be cleared [in liver cirrhosis the amount cleared was greatly decreased (Halpern *et al.*, 1959)]. At suitable concentrations the particles or particulate antigens were eliminated exponentially from the circulation. A suitable mathematical formula has been applied to estimate the phagocytic activity (i.e., phagocytic index). As the concentration of particles increased, the index decreased—an indication of saturation of the capacity of the RES to take up particles. The clearance of bacteria (Benacerraf *et al.*, 1959; Wardlaw and Howard, 1959; Biozzi *et al.*, 1960), viruses (Mims, 1959), and foreign red cells was also studied. Among other tests, the rate of blood clearance indicated that uptake of particles was (1) a radioresistant process, (2) increased by estrogen treatment, (3) decreased by cortisone treatment, (4) increased after active infection with tubercule bacillus (Biozzi *et al.*, 1954) or after injection with mycobacterial products or gram-negative endotoxin. All these effects can be easily explained. Macrophages are radioresistant and are easily activated during delayed hypersensitivity reactions.

Macrophages take up a variety of colloidal substances, proteins,

microorganisms, and red cells; although many of these need to be opsonized by antibody and C first. The molecular basis of the uptake is poorly understood. The uptake of an antigen, either particulate or soluble, by macrophages depends upon two main considerations: (1) whether the antigen can interact with the macrophage membrane "directly," i.e., without serum antibody or C, and (2) whether the antigen is complexed to antibody and/or C (or other serum proteins).

Not all antigens interact well with the macrophage surface. Some bacteria, many of which are virulent, do not bind with macrophage and, hence, are not phagocytized. These include pneumococci (Wood, 1951), some strains of *Salmonella* (C. Jenkin and Benacerraf, 1960), and staphylococci (MacKaness, 1960; see, also, Allen and Cook, 1970; Brumfitt *et al.*, 1965). These bacteria usually have a thick capsule of carbohydrate. It would appear that interactions between capsular polysaccharides and macrophages are weak or poor resulting in poor attachment. However, Wood's classic experiment on phagocytosis of pneumococci indicated that, under suitable conditions, macrophages were able to take up some encapsulated organisms in the absence of antibody (Wood *et al.*, 1946), particularly in the presence of inert material. The macrophages were thought to "corner" the organisms against inert substances and in this way take up some organisms. Bacteria that were poorly taken up by macrophages were easily phagocytized if complexed to specific antibody and/or to serum C. This phenomenon is described in detail below.

Macrophages can take up a variety of foreign erythrocytes without the aid of antibody. Moreover, Perkins and Leonard have indicated that there was preferential uptake of some species of erythrocytes (1963). For example, mouse macrophages preferred chicken red cells over sheep or rabbit red cells. In their experiments, in which uptake of non-opsonized erythrocytes was small, about 15 to 20% of macrophages would take up both types of red cells. Macrophages did not take up autologous red cells unless these became effete (Vaughan and Boyden, 1964; Lee and Cooper, 1966). Trypsin treatment of the macrophages removed the receptor for effete red cells (Vaughan, 1965); those chemically modified by glutaraldehyde readily bound to macrophages (Rabinovitch, 1967a,b).

Most protein antigens that have been examined can be taken up by macrophages directly. The degree of uptake in part depends upon the state of aggregation of the protein. Evidence that some protein antigens can interact "directly" with macrophages at least without the intervention of antibody is based on both *in vivo* and *in vitro* observations. Some antigens injected into animals could be taken up by macrophages in the absence of serum or cell-bound antibodies. Injection of hemocyanin, aggregated albumins, or Ig into an unimmunized animal resulted in their

rapid uptake by the perifollicular macrophages of lymph nodes and spleen (see Section II,A,1). Such large or highly aggregated proteins, if they reached the circulation, were cleared very rapidly by macrophages of the liver and spleen. However, in these *in vivo* experiments the possible presence of small, undetectable amounts of natural serum antibodies could not be completely ruled out. The experiments of Humphrey and Frank (1967) described earlier, using hemocyanin or albumin in rabbits, indicated strongly that natural or acquired antibodies were not playing a role in the uptake of antigen by lymph node macrophages. (The rabbits were immunologically tolerant to the antigens and the presence of antibodies was ruled out by the absence of follicular trapping of the antigen.)

By tissue culture experimentation this problem has been evaluated directly. In some of our own experiments macrophages exposed *in vitro* to protein antigens, in the absence of serum, took up the antigen efficiently (Unanue *et al.*, 1972; Schmidtke and Unanue, 1971a). It seemed unlikely that small amounts of membrane-bound antibody were involved. This possibility was then eliminated by incubating macrophages with anti-Ig antibody before contact with the antigen (Unanue *et al.*, 1972; Schmidtke and Unanue, 1971a). Murine macrophages were washed well, isolated, and planted in culture dishes; different antibodies to mouse Fab and class determinants were added to the dish; at a later stage radioactive hemocyanin or aggregated albumin was added. Treated and untreated macrophages took up equal amounts of the radiolabeled antigen. If antibody was on the membrane of the macrophage, then addition of antibody to Ig should hinder its combination with antigen. Similar antibodies, for example, could block the uptake of the same antigens by B-type lymphocytes which are known to have membrane-bound Ig (Unanue *et al.*, 1972; Warner and Byrt, 1970) (Figs. 4 and 5 are representative experiments). However, if such macrophages were treated with AMS, which presumably covers part of the surface, then uptake of the antigen was greatly impaired. In accordance with these results, treatment of macrophages with trypsin (which removes any surface-bound Ig) did not impair their capacity to take up labeled antigens. Hence, a variety of antigens could, indeed, be taken up in the absence of extracellular or cellular-bound specific antibody.

Aggregation or polymerization of many protein antigens favors interaction with the macrophage surface. This has been studied best with albumins or Ig. For example, the rate of elimination from the circulation of these radiolabeled proteins in their polymeric or monomeric forms is strikingly different. Polymers are cleared in minutes by the RES of liver and spleen, whereas monomers circulate with a half-life similar to that of comparable autologous proteins (i.e., 2-3 days).

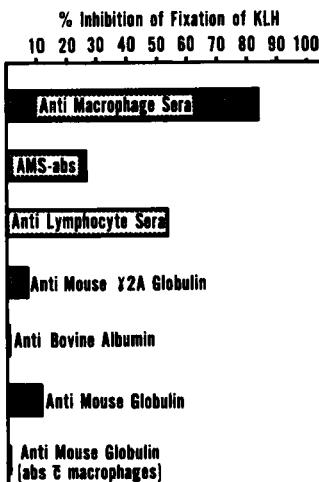


FIG. 4. Results of an experiment in which macrophages were incubated with a series of antisera prior to exposure to radioiodinated keyhole limpet hemocyanin (KLH). The uptake of KLH was estimated by counting the cells. Note that incubation of the macrophages with antimouse Ig (i.e., an antimouse IgG_{2a} or a polyvalent antimouse Ig) had little effect on the binding of KLH (the small effect produced by antimouse globulin was abolished by absorption of the antisera to cells—see last item in figure). As expected, antimacrophage sera (AMS) blocked the effect—the absorption of AMS by macrophages reduced its binding to macrophages and its capacity to block KLH uptake. Antilymphocyte sera which cross-react with macrophages also block the uptake of KLH. Compare these results with the effects of anti Ig in uptake of KLH by lymphocytes in Fig. 5. (From Unanue *et al.*, 1972).

The handling of polymerized and monomer albumins was also studied in tissue culture experiments (Schmidtke and Unanue, 1971a). It was found that monomer human albumin was taken up to a small extent by murine macrophages and that it readily dissociated from the cell surface. In contrast, polymer human albumin was taken up to a much larger extent and, after uptake, a great part of the molecule was retained on the membrane (and subsequently endocytized). Apparently macrophages take up autologous albumin (Schmidtke and Unanue, 1971a) and Ig (Herd and Ada, 1969a) to the same extent as their foreign counterparts, the main consideration being, as with the foreign material, the degree of polymerization. All these results bring out two important considerations: first they suggest that any polymeric antigen with multiple points of attachment to a membrane forms, as expected, a stronger interaction with the cell than that of a monomeric antigen with few points of contact; second, they indicate the poor capacity of the macrophage to discriminate foreign vs. nonforeign substances.

There is little information on the nature and specificity of the inter-

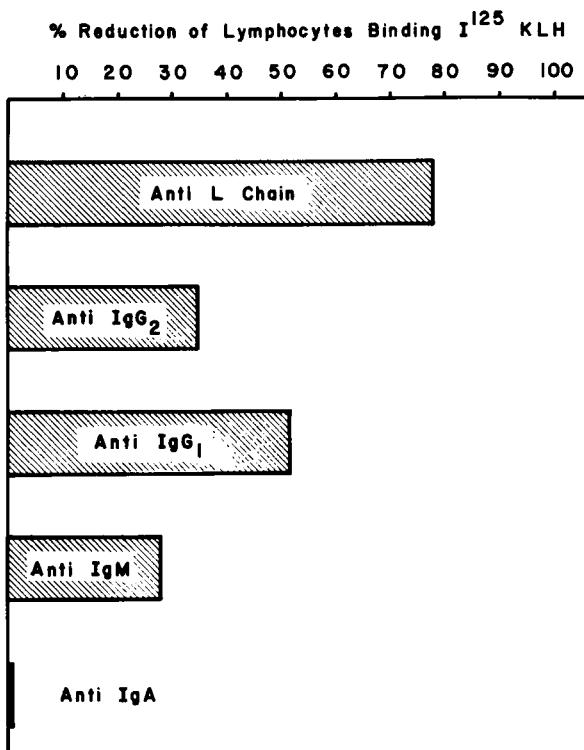


FIG. 5. Results of an experiment in which lymphocytes were incubated with keyhole limpet hemocyanin (KLH)- ^{125}I , and the number binding KLH was determined by radioautography. Prior incubation of lymphocytes with anti-Ig antibodies reduced the number of cells binding KLH—an indication that class-specific Ig was mediating the uptake. Compare with results of experiments of Fig. 4. (From Unanue *et al.*, 1972).

action between different antigens and the macrophage membrane. We do know that protein antigens bind to areas of the membrane surface distinct from those to which Ig and C bind. However, the interaction or possible competition of different surface areas of the cell with the various proteins has not been studied critically as yet.

Are there populations of macrophages differing in their capacity to discriminate among various antigens? All indications are that such populations do not exist and that any macrophage under optimal conditions can take up any antigen. There can be, however, a difference in the degree of uptake of antigen between monocytes and macrophages. About two-thirds or more of the peritoneal macrophages take up protein antigens. A single macrophage can take up two different protein antigens (Rhodes *et al.*, 1969). Perkins and Leonard (1963), however, had some

indication that selected macrophages preferred to take up foreign red cells, but whether this could be explained by the state of cell maturation was not ascertained.

Macrophages from tolerant animals take up the tolerogen as efficiently as macrophages from normal, nontolerant animals (Mitchison, 1969b; Humphrey and Frank, 1967).

1. Immunoglobulin Receptor on Macrophage

Antigen complexed to antibody binds avidly to macrophages as a result of the interaction of the antibody molecule with a specific surface receptor on the macrophage membrane. This antibody has been termed cytophilic (Boyden, 1963). It is well known that aggregated antigens, red cells, or bacteria in the presence of antibody were immediately cleared from the circulation by the RES of liver and spleen. In 1963-1964, Boyden developed an *in vitro* assay to study binding of antigen-antibody complexes (1963, 1964). He employed red blood cells which in the presence of antibody adhered around the macrophage—the antigen (red cell)-antibody complex was easily visualized as a rosette around a central macrophage. Bacteria coated with antibody were also used in this type of test (Uhr, 1965; Auzins and Rowley, 1963). The *in vitro* assay has two varieties: in one, a monolayer of macrophages is exposed to antisera, usually at room temperature, then washed and exposed to the antigen; a second consists of adding antigen-antibody complexes directly to the macrophage monolayer (Jonas *et al.*, 1965; Berken and Benacerraf, 1966). (The complexes could be formed by immunological reaction of antigen with specific antibody or by chemically coupling the antigen to any Ig.) As one would expect, the latter assay was more sensitive. Ultrastructural studies on rosettes of red cells on macrophages showed adherence and many interdigitations of the two-cell surfaces (Abramson *et al.*, 1970a). It is interesting to note that the red cells after adhering to macrophages became spherical (LoBuglio *et al.*, 1967) for no apparent reason. It should be made clear that the attachment of opsonized particles (i.e., particles coated with antibody) to the membrane without their interiorization is a result of working at room temperature. Usually increasing the temperature to 37°C results in rapid interiorization of the antigen (Berken and Benacerraf, 1966).

Only some classes of Ig can interact with the macrophage surface. This can be studied with the rosette test by either isolating specific anti-red-cell antibodies of a particular Ig class or chemically coupling a purified Ig class, usually myeloma proteins, to red cells. A different approach is to perform inhibition tests with a purified class of Ig by reacting macrophages with soluble Ig before the reaction with opsonized red cells

occurs. In guinea pigs, where it was first shown, γ_2 antibodies to red cells formed rosettes on the macrophages, but γ_1 antibodies of a comparable antibody titer did not (Berken and Benacerraf, 1966).

In humans, IgG antibodies are cytophilic (Huber and Fudenberg, 1968; Abramson *et al.*, 1970b; Inchley *et al.*, 1970). Among the IgG subclasses, only IgG₁ and IgG₃ bind to macrophages (Huber and Fudenberg, 1968; Abramson *et al.*, 1970b). In mice, IgG and IgM are cytophilic (Lay and Nussenzweig, 1969). Cytophilic Ig's are specific in that their attachment is blocked only by homologous Ig (Inchley *et al.*, 1970; Huber and Fudenberg, 1968; Huber *et al.*, 1969; Abramson *et al.*, 1970b). Note that IgM is not cytophilic in all species: mouse IgM binds to macrophages, but human IgM does not. As expected, cytophilic Ig binds to the macrophage through the Fc part of the molecule. Red cells coated with Fab antibodies do not form rosettes on macrophages (Berken and Benacerraf, 1966); purified Fc but not Fab inhibits the rosette reaction (Inchley *et al.*, 1970; Abramson *et al.*, 1970b). A word of caution must be expressed concerning cytophilic tests in which macrophages and antibodies derive from different species. Indeed, not all Ig reacts equally with macrophages of every species. For example, Kossard and Nelson (1968a) found that guinea pig antibodies were taken up to a limited extent, if at all, by mouse and rabbit macrophages, although guinea pig macrophages did react well with mouse and rabbit antibodies.

The nature of the cytophilic receptor on macrophages is not known. Treatment of the macrophages with trypsin does not affect the binding of red cells coated with IgG antibodies (Kossard and Nelson, 1968b; Howard and Benacerraf, 1966) but does affect the binding of red cells coated with IgM antibodies (Lay and Nussenzweig, 1969). Free sulfhydryl (SH) groups form part of the receptor site, since treatment of macrophages with reagents that react with free SH groups (such as iodoacetamide) stops the attachment of opsonized red cells. The number of Ig receptors has not been definitely established. Claims have been made that an adult macrophage may have about 2×10^6 sites. These claims are based on the amounts of radiolabeled Ig that binds to saturation on a macrophage. These figures may be an overestimate in that the Fab portion of the Ig may interact with the macrophage on a site different from that where the Fc region acts.

The mechanisms of binding of Ig in free form or as an immune complex to the macrophage membrane have been the subject of some controversy. It was speculated that when antibody bound to antigen it suffered some conformational change in its Fc region and that this change was responsible for attachment to the cell. The observed facts, however, do not support this argument. Soluble Ig devoid of aggregates binds to

the macrophage. This can be tested by showing the binding of radio-iodinated Ig to macrophages and, best of all, by showing that Ig can block to some extent the binding of antigen-antibody complexes to the macrophage. It is apparent, however, that the interaction of monomeric Ig with the macrophage is weak and dissociates readily. However, antibody complexed to antigen or aggregated strongly interacts with the macrophage. Phillips-Quagliata and coworkers (1969, 1971) studied the interaction of macrophages with monovalent, bivalent, and polyvalent haptens bound to antibodies. Polyvalent and bivalent haptens complexed at equivalence with antibody were avidly taken up by macrophages. However, the same haptens in antigen excess were unable to enhance this uptake. Moreover, uptake by macrophages of soluble antibody or antibody bound to a monovalent hapten was comparable. The authors as well as others hypothesized (and we agree) that the increased interaction of antibody with the macrophage, in the presence of antigen, is not the result of an allosteric change in the Ig molecule but probably results from additional binding sites from each Ig molecule forming the complex.

2. Complement Receptor on Macrophages

Macrophages have a receptor on their membranes for C (Lay and Nussenzweig, 1968; Huber *et al.*, 1968; Henson, 1969). Lay and Nussenzweig (1968) found that red cells coated with rabbit γ M antibodies did not bind to mouse macrophages unless serum was added. This binding was dependent on divalent cations and could be reversed by addition of ethylenediaminetetraacetate (EDTA). The receptor on the macrophage was sensitive to treatment with trypsin. Huber *et al.* (1968) showed that the binding of antigen-antibody-C complexes to human macrophages was due to the presence of bound C3. Thus red cells that had reacted with antibody and C1, C14, or C142 did not attach to macrophages; however, red cells that had reacted with antibody and C1423 attached to the macrophages. Soluble Ig or serum did not interfere with the binding of antigen-antibody-C1423 complexes to macrophages. As would be expected from these results, serum from animals deficient in C5 or C6 could be used as a source of C in this test (Lay and Nussenzweig, 1968). The role of macrophage-bound C3 may be of considerable importance in promoting phagocytosis. For example, complexes of human IgG with antigen could be easily blocked from attaching to macrophages by soluble Ig *in vitro*. Still, soluble Ig or serum did not block the binding of antigen-antibody-C complexes to macrophages. Spiegelberg *et al.* (1963) have shown the importance of C in the clearance of bacteria and red cells from the circulation. Decomplemented mice were no longer able to eliminate antibody-coated *Escherichia coli* and rat erythrocytes from

the circulation rapidly, yet they quickly eliminated *E. coli* previously opsonized with antibody and fresh mouse serum (as a C source).

The IgG and C3 receptors on the macrophages enjoy cooperative interaction (Huber *et al.*, 1968). About 100 molecules of C3 lead to ingestion of IgG antibody-antigen complexes; however, tenfold more C3 molecules are needed to favor ingestion of complexes made by IgM (which by itself has no attachment site for human monocytes). The C3 receptor on macrophages is probably similar to the immune adherence receptor described previously (Nelson, 1963). Granulocytes also possess a receptor for C3 (Shin *et al.*, 1969; Gigli and Nelson, 1968).

There is suggestive evidence that C5 may also be involved in the promotion of phagocytosis, at least by granulocytes. Phagocytosis of *Candida albicans* (Morelli and Rosenberg, 1971) and *Pneumococcus* (Shin *et al.*, 1969) was somewhat depressed in serum from congenitally C5-deficient mice. M. E. Miller and Nilsson (1970) have described a hereditary dysfunction of C5 which results in deficient phagocytosis of yeast by granulocytes.

In summary, although we do not understand the basic molecular interactions involved in the uptake of a variety of antigenic and non-antigenic particles, nevertheless certain conclusions can be reached insofar as the process of antigen recognition by macrophages is concerned. Although it has become quite apparent that only few lymphocytes can interact with a single antigen (Naor and Sulitzeanu, 1967; Ada and Byrt, 1969; Humphrey and Keller, 1970), presumably these being a selected clonal population, specific immune recognition by macrophages is nonexistent (unless the antigen is itself coated with antibody and/or antibody and C). Lymphocytes bind to antigen as a result of antibody receptors synthesized by the cell; macrophages bind antigen directly or because the antigen is coated by antibody (made by a lymphocyte). Thus, macrophages take up a wide variety of substances regardless of whether these are immunogenic or nonimmunogenic. The responsibility to recognize foreignness in an immune response lies in the lymphocyte population—if antigens do not reach lymphocytes which are specific for them, then there will be no recognition (Ada and Byrt, 1969; Humphrey and Keller, 1970) and no immune process.

B. THE IMMUNOGENIC MOIETY

Most of the antigen taken up by macrophages is degraded and does not participate in immune induction. Of the total antigen taken up by the cell, only a small fraction escapes complete digestion and becomes available for lymphocyte recognition. Various experimental approaches have been used for identifying immunogenic antigen and its locus on the

macrophage. In some experiments the antigens have been followed by using live macrophages in a transfer system. In other experiments, cells have been disrupted and the fractions tested for immunogenicity. Obviously this approach offers no certainty that any extracted immunogenic material is functional *in vivo*. A combination of tissue culture methods plus the macrophage transfer system, although showing that a great part of the antigen is lost as a result of catabolism, has indicated two possible pathways by which antigen may be presented to lymphocytes for immune recognition: one is by retention of nonpinocytized antigen on the surface membrane, and the second is by release of intracellular antigen, some of which may reattach to the surface of macrophages.

Most antigens that bind to the plasma membrane are interiorized in vesicles. Attachment, however, is not necessarily followed by endocytosis. Chemically treated red cells bind to the macrophage but are not endocytized unless specific antibody is added (Rabinovitch, 1967a,b). A similar example was seen with *Mycoplasma pulmonis* in tissue culture; the organisms attached to the macrophages but were not phagocytized until antibody was added to the culture (Jones and Hirsch, 1971). Presumably there was need for linkage of several close attachment sites for interiorization of the membrane. The antibody may be linking two red cells (or organisms) or linking one of them to the membrane site where the antibody Fc piece is attached. Endocytosis is a temperature- and energy-dependent process (Cohn, 1968), and the metabolic requirements vary depending upon the size of the interiorized vesicle (Cohn, 1968). After endocytosis, the endocytic vesicle fuses with primary lysosomes. The antigen is then exposed to the hydrolytic enzymes of the lysosomes and degraded (Cohn, 1968; Rabinovitch, 1968; Weissman and Dukor, 1970). Most of the endocytized antigen is extensively catabolized. For example, in tissue culture, macrophages digested most pinocytized ¹²⁵I-human albumin down to the level of amino acids (Ehrenreich and Cohn, 1967). Similar results were found with hemoglobin (Ehrenreich and Cohn, 1968). Also, many types of bacteria are well known to be extensively degraded by macrophages (Gill and Cole, 1965). However, synthetic polypeptides formed by D-amino acids or some carbohydrates, such as pneumococcal polysaccharides, were taken up by macrophages but were not digested. Presumably the macrophages did not have the enzymes necessary to catabolize these antigens. The effects of lysosomal enzymes on different materials will not be reviewed since Weissman and Dukor have recently examined this topic (1970).

Cohn in 1962 attempted to correlate immunogenicity with catabolism of antigen. He studied the degradation of ³²P-labeled *Escherichia coli* by rabbit macrophages and polymorphonuclear leukocytes and found

that the rates of ingestion, killing, and degradation were comparable (Cohn, 1963a,b). However, the immunogenicities of *E. coli* in live peritoneal macrophages and polymorphonuclear leukocytes (as assayed in a transfer system) were quite different. Although the immune response to *E. coli* ingested by macrophages persisted and was relatively unaltered for up to 2 hours, it was quickly destroyed by ingestion by polymorphonuclear leukocytes (Cohn, 1963b). The conclusion was that in some way immunogens from *E. coli* had resisted digestion by peritoneal macrophages and were available for immune recognition. [It had previously been shown that injection of *Salmonella typhi* into serous cavities of rabbits with a macrophage-rich exudate or a polymorphonuclear leukocyte-rich exudate led to immune responses only in the former case (Walsh and Smith, 1951).]

1. Protein Antigens on the Plasma Membrane

Some molecules of antigen could be retained on the surface of the macrophage for long periods of time. In one of our experiments, mouse macrophages were exposed to ^{125}I -labeled hemocyanin and, after uptake, were placed in suspension culture (Unanue and Askonas, 1968a). At various time intervals the amount of radioactivity associated with macrophages and in the culture fluid was determined; the live cells were then transferred to syngeneic hosts in order to study their immunogenicity. It was found that most of the protein radioactivity was readily lost from the macrophage and appeared in the fluid as protein-free iodine; however, after a few hours, a small residual amount of radioactivity persisted in the cells for up to 2 days of culture. Interestingly enough, the immune response to a constant number of macrophages in a sensitive system was comparable whether macrophages were transferred early or late after ingestion of the protein. (The immune response was comparable if macrophages were transferred early with 100% of the antigen initially taken up or late with about 10% of the antigen remaining on the cell.) Thus, the immune response must be directed to residual antigen (represented by, at the most, 10% of the total) and not to the 90% catabolized.

Some of the persisting protein antigen has been identified on the plasma membrane. Indications are that this plasma membrane-bound antigen mostly comprises molecules of sizes comparable to native antigen that has eluded endocytosis. The evidence is based on the following findings:

1. By electron microscopy using ^{125}I -labeled antigen, it was possible to detect a few molecules of antigen on the surface of the macrophage, although most of the antigen was associated with lysosomes (Unanue *et al.*, 1969b) (Fig. 6). (No evidence was obtained for two populations

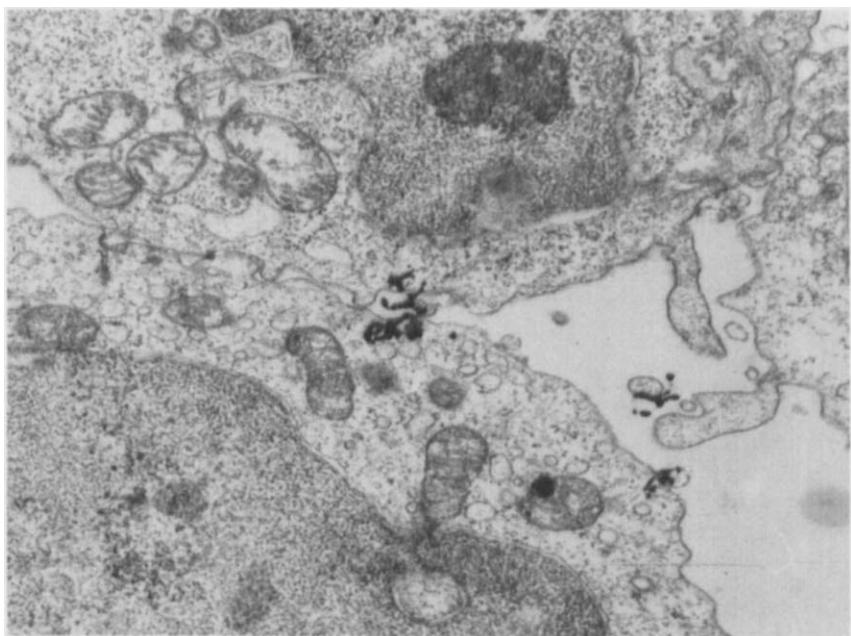


FIG. 6. An electron photomicrograph of a macrophage several hours after uptake of *Maia squinado* hemocyanin (MSH)-¹²⁵I. Grains are on the membrane of the cell. (From Unanue *et al.*, 1969b.)

of functional macrophages; most macrophages had antigen on the plasma membrane as well as in intracellular vesicles.)

2. Macrophages that had membrane-bound antigen were capable of reacting with specific antibodies or antibody fragments (Unanue *et al.*, 1969b; Unanue and Cerottini, 1970).

3. Treatment with trypsin or EDTA of macrophages that had taken up antigen removed a small amount of antigen from the surface of the cell (Unanue and Cerottini, 1970).

4. The antigen removed from the membrane was of a size comparable to the native molecule.

5. Removal of surface antigen on macrophages by treatment with trypsin or by covering with antibody abrogated most of the immune response to it (Unanue and Cerottini, 1970); see Table IV.

Other investigators have confirmed these observations. With EDTA, Askonas and Jaroskova (1970) removed *Maia squinado* hemocyanin that was retained on macrophages and thus abrogated a great part of their capacity to induce a response in the macrophage transfer system. Recently, Garcia found that macrophages exposed to diphtheria toxoid retained a few molecules on their membranes; such macrophages when

TABLE IV
EFFECT OF TREATING MACROPHAGES WITH ANTIBODY OR TRYPSIN^{a,b}

	HSA ABC-33	KLH ABC-33 (control/experimental)	MSH HA titer
Trypsin	18.6/0.0 ^c	9.4/2.4	Not done
Antibody	Not done	11.3/1.8	80/neg.

^a From Unanue *et al.* (1972).

^b Treatment of macrophages *in vitro* with trypsin or antibody markedly reduced the immune response to macrophage bound antigen. The immune response to human serum albumin (HSA) was determined 2 weeks after transfer of macrophages containing 4 µg. of HSA (titers are expressed as micrograms of HSA N bound per milliliter of serum $\times 10^3$). The immune response to keyhole limpet hemocyanin (KLH) represents the secondary antibody titers. In these experiments the mice were primed with 4 µg. of KLH. The immune response to *Maia squinado* hemocyanin (MSH) represents secondary hemagglutination titers. Mice were given 1 µg. of MSH in 4.5×10^6 cells, either treated or untreated with antibody, and challenged 4 weeks later with 1 µg. of free MSH i.p.

^c Titers after transfer of control; untreated cells/titers after transfer of treated cells.

added to lymph node cell fragments *in vitro* carried antigens and initiated a strong immune response (Garcia *et al.*, 1972). Removal of the membrane-bound antigen completely abrogated the immune response. Harris claimed the presence of membrane antigen in macrophages during *in vitro* DNA synthesis by lymphocytes (1965). Schmidtke and Dixon (1972) correlated the antigen retained on the membranes of X-irradiated macrophages with the immune response to macrophage-bound antigen.

The amount of antigen remaining on the plasma membrane in tissue culture experiments varied depending upon the type of antigen and state of activation of the macrophage. For example, 1-2% of hemocyanin molecules were retained for prolonged periods. In the case of polymeric albumin, although the amount was about the same, the retained molecules dissociated faster (half-life of about 1 day). Monomer albumin was readily lost from the membrane (Schmidtke and Unanue, 1971a). It is clear that different antigens have to be analyzed critically before generalizations are made. Concerning the state of activation of macrophages, the amount of the hemocyanin on the membranes of macrophages activated by *Listeria monocytogenes* infection was about 5 to 10 times more than in macrophages isolated from the unstimulated peritoneal cavity—as would be expected, the activated macrophages induced, when transferred, a somewhat higher immune response (Lane and Unanue, 1972).

The origin and persistence of the few molecules of protein antigens on the membrane of macrophages, a cell that displays strong endocytotic

activity, needs explaining. The evidence appears reasonably clear that most of the molecules derived from the first contact with extracellular antigen (Unanue and Cerottini, 1970; Schmidtke and Unanue, 1971a). This conclusion was reached after removing the surface-bound antigen by trypsin and then observing that new antigen did not appear on the membrane. No relationship was apparent between the antigen in intracellular vesicles and the antigen on the membrane (Unanue and Cerottini, 1970; Schmidtke and Unanue, 1971a). One explanation for the persistence of these few molecules may be that they were held on surface areas having little endocytotic activity. This would imply that the plasma membrane of macrophages is not homogeneous but is made up of different functional areas insofar as endocytosis is concerned. In fact, stimulating pinocytosis on macrophages that have retained antigen on their membranes did not result in the interiorization of the retained antigen [i.e., macrophages were exposed to antigen and cultured for a day at which time a small amount of antigen was retained on the surface whereas the bulk had been pinocytized and catabolized; the culture conditions were then changed to increase endocytosis (Unanue *et al.*, 1972)]. The recent experiments of Tsan and Berlin (1971) tend to support this explanation of different functional areas on the macrophage membrane. They found that the sites for amino acid transport on the macrophage remained the same before and after phagocytosis, i.e., despite interiorization of membrane as a result of endocytosis the transport sites were preserved. They envisioned the membrane as "mosaic in character with geographically separate transport and phagocytic sites."

2. Antigen in Intracellular Vesicles

Information on the immunogenicity of complex particulate antigens, such as red blood cells or microorganisms, when taken up by macrophages is confused by the chemical complexity of the particulate antigen and the poorly understood nature of immunogens. Apart from this, particulate antigens are not always easy to separate from macrophages, and there is always the possibility that tests of macrophage-associated antigens are contaminated with free nonphagocytized antigens. Some experiments showing immunogenicity of particulate antigens bound to live macrophages appear in Table I. In most of these experiments the antigen presumably had been interiorized by the macrophage, although there was no conclusive proof of this. It is possible, although unlikely, that these antigens remained on the surface membrane without being endocytized for some time (evidence for intact SRBC in or around macrophages has been brought forth in Mishell-Dutton *in vitro* responses).

Cruchaud and I attempted to follow some antigens of foreign red cells to obtain some idea of the possible fate of the endocytized immunogen (Cruchaud and Unanue, 1971a). We used SRBC the stroma of which were labeled with ^{125}I or to which specific IgG- ^{125}I was bound. The macrophages were exposed to SRBC for a period of time after which the nonphagocytized SRBC were eliminated. The cells were then placed in culture for up to 1-3 days. It was found that, after the period of culture, partially degraded material appeared in the culture fluid. In the case of ^{125}I -labeled antibody the released material was a fragment of the Ig molecule. We concluded that small amounts of the endocytized antigens had escaped complete degradation, were released, and, hence, had become available for immune recognition. Evidence also indicated that the antigens from particulate substances liberated by the macrophage could bind *de novo* to their surface membrane for presentation to lymphocytes. Indeed, treatment of macrophages that had taken up SRBC with trypsin or antibody curbed their immunogenicity (as assayed in the macrophage transfer system). The exact pathway that the material followed in the cell and the conditions determining its release have not been established. Indeed, there is not much information available on the final intracellular fates of different phagocytic vesicles. Is it possible that some phagocytic vesicles are channeled into a different pathway, away from typical primary lysosomes, or that some vesicles fuse with lysosomes that have a small content of hydrolases? Can the digestive process in a phagolysosome be uneven with a resulting pool in the vesicle of undigested or partially digested material?

Many other experiments have demonstrated that immunogenic material could be extracted from intracellular vesicles for some time after uptake. Uhr and Weissman isolated a "large granule" fraction from liver macrophages that had taken up bacteriophages and which was immunogenic as well as containing active phage (Uhr and Weissman, 1965, 1968). Franzl (1962) isolated "lysosomal" fractions, which were also immunogenic, from spleens of mice injected 1 and 3 days previously with SRBC. Kolsch and Mitchison (1968) identified antigen in two cell compartments: one was present in a light sedimenting fraction with a rapid turnover to which 90% of the antigen was channeled; the other was present in a dense sedimenting fraction of slow turnover and associated with the remaining 10% of the antigen. This last dense, sedimenting compartment was heterogeneous and most likely contained cell membrane, nuclei, and debris. These same investigators also found that antigen bound to live macrophages was more immunogenic than the antigen extracted from the cytoplasm. The SRBC antigens extracted from macrophages also induced strong delayed reactions (Pearson and Raffel, 1971).

Antigen extracted from macrophages has been found complexed with RNA (see below).

The release process of intracellular antigen is undetermined. It appears clear that soluble antigens do not cross the membrane of the secondary lysosome under normal circumstances. The extent to which the lysosomal membrane is permeable to small products of catabolism was investigated by Ehrenreich and Cohn (1969). They exposed macrophages to di- or tripeptides made of D-amino acids which were not degradable by macrophage enzymes. The macrophages took up the peptides and stored them in secondary lysosomes for some periods of time—the distended secondary lysosomes containing the peptides were easily visualized as translucent vesicles by phase microscopy. In contrast, small peptides of L-amino acids were degraded, easily crossed the membrane, and left the cell. The conclusion was that the lysosomal membrane was only permeable to small peptides up to the level of 2 or 3 amino acids.

Antigen could be released from a cell as a result of death, by channels connecting the phagocytic vesicle with the surface of the cell or by exocytosis. Death of cells is known to lead to the release of cytoplasmic material and could account for release of intracellular antigens. Yet in the experiments of Cruchaud and Unanue (1971a) the estimated amount of cell death could not account for the release of antigen, although direct measurement of other cell cytoplasmic components in the culture fluids was not done. It should be pointed out that this release of antigen from macrophage may be akin to the release of lysosomal enzymes that has been observed to take place after phagocytosis of large particles by both macrophages (Weissman *et al.*, 1971b) and polymorphonuclear leukocytes (Henson, 1971; Weissman *et al.*, 1971b). Weissman *et al.* (1971b) found that macrophages which had taken up zymosan particles released lysosomal β -glucuronidase into the culture fluid but did not release lactic dehydrogenase, a cytoplasmic enzyme. To this effect both Henson (1971) and Karnovsky and Karnovsky (1971) have ultrastructural evidence in polymorphonuclear leukocytes for openings or channels from the phagocytic vesicles to the surface through which the enzymes could be released. The final possible pathway of release of intracellular antigen is by reversed pinocytosis or exocytosis. Exocytosis of antigen is a possibility that has been raised to explain the continuous presence of small amounts of undigested antigens in body fluids (Janeway and Humphrey, 1969). Such antigens are taken up and stored by the RES. There is at present no morphological documentation of exocytosis by macrophages.

3. RNA-Antigen Complexes

In early 1960, Fishman (1961) and Fishman and Adler (1963b) reported that lymphocytes responded immunologically to extracts of peri-

toneal exudate cells from rats injected a few minutes previously with T2 phage. The experimental system consisted of culturing lymph node cells or, alternatively, of placing lymph node cells in diffusion chambers and implanting them into X-irradiated rats (reviewed in Fishman and Adler, 1963a). The filtrate from peritoneal cells exposed to T2 phage induced neutralizing antibodies by 8 days of culture; T2 phage by itself or normal extract of macrophage (i.e., not exposed to T2 phage) did not induce antibody. The antibody response was specific in that the cultures did not contain neutralizing antibodies to T1 or T5 phage. The same authors demonstrated that the extract was sensitive to ribonuclease (RNase) treatment; indeed, extracting RNA with cold phenol yielded active material which sedimented as a light molecular weight RNA. It was these kinds of experimental results that led many to conclude that for an antigen to be immunogenic it had to be "processed" by macrophages. The system itself has been criticized by those familiar with tissue culture assays because some of the early results were not reproducible, and antibody titers were low.

It was subsequently found that antigen—or still better, a fragment of it—was associated with the RNA extract. Askonas and Rhodes (1965a,b) isolated RNA (by cold phenol extraction) from murine macrophages that had taken up ^{125}I -labeled hemocyanin. A small amount of the iodinated protein (about 0.01% of the total taken by the macrophage) was found associated with RNA. The material, when injected into mice, was discovered to be highly immunogenic. Friedman *et al.* (1965) incubated peritoneal exudate cells, *in vitro*, with T2 phage, isolated RNA, and found, by using complement fixation assays, antigens of head, tail, and internal protein of the phage in the extract. Subsequent studies have confirmed these two early findings; indeed, after uptake of many antigens by macrophages, a complex of RNA with antigen can be isolated from these cells (Gottlieb and Doty, 1967; Roelants and Goodman, 1968; Herscowitz and Stelos, 1970a,b; Bishop and Gottlieb, 1971). The biological significance of this finding is in doubt, however, and strong evidence has been presented that the phenomenon may even be a laboratory artifact (Roelants and Goodman, 1969; Goodman, 1972).

Some of the characteristics of the RNA-antigen complex are known. The complex isolated from macrophages recently exposed to the antigen (Fishman and Adler, 1963a) did not involve newly synthesized RNA (Gottlieb and Doty, 1967). In one such experiment, macrophages were pulsed with tritiated uridine at the same time that T2 phage was added to the cells. The RNA was extracted and analyzed in a gradient. The fraction containing the immunogenic moiety was not associated with the radioactive label. The size of the RNA-antigen complex appeared to be heterogeneous probably because of the different sizes of the antigen

associated with the RNA (Saha *et al.*, 1964; Roelants and Goodman, 1968; Roelants *et al.*, 1971). Gottlieb, however, found the RNA banding in cesium sulfate gradients at an invariable density of 1.588 (Gottlieb, 1969; Gottlieb and Straus, 1969); this band disappeared after treatment with proteolytic enzymes (Gottlieb and Doty, 1967). Roelants and Goodman (1969) estimated that their RNA-antigen complex had an S value of about 4.

Many antigens taken up by macrophages could complex with RNA—the common denominator of these antigens was the presence of charged groups in the molecule (Roelants and Goodman, 1969). Uncharged antigens, although immunogenic *in vivo*, were not found to be associated with RNA from macrophages. Mixing RNA with antigen *in vitro* did not result in complex formation (Roelants and Goodman, 1968). Roelants and Goodman (1969) studied the binding of pure RNA to negatively charged antigen and found that the presence of Mg salts complemented binding; they also found that the RNA-antigen complex dissociated after exhaustive dialysis against buffers containing chelating agents. Their explanation was that antigen chelated to Mg would bind and aggregate a transfer-type RNA present as a precursor in an unspun or expanded form. Thus, the complex may be entirely a laboratory artifact resulting from cellular disruption which allows RNA to mix with antigenic fragments. Indeed, the lysosomal membrane was highly impermeable to small antigen fragments of up to a size of 2 or 3 amino acids (Ehrenreich and Cohn, 1969). It cannot be ruled out, however, that local combination of RNA with antigen fragments can occur as a result of cell death in tissues. Garvey *et al.* (1967) have reported on the presence of RNA complexed to antigen in urine.

Is the RNA-antigen complex specific to macrophages? Not all investigators agree here. Gottlieb contended that the complex was, indeed, peculiar to macrophages since he was unable to find it among other types of cells. However, the recent experiments of Roelants *et al.* (1971) clearly indicated that *in vitro* complexing of antigen to RNA of other cells like HeLa or even bacteria (*Escherichia coli*) could occur. Moreover, in their hands, these complexes formed with RNA of other nonmacrophagic cells were highly immunogenic. Whether their antigen-RNA complexes are identical to those reported by Gottlieb has not been ascertained. Finally, it is also clear that there was no relationship between complexing of antigen to RNA and its immunogenic capacity when injected *in vivo* (Roelants and Goodman, 1969). For example, a poly- γ -D-glutamyl polypeptide was found to complex with 4 S RNA of macrophages. Such a polypeptide, however, was nonimmunogenic when administered *in vivo* (unless complexed with methylated albumin) (Roelants and Goodman, 1968).

In summary, although the complexing of antigen with RNA may result in a strong immunogenic complex *in vitro*, we still have no indication that such a complex operates *in vivo*. Roelants and Goodman's experiments indicate that when a cell containing antigen in the vesicles is disrupted, it is practically unavoidable for the RNA and antigen to mix and not form a complex. Hence, the existence of complexes preceding cell disruption is questionable. As mentioned previously, however, such complexes could conceivably form *in vivo* in areas where cell death readily occurs after phagocytosis. The reasons for the high immunogenicity of the RNA-antigen complex are not known, but complexing of antigen to many chemicals may enhance immunogenicity by means not yet understood. It should be remembered that synthetic polynucleotide complexes, such as polyadenilic and polyuridilic acids, are potent adjuvants to antigens administered simultaneously (Braun *et al.*, 1968; Schmidtko and Johnson, 1971).

Some observations on RNA from macrophages are not explicable on the basis of an RNA-antigen complex. In these RNA extracted from peritoneal exudate cells exposed to phage from rabbits of a given allotype was mixed with normal lymphocytes of rabbits of a different allotype. Part of the antibody molecules (the IgM) carried the allotypic specificities of the donor (Adler *et al.*, 1968). Similar experiments with extracts from lymphocytes (Bell and Dray, 1969, 1970) suggest a messenger-type function in the RNA extract, the significance of which is not known. These results have been difficult to reproduce (Bluestein *et al.*, 1970) and need to be confirmed by other investigators. Furthermore, this phenomenon may merely represent a laboratory exercise with no relevance to *in vivo* experiments. Recently, Goodman (1972) has discussed these experiments at length.

IV. Macrophage-Lymphocyte Contact

If one of the functions of macrophages is to present some molecules of antigen to lymphocytes for immune recognition, it is reasonable to expect that there should be a close anatomical association of both cells at some time. Clusters of lymphocytes and plasma cells around macrophages are frequently observed in the perifollicular areas of lymph nodes (Thiery, 1962; H. R. P. Miller and Avrameas, 1971). Needless to say, the association of lymphocytes around dendritic cells of the follicles is a very close one. There is one ultrastructural study (Schoenberg *et al.*, 1964) on cell fusion between macrophages and lymphocytes of the spleen, but this phenomenon, if present, appears to be the exception rather than the rule. In the Mishell-Dutton tissue culture system, lymphocytes readily cluster around macrophages; the importance of this clustering for immune induction has been discussed previously (Mosier,

1967; Pierce and Benacerraf, 1969). Electron microscopy of these clusters reveals close approximation of lymphocytes with macrophages but no cytoplasmic fusion (McIntyre *et al.*, 1971). In tissue cultures employing diffusion chambers, it was possible to identify cytologically the lymphocytes clustering around a central macrophage containing the antigen (Sulitzeanu *et al.*, 1971). Lymphocytes have also been shown to cluster around blastlike large cells of unknown origin (Sulitzeanu *et al.*, 1971). McFarland and colleagues have studied the movement of lymphocytes around macrophages (or other cells) and have identified a special area in one pole of the lymphocytes—the uropod—which may be important in cell-to-cell contact (Fig. 7) (McFarland and Heilman, 1965; McFarland *et al.*, 1966; McFarland, 1969). Recently it has been shown that B lymphocytes tend to attach preferentially to macrophages (Schmidtke and Unanue, 1971b). This adherence resulted from interaction of Ig receptors on the B lymphocytes with the Fc cytophilic receptor on macrophages, since it was blocked by aggregated Ig. Moreover, B lymphocytes did not attach preferentially to fibroblasts, cells lacking receptors for Ig. There is a report of adherence of guinea pig thymocytes to macrophages—adherence was increased by addition of guinea pig serum and abolished by trypsinization of macrophages. Heterologous thymocytes did not adhere to the guinea pig macrophages (Siegel, 1970).

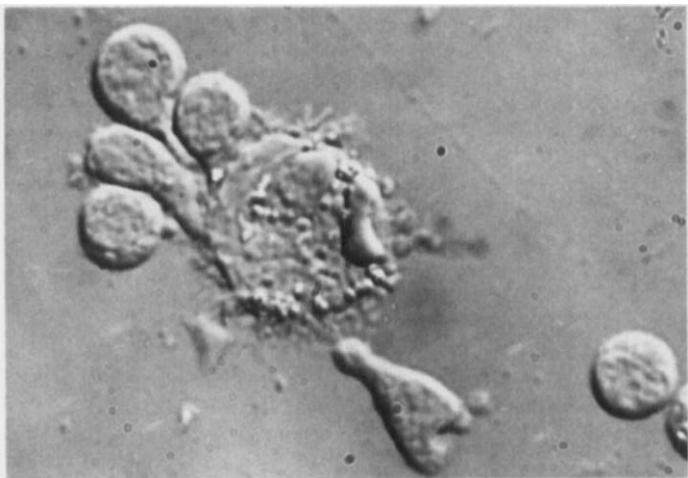


FIG. 7. Photomicrograph of a mixed leukocyte culture. Lymphocytes are in close approximation to a macrophage. The typical appearance of two lymphocytes forming the uropod is clearly seen. Original photographs were taken with Nomarski interference microscopy at an approximate magnification of 600. I thank Dr. W. McFarland (Veterans Administration Hospital, Washington, D. C.) for allowing us to publish this photograph.

V. Macrophages and Adjuvants

In recent years it was speculated that macrophages might be involved in the heightened immune response produced by adjuvants, although nowhere has direct, clear evidence been introduced.

1. Many adjuvants such as the water-in-oil emulsions or alum emulsions, devised originally by Freund, produced a marked inflammatory reaction at the tissue of inoculation or at distinct sites characterized by a heavy macrophage infiltrate (White *et al.*, 1955; French *et al.*, 1970; Steinberg *et al.*, 1970). The macrophages of this inflammatory reaction formed epithelioid and giant cells. Plasma cells secreting specific antibody were abundant in the granulomatous tissue. White and collaborators, when studying the immunological response in chickens to human albumin mixed with water-in-oil emulsions (French *et al.*, 1970; Steinberg *et al.*, 1970) observed that there was a small early peak of antibody synthesis (which was also present in animals given albumin without adjuvants) followed by a very large peak about 40 days after injection. The onset of the last peak coincided with the development of a large granulomatous reaction at the site of the adjuvant-antigen depot. The granuloma was characterized by abundant plasma cells secreting specific antibody in contrast to the spleen which showed a marked paucity of plasma cells. Interestingly, thymectomy but not bursectomy of the chicken resulted in marked reduction of the granuloma. Bursectomy resulted in a reduced peak antibody response and in fewer plasma cells in the granuloma; the hyperactive macrophages were still abundant in the granuloma of these chickens. The conclusion, which seems reasonably clear, was that the late granuloma reaction (with the hyperactivated macrophages) was associated with the presence of an intact thymus—perhaps abundant macrophages at the granuloma contributed in some way to the heightened immune reaction. In fact, Humphrey and Turk, in 1963, reported and Leskowitz (1970) confirmed that injection of diphtheria toxoid at the skin site of a delayed hypersensitivity reaction to tuberculin (which is full of macrophages) resulted in 2 to 4 times more antitoxoid antibody production.

2. A series of adjuvants have marked effects on lysosomal membranes. These adjuvants include silica (Pernis and Paronetto, 1962), vitamin A or retinol (Dresser, 1968; Spitznagel and Allison, 1970a), endotoxin (Spitznagel and Allison, 1970a), beryllium (Unanue *et al.*, 1969a), and *Bordetella pertussis* vaccine (Unanue *et al.*, 1969a). Culture with these adjuvants led to marked effects on macrophages (Kessel *et al.*, 1963; Kessel and Braun, 1965; Allison *et al.*, 1966; Wiener and Levanon, 1968; Heilman and Robert, 1967; Heilman, 1965) (Fig. 8) characterized

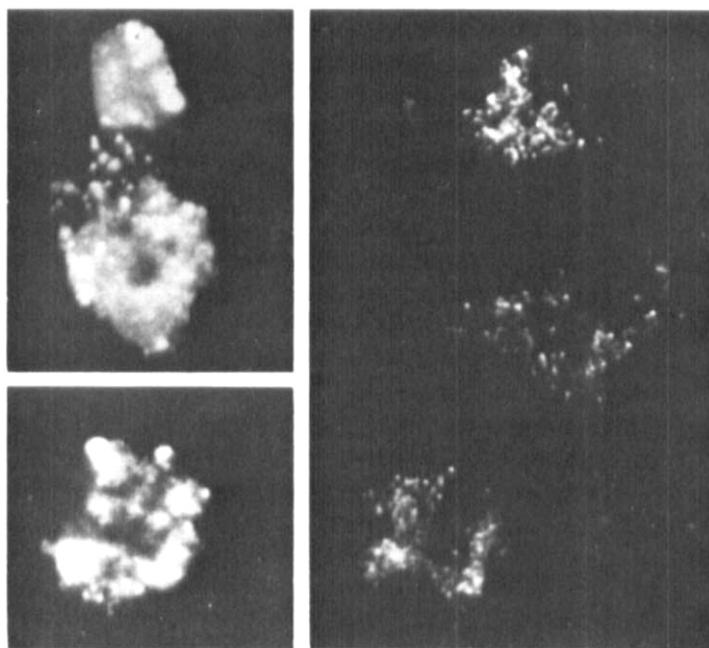


FIG. 8. Changes in appearance of macrophages and number of lysosomes after exposure to immunological adjuvants. Lysosomes were visualized by exposing the cells to the fluorescent dye, Euchrysine. The treated macrophages to the left show large Euchrysine-positive vacuoles; the macrophages on the right are controls. Results similar to these can be obtained by incubating macrophages with endotoxin, beryllium, *Bordetella pertussis*, etc. [see *J. Immunol.* 103, 7 (1969). This technique of staining live cells with Euchrysine is described by Allison and Young (1964).]

by formation of large lysosomes, increased hydrolytic enzymes, and, at large doses, death. Macrophages containing antigen and treated with adjuvants were studied in the macrophage transfer system. It was found that mice made a stronger immune response to hemocyanin bound to beryllium- or *B. pertussis*-treated macrophages than to normal macrophages. This heightened immune response was unrelated to an effect on the handling of antigen, since transfer of macrophages containing antigen together with macrophages treated with the adjuvant resulted in an increased response. In essence, part of the adjuvant effect could be transferred by macrophages exposed to these adjuvants. However, the nature of this effect was not determined. Adjuvants increased the formation of lysophosphatides and free fatty acids in macrophages, and some have speculated that these surface active substances may mediate the adjuvant effect (Munder *et al.*, 1970).

It has been shown that beryllium (Schmidtke and Unanue, in preparation), or vitamin A (Dresser *et al.*, 1970), which were so avidly taken up by tissue macrophages, could produce marked effects on the distribution pattern of lymphoid cells in spleen and lymph nodes. These effects were seen early (usually within the first 24 hours) and were characterized by transitory increase of lymphocytes in the paracortex. Injection of adjuvant with ^{51}Cr -labeled lymphocytes led to their increased entrapment in the spleen.

3. There was a marked adjuvant effect when antigen was administered together with lactic dehydrogenase virus (Notkins *et al.*, 1966; Mergenhagen *et al.*, 1967) or *Listeria monocytogenes* (Lane and Unanue, 1972). Lactic dehydrogenase virus is readily taken up by macrophages (DuBuy and Johnson, 1969), whereas listeriosis is known to produce marked macrophage activation.

The mechanisms of adjuvant action and the role of the macrophage were not established in any of these three situations. The evidence for some role of macrophages—perhaps by antigen presentation, by affecting in some way neighboring cells through release of inflammatory factors, or by affecting the traffic of cells in a lymphoid organ—is circumstantial but suggestive enough to warrant close attention and further experimentation.

VI. Summary

The immune response to many antigens involves the participation of two lymphocytes (T and B) that are specifically committed to react with different but closely spaced antigenic determinants (J. A. F. P. Miller *et al.*, 1971). These lymphocytes appear to have Ig receptors on their membranes and can bind antigen. Participating in this interaction between antigen and T and B lymphocytes is the macrophage, a cell line endowed with the properties of taking up, degrading, and retaining a wide range of antigens. There is agreement that the specificity of the immune response lies in the T and B lymphocytes—the macrophage handles a variety of materials either immunogenic or not without much immunological discrimination. The macrophage does not instruct the lymphocyte to react wth antigen, but rather it is the committed lymphocyte that selects the molecule of antigen with which it can react.

The experimental results that show immunogenic material associated with macrophages appear reasonably clear, although the molecular basis for macrophage-antigen-lymphocyte interaction and for lymphocyte triggering needs to be determined. The participation of macrophages in the context of the lymph node and spleen histology is not resolved. Further work needs to be done in order to clarify the origin and func-

tion of the elusive dendritic cells of the lymphoid follicles. The pathways for presentation of immunogenic moieties by macrophages, although outlined, need further exploration with several types of antigen. Finally, improvement of and experience with tissue culture methodology should make possible clarification of many of the problems associated with cellular interactions involving macrophages and lymphocytes.

The effects of macrophages in immune induction can be discussed along three separate lines: (1) the capacity of the macrophage to remove and eliminate extracellular antigen; (2) their property of holding a few immunogenic moieties; and (3) their ability to degrade antigen.

1. The macrophage of both lymphoid and nonlymphoid tissues participates in the removal and degradation of soluble or particulate antigen molecules from the circulation and extracellular fluids, a process which is critical for immune induction. The presence of disseminated antigen molecules might eventually lead to their interaction with isolated T or B lymphocytes. Such an isolated interaction (without other cells participating) might result in an ineffective process. On the one hand, a T lymphocyte that meets antigen outside the lymphoid tissues, even if triggered to proliferate, does not have a responding B lymphocyte nearby and, therefore, part of its biological function is lost; on the other hand, a B lymphocyte that meets antigen will not be triggered unless a T cell is in close proximity (see Katz and Benacerraf, 1972). Critical experiments showing the deleterious effects of a large pool of unconcentrated antigen are that (*a*) the injection of molecules of monomer albumin or Ig leads to tolerance, whereas injection of polymeric molecules that leave the circulation produces immunity (Section II,A,3); (*b*) injection of free antigen together with macrophage-bound antigen reduces the response to the latter—in essence, a competition for the lymphocyte between the extracellular diffusible antigen and the concentrated moieties exists (Section II,A,5); and, very important, (*c*), in cases where the RES cannot degrade antigen, these molecules persist throughout the life of the animal and lead to life-long tolerance (here the RES may even contribute to tolerance by continuous release of the undegraded antigen) (Howard and Siskind, 1969; Janeway and Humphrey, 1969; Medlin *et al.*, 1970). In these experiments we also have to consider the possibility that “monomeric” molecules by themselves may not trigger the T and B lymphocyte and the “polymeric” forms, whether macrophage-bound or free, may be needed to induce the cells to proliferate—analysis of the mechanism of cell triggering will presumably solve this problem.

2. We conclude from the preceding discussion that the diffusion of isolated molecules of antigen throughout tissues may be detrimental and that for antigen to initiate an immune process effectively it should

be concentrated in critical sites. There are indications for a homeostatic process that concentrates antigen and lymphocytes in the lymphoid tissues. Antigen is concentrated in part or totally by macrophage uptake (Section II,A,1); after antigen injection, circulating lymphocytes pool into a lymph node or the spleen by mechanisms yet unknown (Zatz and Lance, 1971). Hence, the proposed helper role of the macrophage is to favor the meeting of antigen with the few committed T and B lymphocytes at one point. This statement does not rule out that free molecules of some antigens in a lymphoid tissue, if available, may lead to effective induction of immunity. Clearly, antigen bound to macrophages in the absence of free extracellular antigen may be a very effective pathway for immunogenicity, as was discussed in Section II,A,5 where it was mentioned that both T and B cells may need to interact with some macrophage-bound antigen. The length of time that antigen bound to macrophages helps in the inductive process is not clearly determined (see Section II,A,2).

3. The final point concerns the need for processing or for changing the structure of the antigen in order that it can be recognized by the lymphocytes. This point can be analyzed with respect to different kinds of antigen. Is there a need for degradation or change of polypeptides or proteins? It is apparent that such antigens are recognized before degradation, since most of the antibodies that they elicit are directed against steric or conformational determinants (Sela *et al.*, 1967; Sela, 1969). Moreover, the need for two close determinants (a "hapten" and a "carrier") for immune induction precludes any breakdown or digestion of the antigen before recognition. The need to maintain native configuration does not rule out participation of macrophages in induction of antibody. We now know that the immune response to proteins bound to macrophages is directed against molecules, although few in number, which have not been digested (Unanue and Askonas, 1968a; Unanue and Cerottini, 1970). To this end the response to hapten carriers can take place after uptake of antigen by macrophages (Hamaoka *et al.*, 1971)—another indication that the immunogenic protein moieties in macrophages are represented by nondegradable antigen. It is also clear that native proteins can bind to lymphocytes (Naor and Sulitzeanu, 1967; Ada and Byrt, 1969; Humphrey and Keller, 1970) and, in fact, can trigger the cells under appropriate circumstances (Britton *et al.*, 1971). Hence, the role of the macrophages related to protein antigens may be explained mainly by their capacity to concentrate the antigen and allow for cell interactions as discussed above.

Insofar as large particulate antigens are concerned, the information available is neither extensive nor conclusive. Lymphocytes can react

TABLE V
ROLE OF MACROPHAGE IN IMMUNE RESPONSE TO FOUR KINDS OF ANTIGEN

Antigen	Macrophage function in immune response*		
	Removal of antigen from extracellular fluids	Concentration and presentation of antigen	Change of antigen
1. Soluble antigens: mainly proteins (extent of uptake may depend upon degree of polymerization)	Very important in nonimmune individuals (1, 2). In immune individuals antibody may aid in eliminating antigen.	Important depending upon number of specific T and B lymphocytes (1). No information available on the extent to which antigen is presented by macrophages as compared to other pathways.	Macrophage does not alter the molecule but plays a passive role by presentation of "native" molecule (3, 4). More information needed in molecular mechanisms of lymphocyte triggering.
2. Particulate antigens: bacteria and red cells (highly phagocytized by macrophages)	Important depending upon site of entry of antigen and upon the amount of antigen administered—nonlymphoid macrophages may reduce the amount of antigen available to lymphoid organs and, therefore, reduce immune response (5).	Important (6)—depends upon number of T and B lymphocytes.	Some experiments indicate that partial degradation may be necessary (7, 8).

3. T independent antigens, such as pneumococcal polysaccharide or polymerized flagellin (antigens with repeating antigen determinants)	Important—in the case of S _{III} (or other noncatabolizable antigens) the lack of removal may lead to persistence of tolerance (9, 10)	May depend upon number of B lymphocytes—no information available.	Not necessary (7)
4. Tissue or cell antigens, such as tumors or grafts (no diffusible antigen)	Not necessary	Not necessary	May not be necessary

* Key to references:

- 1. Mitchison, 1969b
- 2. Spitznagel and Allison, 1970b
- 3. Unanue and Cerottini, 1970
- 4. Unanue *et al.*, 1969
- 5. Perkins, 1970
- 6. Goreczynski *et al.*, 1971
- 7. Shortman and Palmer, 1971
- 8. M. Feldman and Palmer, 1971
- 9. Howard and Siskind, 1969
- 10. Medlin *et al.*, 1970

The references refer to key papers that have dealt with the particular problem—the conclusions in this Table are mine and do not necessarily reflect that of the authors of the reference.

with red cells or *Shigella*, for example, but we do not know if that interaction can trigger the lymphoid cell. Some experiments suggest that breakdown by macrophages of the SRBC (Shortman and Palmer, 1971; M. Feldman and Palmer, 1971) or *Shigella* (Gallily and Feldman, 1967) may be needed for immune induction. These interesting observations need confirmation and should be extended to other large particulate antigens. Apart from this point, there is indirect information that there may be *in vivo* immune recognition of "hidden" bacterial antigenic determinants which most likely have been exposed as a result of partial degradation and release by macrophages (Schwab and Brown, 1968; Schwab and Ohanian, 1967). In this case the macrophage may contribute to the heterogeneity of antibody by releasing these hidden antigens; along these lines the RNA-antigen complex, whether formed before or after cell death, may add to the pool of immunogenic moieties.

The extent to which the three macrophage processes—antigen removal, antigen concentration and presentation, and antigen change— influence the immune response depends upon (1) amount of antigen, (2) physicochemistry of the antigen, (3) number of reactive T and B lymphocytes, (4) T dependency of the antigen, (5) presence or absence of adjuvant-type effects, (6) the anatomical site of antigen arrival, and (7) the molecular mechanisms of lymphocyte triggering. Some of these variables have been considered. It is apparent that the degree to which the immune response needs macrophage participation may be very different in the case of a soluble antigen and in the case of an antigen forming part of a cell membrane, such as a tumor cell. In the latter case there is no need for antigen removal nor for concentration (i.e., the antigens are already concentrated on a membrane and there is no extracellular diffusible antigen); also, the lymphocyte apparently can recognize the concentrated tissue antigen without any need of macrophages. In contrast, in the case of soluble antigen, macrophage participation is clearly present but is influenced by the number of reactive lymphocytes, amount of antibody, or by concomitant injection of adjuvants. In judging such a complicated process as immune induction, we must take into consideration each variable. Table V is an attempt to summarize these points with few different antigenic stimuli and with the incomplete series of observations now at hand. Perhaps in the future when more data will be available these points can be better documented.

ACKNOWLEDGMENTS

The work reported here was done in collaboration with many colleagues to whom I am deeply indebted. I wish to thank Brigitte A. Askonas, John H. Humphrey, Frank J. Dixon, and Baruj Benacerraf in whose laboratories I have had the opportunity to work and discuss many problems in the field of cellular immunology and

to express my appreciation to those investigators who sent me unpublished observations or who authorized me to reproduce some of their material. I thank Miss Karen Ellis for excellent secretarial assistance.

REFERENCES

- Abramson, N., LoBuglio, A. F., Jandl, J. H., and Cotran, R. S. (1970a). *J. Exp. Med.* **132**, 1191.
- Abramson, N., Gelfand, E. W., Jandl, J. H., and Rosen, F. S. (1970b). *J. Exp. Med.* **132**, 1207.
- Ada, G. L., and Byrt, P. (1969). *Nature (London)* **222**, 1291.
- Ada, G. L., and Lang, P. G. (1966). *Immunology* **10**, 431.
- Ada, G. L., Nossal, G. J. V., and Austin, C. M. (1964). *Aust. J. Exp. Biol. Med. Sci.* **42**, 331.
- Ada, G. L., Humphrey, J. H., Askonas, B. A., McDevitt, H. O., and Nossal, G. J. V. (1966). *Exp. Cell Res.* **41**, 557.
- Adler, F. L., Fishman, M., and Dray, S. (1968). *J. Immunol.* **97**, 554.
- Allen, J. M., and Cook, G. M. W. (1970). *Exp. Cell Res.* **59**, 105.
- Allison, A. C., and Young, D. D. (1964). *Life Sci.* **3**, 1407.
- Allison, A. C., Harrington, J. S., and Birbeck, M. (1966). *J. Exp. Med.* **124**, 141.
- Argyris, B. F. (1967). *J. Immunol.* **99**, 744.
- Argyris, B. F., and Askonas, B. A. (1968). *Immunology* **14**, 379.
- Argyris, B. F., and Plotkin, D. H. (1969). *J. Immunol.* **103**, 372.
- Askonas, B. A., and Jaroskova, L. (1970). In "Developmental Aspects of Antibody Formation and Structure" (J. Sterzl and I. Riha, eds.), 2nd ed., Vol. 1. Academic Press, New York.
- Askonas, B. A., and Rhodes, J. M. (1965a). *Proc. Symp. Mol. Cell. Basis Antibody Formation*, 1964, p. 503.
- Askonas, B. A., and Rhodes, J. M. (1965b). *Nature (London)* **205**, 470.
- Auzins, I., and Rowley, D. (1963). *Aust. J. Exp. Biol. Med. Sci.* **41**, 539.
- Bach, F. H., Alter, B. J., Solliday, S., Zoschke, D. C., and Janis, M. (1970). *Cell. Immunol.* **1**, 219.
- Balfour, B. M., and Humphrey, J. H. (1966). In "Germinal Centres in Immune Responses" (H. Cottier *et al.*, eds.), pp. 80-85. Springer-Verlag, Berlin and New York.
- Bell, C., and Dray, S. (1969). *J. Immunol.* **103**, 1196.
- Bell, C., and Dray, S. (1970). *J. Immunol.* **105**, 541.
- Benacerraf, B. (1964). In "The Liver" (C. Rouiller, ed.), Vol. 2, p. 37. Academic Press, New York.
- Benacerraf, B., and Green, I. (1969). *Annu. Rev. Med.* **20**, 141.
- Benacerraf, B., Stiffel, C., Biozzi, G., and Halpern, B. M. (1954). *C. R. Soc. Biol.* **148**, 486.
- Benacerraf, B., Biozzi, G., Cuendet, A., and Halpern, B. M. (1955a). *J. Physiol. (London)* **128**, 1.
- Benacerraf, B., Halpern, B. N., Stiffel, C., Cruchaud, S., and Biozzi, G. (1955b). *Ann. Inst. Pasteur, Paris* **89**, 601.
- Benacerraf, B., Stiffel, C., and Biozzi, G. (1956). *C. R. Soc. Biol.* **150**, 1161.
- Benacerraf, B., Biozzi, G., Halpern, B. N., and Stiffel, C. (1957a). In "Physiopathology of the Reticuloendothelial System" (B. N. Halpern, B. Benacerraf, and J. F. Delafresnaye, eds.), p. 52. Thomas, Springfield, Illinois.

- Benacerraf, B., Biozzi, G., Halpern, B. N., Stiffel, C., and Mouton, D. (1957b). *Brit. J. Exp. Pathol.* **38**, 35.
- Benacerraf, B., Sebestyen, M. M., and Schlossman, S. (1959). *J. Exp. Med.* **110**, 27.
- Berken, A., and Benacerraf, B. (1966). *J. Exp. Med.* **123**, 119.
- Bianco, C., Patrick, R., and Nussenzweig, V. (1970). *J. Exp. Med.* **132**, 702.
- Biozzi, G., Benacerraf, B., Grumbach, F., Halpern, B. N., Levaditi, J., and Rist, N. (1954). *Ann. Inst. Pasteur, Paris* **87**, 291.
- Biozzi, G., Halpern, B. N., Benacerraf, B., and Stiffel, C. (1957). In "Physiopathology of the Reticuloendothelial System" (B. N. Halpern, B. Benacerraf, and J. F. Delafresnaye, eds.), p. 205. Thomas, Springfield, Illinois.
- Biozzi, G., Benacerraf, B., Halpern, B. N., Stiffel, C., and Hillemand, B. (1958). *J. Lab. Clin. Med.* **51**, 230.
- Biozzi, G., Howard, J. G., Halpern, B. N., Stiffel, C., and Mouton, D. (1960). *Immunology* **3**, 74.
- Biro, C. E., and Garcia, G. (1965). *Immunology* **8**, 411.
- Bishop, D. C., and Gottlieb, A. A. (1971). *J. Immunol.* **107**, 269.
- Bluestein, H. G., Green, I., and Benacerraf, B. (1970). *Proc. Soc. Exp. Biol. Med.* **135**, 146.
- Boros, D. L., and Warren, K. S. (1971). *J. Immunol.* **107**, 534.
- Boyden, S. V. (1963). In "Cell-Bound Antibodies" (B. Amos and H. Koprowski, eds.), p. 7. Wistar Inst. Press, Philadelphia, Pennsylvania.
- Boyden, S. V. (1964). *Immunology* **7**, 474.
- Braun, W., Nakano, M., Jaraskova, L., Yajima, Y., and Jimenez, L. (1968). In "Nucleic Acids in Immunology" (O. J. Plescia and W. Braun, eds.), p. 347. Springer Publ., New York.
- Britton, S., and Celada, F. (1968). *Immunology* **14**, 503.
- Britton, S., and Möller, G. (1968). *J. Immunol.* **100**, 1320.
- Britton, S., Wepsic, T., and Möller, G. (1968). *Immunology* **14**, 491.
- Britton, S., Mitchison, N. A., and Rajewsky, K. (1971). *Eur. J. Immunol.* **1**, 65.
- Brumfitt, W., Glynn, A. A., and Percival, A. (1965). *Brit. J. Exp. Pathol.* **46**, 213.
- Campbell, D. H., and Garvey, J. S. (1961). *Lab. Invest.* **10**, 1126.
- Campbell, D. H., and Garvey, J. S. (1963). *Advan. Immunol.* **3**, 261-313.
- Cannon, P. R., Baer, R. B., Sullivan, F. L., and Webster, J. R. (1929). *J. Immunol.* **17**, 441.
- Cayeux, P., Panjel, J., Cluzan, R., and Levillain, R. (1966). *Nature (London)* **212**, 688.
- Celada, F. (1967). *J. Exp. Med.* **125**, 199.
- Cerottini, J.-C., and Trnka, Z. (1970). *Int. Arch. Allergy Appl. Immunol.* **38**, 37.
- Cerottini, J.-C., and Unanue, E. R. (1971). *J. Immunol.* **106**, 732.
- Chiller, J. M., Habicht, G. S., and Weigle, W. O. (1971). *Science* **171**, 813.
- Claman, H. N. (1963). *J. Immunol.* **91**, 833.
- Cline, M. J., and Sweet, V. C. (1968). *J. Exp. Med.* **128**, 1309.
- Cochrane, C. G., Unanue, E. R., and Dixon, F. J. (1965). *J. Exp. Med.* **122**, 99.
- Cohn, Z. A. (1962). *Nature (London)* **196**, 1066.
- Cohn, Z. A. (1963a). *J. Exp. Med.* **117**, 27.
- Cohn, Z. A. (1963b). *J. Exp. Med.* **117**, 43.
- Cohn, Z. A. (1968). *Advan. Immunol.* **9**, 163-214.
- Cohn, Z. A., and Parks, E. (1967). *J. Exp. Med.* **125**, 1091.
- Cottier, H., and Sordat, B. (1971). *Advan. Exp. Med. Biol.* **12**, 203.
- Cruchaud, A. (1968). *Lab. Invest.* **19**, 15.

- Cruchaud, A., and Unanue, E. R. (1971a). *J. Immunol.* **107**, 1329.
Cruchaud, A., and Unanue, E. R. (1971b). In "Morphological and Functional Aspects of Immunity" (K. Lindahl-Kiessling, G. Alm, and M. G. Hanna, Jr., eds.), pp. 187-195. Plenum, New York.
Cruchaud, A., Despont, J. P., Girard, J. P., and Mach, B. (1970). *J. Immunol.* **104**, 1256.
Despont, J. P., and Cruchaud, A. (1969). *Nature (London)* **223**, 838.
Dobson, E. L. (1957). In "Physiopathology of the Reticuloendothelial System" (B. N. Halpern, B. Benacerraf, and J. F. Delafresnaye, eds.), p. 80. Thomas, Springfield, Illinois.
Dobson, E. L., and Jones, H. B. (1951). *Acta Med. Scand.* **145**, Suppl. 273, 1.
Dresser, D. W. (1962). *Immunology* **5**, 378.
Dresser, D. W. (1968). *Nature (London)* **217**, 527.
Dresser, D. W., and Mitchison, N. A. (1968). *Advan. Immunol.* **8**, 129-181.
Dresser, D. W., Taub, R. N., and Krantz, A. R. (1970). *Immunology* **18**, 663.
DuBuy, H. G., and Johnson, M. L. (1969). *Proc. Soc. Exp. Biol. Med.* **128**, 1210.
Dutton, R. W. (1967). *Advan. Immunol.* **6**, 253-336.
Dutton, R. W., McCarthy, M. M., Mishell, R. I., and Raidt, D. J. (1970). *Cell. Immunol.* **1**, 196.
Dvorak, H. F., and Bast, R. C., Jr. (1970). *Immunochemistry* **7**, 118.
Ehrenreich, B. A., and Cohn, Z. A. (1967). *J. Exp. Med.* **126**, 941.
Ehrenreich, B. A., and Cohn, Z. A. (1968). *J. Cell Biol.* **38**, 244.
Ehrenreich, B. A., and Cohn, Z. A. (1969). *J. Exp. Med.* **129**, 227.
Fauci, A. S., Benacerraf, B., and Wolff, S. M. (1971). *Proc. Soc. Exp. Biol. Med.* **136**, 264.
Feldman, J. D., and Unanue, E. R. (1971). *Cell. Immunol.* **2**, 275.
Feldman, M., and Palmer, J. (1971). *Immunology* **21**, 685.
Fischer, H., Ax, W., Freund-Molbert, E., Holub, M., Krusman, W. F., and Matthes, M. L. (1970). In "Mononuclear Phagocytes" (R. Van Furth, ed.), p. 528. Davis, Philadelphia, Pennsylvania.
Fisher, S. (1966). *Immunology* **11**, 127.
Fishman, M. (1961). *J. Exp. Med.* **114**, 837.
Fishman, M., and Adler, F. L. (1963a). *Proc. Int. Symp. Immunopathol.*, 3rd, 1962, p. 79.
Fishman, M., and Adler, F. L. (1963b). *J. Exp. Med.* **117**, 595.
Fishman, M., and Adler, F. L. (1970). In "Mononuclear Phagocytes" (R. Van Furth, ed.), p. 581. Davis, Philadelphia, Pennsylvania.
Ford, W. L., Gowans, J. L., and McCullagh, P. J. (1966). *Thymus: Exp. Clin. Stud., Ciba Found. Symp.*, 1965, p. 58.
Franzl, R. E. (1962). *Nature (London)* **195**, 457.
Franzl, R. E., and Morrello, J. A. (1966). *J. Reticuloendothel. Soc.* **3**, 351.
Frei, P. C., Benacerraf, B., and Thorbecke, G. J. (1965). *Proc. Nat. Acad. Sci. U. S.* **53**, 20.
French, V. I., Stark, J. M., and White, R. G. (1970). *Immunology* **18**, 645.
Friedman, H. P., Stavitsky, A. B., and Solomon, J. M. (1965). *Science* **149**, 1106.
Gajl-Peczalska, K. J., Fish, A. J., Meuwissen, H. J., Frommel, D., and Good, R. A. (1969). *J. Exp. Med.* **130**, 1367.
Gallily, R., and Feldman, M. (1967). *Immunology* **12**, 197.
Garcia, D. A., Little, J. B., and Ourth, D. D. (1972). *J. Immunol.* (Submitted).
Garvey, J. S., Campbell, D. H., and Das, M. L. (1967). *J. Exp. Med.* **125**, 111.

- Gay, F. P., and Clark, A. R. (1924). *J. Amer. Med. Ass.* **83**, 1296.
Gigli, I., and Nelson, R. A., Jr. (1968). *Exp. Cell Res.* **51**, 45.
Gill, F. A., and Cole, R. M. (1965). *J. Immunol.* **94**, 898.
Gillette, R. W., and Lance, E. M. (1971). *J. Reticuloendothel. Soc.* **10**, 223.
Colub, E. S., and Weigle, W. O. (1969). *J. Immunol.* **102**, 389.
Goodman, J. W. (1972). In "Contemporary Topics in Immunochemistry" (F. P. Inman, ed.). Plenum, New York (in press).
Gorcynski, R. M., Miller, R. G., and Phillips, R. A. (1971). *J. Exp. Med.* **134**, 1201.
Gordon, J. (1968). *Proc. Soc. Exp. Biol. Med.* **127**, 30.
Gottlieb, A. A. (1969). *Biochemistry* **8**, 2111.
Gottlieb, A. A., and Doty, P. (1967). *Proc. Nat. Acad. Sci. U. S.* **57**, 1849.
Gottlieb, A. A., and Straus, D. S. (1969). *J. Biol. Chem.* **244**, 3324.
Graf, M. W., and Uhr, J. W. (1969). *J. Exp. Med.* **130**, 1175.
Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963). *Biochem. J.* **89**, 114.
Halpern, B. N., Biozzi, G., Benacerraf, B., and Stiffel, C. (1957). *Amer. J. Physiol.* **189**, 520.
Halpern, B. N., Biozzi, G., Pequignot, G., Delaloye, B., Stiffel, C., and Mouton, D. (1959). *Pathol. Biol.* **7**, 1637.
Hamaoka, T., Takatsu, K., and Kitagawa, M. (1971). *Immunology* **21**, 259.
Hanna, M. G., Jr., and Hunter, R. L. (1971). In "Morphological and Functional Aspects of Immunity" (K. Lindahl-Kiessling, G. Alm, and M. Hanna, eds.), p. 257. Plenum, New York.
Hanna, M. G., Jr., Francis, M. W., and Peters, L. C. (1968). *Immunology* **15**, 75.
Harris, G. (1965). *Immunology* **9**, 529.
Harris, G. (1967). *Immunology* **12**, 159.
Hartmann, K.-U., Dutton, R. W., McCarthy, M. M., and Mishell, R. I. (1970). *Cell. Immunol.* **1**, 183.
Haskill, J. S., Byrt, P. N., and Marbrook, J. (1970). *J. Exp. Med.* **131**, 57.
Heilman, D. H. (1965). *Int. Arch. Allergy Appl. Immunol.* **26**, 63.
Heilman, D. H., and Robert, C. B. (1967). *J. Bacteriol.* **93**, 15.
Heise, E. R., and Weiser, R. S. (1970). *J. Immunol.* **104**, 704.
Henson, P. M. (1969). *Immunology* **16**, 107.
Henson, P. M. (1971). *J. Exp. Med.* **134**, No. 3, Part 2, 1145.
Herd, Z. L., and Ada, G. L. (1969a). *Aust. J. Exp. Biol. Med. Sci.* **47**, 63.
Herd, Z. L., and Ada, G. L. (1969b). *Aust. J. Exp. Biol. Med. Sci.* **47**, 73.
Herscowitz, H. B., and Stelos, P. (1970a). *J. Immunol.* **105**, 771.
Herscowitz, H. B., and Stelos, P. (1970b). *J. Immunol.* **105**, 779.
Hersh, E. M., and Harris, J. E. (1968). *J. Immunol.* **100**, 1184.
Hirsch, M. S., Gary, G. W., and Murphy, F. A. (1969). *J. Immunol.* **102**, 656.
Hoffmann, M., and Dutton, R. W. (1971). *Science* **172**, 1047.
Howard, J. G. (1970). In "Mononuclear Phagocytes" (R. Van Furth, ed.), p. 179. Davis, Philadelphia, Pennsylvania.
Howard, J. G., and Benacerraf, B. (1966). *Brit. J. Exp. Pathol.* **47**, 193.
Howard, J. G., and Siskind, G. W. (1969). *Clin. Exp. Immunol.* **4**, 29.
Huber, H., and Fudenberg, H. H. (1968). *Int. Arch. Allergy Appl. Immunol.* **34**, 18.
Huber, H., Polley, M. J., Linsecott, W. D., Fudenberg, H. H., and Müller-Eberhard, H. J. (1968). *Science* **162**, 1281.
Huber, H., Douglas, S. D., and Fudenberg, H. H. (1969). *Immunology* **17**, 7.
Humphrey, J. H. (1955a). *Brit. J. Exp. Pathol.* **36**, 268.
Humphrey, J. H. (1955b). *Brit. J. Exp. Pathol.* **36**, 283.
Humphrey, J. H. (1960). In "Mechanisms of Antibody Formation" (M. Holub and

- L. Jarosková, eds.), p. 44. Academic Press, New York.
- Humphrey, J. H. (1969). *Antibiot. Chemother. (Basel)* **15**, 7.
- Humphrey, J. H., and Frank, M. M. (1967). *Immunology* **13**, 87.
- Humphrey, J. H., and Keller, H. U. (1970). In "Developmental Aspects of Antibody Formation and Structure" (J. Sterzl and I. Riha, eds.), 2nd ed., Vol. 1, p. 181. Academic Press, New York.
- Humphrey, J. H., and Turk, J. L. (1963). *Immunology* **6**, 119.
- Humphrey, J. H., Askonas, B. A., Auzins, I., Schechter, I., and Sela, M. (1967). *Immunology* **13**, 71.
- Inchley, C. J. (1969). *Clin. Exp. Immunol.* **5**, 173.
- Inchley, C. J., Grey, H. M., and Uhr, J. W. (1970). *J. Immunol.* **105**, 362.
- Isa, A. M. (1971). *J. Immunol.* **107**, 595.
- Jacoby, F. (1965). In "Cells and Tissues in Culture" (E. N. Willmer, ed.), Vol. 2, p. 1. Academic Press, New York.
- Janeway, C. A., Jr., and Humphrey, J. H. (1969). *Isr. J. Med. Sci.* **5**, 185.
- Jaroslav, B. M., and Nossal, G. J. V. (1966). *Aust. J. Exp. Biol. Med. Sci.* **44**, 609.
- Jehn, U. W., Musher, D. M., and Weinstein, L. (1970). *Proc. Soc. Exp. Biol. Med.* **134**, 241.
- Jenkin, C., and Benacerraf, B. (1960). *J. Exp. Med.* **112**, 403.
- Jenkin, C. R., Auzins, I., and Reade, P. P. (1965). *Aust. J. Exp. Biol. Med. Sci.* **43**, 607.
- Jennings, J. F., and Hughes, L. A. (1969). *Nature (London)* **221**, 79.
- Jonas, W. E., Gurner, B. W., Nelson, D. S., and Coombs, R. R. A. (1965). *Int. Arch. Allergy Appl. Immunol.* **28**, 86.
- Jones, T. C., and Hirsch, J. G. (1971). *J. Exp. Med.* **133**, 231.
- Kaplan, M. H., Coons, A. H., and Deane, H. W. (1950). *J. Exp. Med.* **91**, 15.
- Karnovsky, M. L., and Karnovsky, M. J. (1971). Unpublished data.
- Katz, D. H., and Benacerraf, B. (1972). *Advan. Immunol.* **15**, 1 (this volume).
- Katz, D. H., and Unanue, E. R. (1971). Unpublished experiments.
- Kessel, R. W. I., and Braun, W. (1965). *Aust. J. Exp. Biol. Med. Sci.* **43**, 511.
- Kessel, R. W. I., Monaco, L., and Marchiso, M. A. (1963). *Brit. J. Exp. Pathol.* **44**, 351.
- Kolsch, E., and Mitchison, N. A. (1968). *J. Exp. Med.* **128**, 1059.
- Kossard, S., and Nelson, D. S. (1968a). *Aust. J. Exp. Biol. Med. Sci.* **46**, 51.
- Kossard, S., and Nelson, D. S. (1968b). *Aust. J. Exp. Biol. Med. Sci.* **46**, 63.
- Lamvik, J. O. (1969). *Scand. J. Haematol.* **6**, 149.
- Lance, E. M. (1970). *Clin. Exp. Immunol.* **6**, 789.
- Lane, F. C., and Unanue, E. R. (1972). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **31**, 610.
- Lang, P. G., and Ada, G. L. (1967). *Immunology* **13**, 523.
- Lay, W. H., and Nussenzweig, V. (1968). *J. Exp. Med.* **128**, 991.
- Lay, W. H., and Nussenzweig, V. (1969). *J. Immunol.* **102**, 1172.
- Leduc, E. H., Arrameas, S., and Bouteille, M. (1968). *J. Exp. Med.* **127**, 109.
- Lee, A., and Cooper, G. N. (1966). *Aust. J. Exp. Biol. Med. Sci.* **44**, 527.
- Leserman, L. D., and Roseman, J. M. (1971). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **30**, 589.
- Leskowitz, S. (1970). In "Developmental Aspects of Antibody Formation and Structure" (J. Sterzl and I. Riha, eds.), 2nd ed., Vol. 1, p. 165. Academic Press, New York.
- Levis, W. R., and Robbins, J. H. (1969). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **28**, 566.

- Lewis, L. A. (1954). *Amer. J. Physiol.* **179**, 285.
- LoBuglio, A. F., Cotran, R. S., and Jandl, J. H. (1967). *Science* **158**, 1582.
- McConahey, P. J., and Dixon, F. J. (1966). *Int. Arch. Allergy Appl. Immunol.* **29**, 185.
- McConahey, P. J., Cerottini, J.-C., and Dixon, F. J. (1968). *J. Exp. Med.* **127**, 1003.
- McCullagh, P. J. (1970). *Aust. J. Exp. Biol. Med. Sci.* **48**, 551.
- McDevitt, H. O., and Benacerraf, B. (1969). *Advan. Immunol.* **2**, 31-74.
- McDevitt, H. O., Askonas, B. A., Humphrey, J. H., Schechter, I., and Sela, M. (1966). *Immunology* **11**, 337.
- McFarland, W. (1969). *Science* **163**, 818.
- McFarland, W., and Heilman, D. H. (1965). *Nature (London)* **205**, 887.
- McFarland, W., Heilman, D. H., and Moorhead, J. F. (1966). *J. Exp. Med.* **124**, 851.
- McIntyre, J. A., Niblack, G. D., Prater, T. F. K., and LaVia, M. F. (1971). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **30**, 649.
- Mackaness, G. B. (1960). *J. Exp. Med.* **112**, 35.
- Mackaness, G. B., and Blanden, R. V. (1967). *Progr. Allergy* **11**, 89.
- Marbrook, J. (1967). *Lancet* **2**, 1279.
- Medlin, J., Humphrey, J. H., and Sela, M. (1970). *Folia Biol. (Praque)* **16**, 156.
- Melnick, H. D., and Friedman, H. (1969). *Proc. Soc. Exp. Biol. Med.* **133**, 423.
- Mergenhagen, S. E., Notkins, A. L., and Dougherty, S. F. (1967). *J. Immunol.* **99**, 576.
- Metchnikoff, E. (1899). *Ann. Inst. Pasteur, Paris* **13**, 47.
- Miller, H. R. P., and Avrameas, S. (1971). *Nature (London)* **229**, 184.
- Miller, J. F. A. P., Basten, A., Sprent, J., and Cheers, C. (1971). *Cell. Immunol.* **2**, 469.
- Miller, M. E., and Nilsson, U. R. (1970). *N. Engl. J. Med.* **282**, 354.
- Mims, C. A. (1959). *Brit. J. Exp. Pathol.* **40**, 533.
- Mishell, R. I., and Dutton, R. W. (1966). *Science* **153**, 1004.
- Mitchell, J., and Abbot, A. (1965). *Nature (London)* **208**, 500.
- Mitchell, J., and Nossal, G. J. V. (1966). *Aust. J. Exp. Biol. Med. Sci.* **44**, 211.
- Mitchison, N. A. (1965). *Immunology* **9**, 129.
- Mitchison, N. A. (1969a). *Isr. J. Med. Sci.* **5**, 230.
- Mitchison, N. A. (1969b). *Immunology* **16**, 1.
- Morelli, R., and Rosenberg, L. T. (1971). *J. Immunol.* **107**, 476.
- Mosier, D. E. (1967). *Science* **151**, 1573.
- Mosier, D. E. (1969). *J. Exp. Med.* **129**, 351.
- Mosier, D. E., Rowley, D. A., and Davies, A. J. S. (1970). *Nature (London)* **255**, 276.
- Muller-Hermelink, H. K., and Muller-Ruchholtz, W. (1971). In "Morphological and Functional Aspects of Immunity" (K. Lindahl-Kiessling, G. Alm, and M. G. Hanna, Jr., eds.), p. 451. Plenum, New York.
- Munder, P. G., Modolell, M., Ferber, E., and Fischer, H. (1970). In "Mononuclear Phagocytes" (R. Van Furth, ed.), p. 445. Davis, Philadelphia, Pennsylvania.
- Naor, D., and Sulitzeanu, D. (1967). *Nature (London)* **214**, 687.
- Nelson, D. S. (1963). *Advan. Immunol.* **3**, 131.
- Nettesheim, P., and Hammons, A. S. (1971). *J. Immunol.* **107**, 518.
- Nossal, G. J. V. (1967). *Gamma Globulins, Nobel Symp.*, 3rd, 1967.
- Nossal, G. J. V., and Ada, G. L. (1971). "Antigens, Lymphoid Cells, and the Immune Response." Academic Press, New York.
- Nossal, G. J. V., Ada, G. L., and Austin, C. M. (1964). *Aust. J. Exp. Biol. Med. Sci.* **42**, 311.

- Nossal, G. J. V., Ada, G. L., Austin, C. M., and Pye, J. (1965). *Immunology* **9**, 349.
- Nossal, G. J. V., Austin, C. M., Pye, J., and Mitchell, J. (1966). *Int. Arch. Allergy Appl. Immunol.* **29**, 368.
- Nossal, G. J. V., Abbot, A., and Mitchell, J. (1968a). *J. Exp. Med.* **127**, 263.
- Nossal, G. J. V., Abbot, A., Mitchell, J., and Lummus, Z. (1968b). *J. Exp. Med.* **127**, 277.
- Notkins, A. L., Mergenhagen, S. E., Rizzo, A. A., Sheele, C., and Waldmann, T. A. (1966). *J. Exp. Med.* **123**, 347.
- Oort, J., and Turk, J. L. (1965). *Brit. J. Exp. Pathol.* **46**, 147.
- Oppenheim, J. J., Hersh, E. M., and Block, J. B. (1966). In "Proceedings of the Symposium: The Biological Effects of Phytohemagglutinin" (M. W. Elves, ed.). England.
- Oppenheim, J. J., Leventhal, B. G., and Hersh, E. M. (1968). *J. Immunol.* **101**, 262.
- Panijel, J., and Cayeux, P. (1968). *Immunology* **14**, 769.
- Parkhouse, R. M. E., and Dutton, R. W. (1966). *J. Immunol.* **97**, 663.
- Pearson, M. N., and Raffel, S. (1971). *J. Exp. Med.* **133**, 494.
- Perkins, E. H. (1970). In "Mononuclear Phagocytes" (R. Van Furth, ed.), p. 35. Davis, Philadelphia, Pennsylvania.
- Perkins, E. H., and Leonard, M. R. (1963). *J. Immunol.* **90**, 228.
- Perkins, E. H., and Makinodan, T. (1964). *J. Immunol.* **92**, 192.
- Pernis, B., and Paronetto, F. (1962). *Proc. Soc. Exp. Biol. Med.* **110**, 390.
- Phillips-Quagliata, J., Levine, B. B., and Uhr, J. W. (1969). *Nature (London)* **222**, 1290.
- Phillips-Quagliata, J. M., Levine, B. B., Quagliata, F., and Uhr, J. W. (1971). *J. Exp. Med.* **133**, 589.
- Pierce, C. W. (1969a). *J. Exp. Med.* **130**, 345.
- Pierce, C. W. (1969b). *J. Exp. Med.* **130**, 365.
- Pierce, C. W., and Benacerraf, B. (1969). *Science* **166**, 1002.
- Pribnow, J. F., and Silverman, M. S. (1967). *J. Immunol.* **98**, 225.
- Rabinovitch, M. (1967a). *J. Immunol.* **99**, 232.
- Rabinovitch, M. (1967b). *J. Immunol.* **99**, 1115.
- Rabinovitch, M. (1968). *Semin. Hematol.* **5**, 134.
- Rhodes, J. M., Lind, I., Birch-Andersen, A., and Rarn, H. (1969). *Immunology* **17**, 445.
- Roelants, G. E., and Goodman, J. W. (1968). *Biochemistry* **7**, 1432.
- Roelants, G. E., and Goodman, J. W. (1969). *J. Exp. Med.* **130**, 557.
- Roelants, G. E., Goodman, J. W., and McDevitt, H. O. (1971). *J. Immunol.* **106**, 1222.
- Roseman, J. M. (1969). *Science* **165**, 1125.
- Roseman, J. M., Fitch, F. W., and Rowley, D. A. (1969). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **28**, 814.
- Roser, B. (1965). *Aust. J. Biol. Med. Sci.* **43**, 553.
- Roser, B. (1968). *J. Reticuloendothel. Soc.* **5**, 455.
- Russell, P., and Roser, B. (1966). *Aust. J. Biol. Med. Sci.* **44**, 629.
- Sabet, T. W., and Friedman, H. (1969). *Proc. Soc. Exp. Biol. Med.* **131**, 1317.
- Sabet, T., Newlin, C., and Friedman, H. (1969). *Immunology* **16**, 433.
- Sabin, F. R. (1923). *Bull. Johns Hopkins Hosp.* **34**, 277.
- Saha, A., Garvey, J. S., and Campbell, D. H. (1964). *Arch. Biochem. Biophys.* **105**, 179.
- Schechter, G. P., and McFarland, W. (1970). *J. Immunol.* **105**, 661.

- Schmidtke, J. R., and Dixon, F. J. (1972). *J. Immunol.* (in press).
- Schmidtke, J. R., and Johnson, A. G. (1971). *J. Immunol.* **106**, 1191.
- Schmidtke, J. R., and Unanue, E. R. (1971a). *J. Immunol.* **107**, 331.
- Schmidtke, J. R., and Unanue, E. R. (1971b). *Nature (London)* **223**, 84.
- Schoenberg, M. D., Mumaw, W. R., Moore, R. D., and Weisberger, A. S. (1964). *Science* **143**, 964.
- Schwab, J. H., and Brown, R. R. (1968). *J. Immunol.* **101**, 930.
- Schwab, J. H., and Ohanian, S. H. (1967). *J. Bacteriol.* **94**, 1346.
- Schwartz, H. J. (1971). Cited in *Progr. Immunol.* (1971) p. 1382.
- Schwartz, H. J., and Leskowitz, S. (1969). *J. Immunol.* **103**, 87.
- Schwartz, R. S., Ryder, R. J. W., and Gottlieb, A. A. (1970). *Progr. Allergy* **14**, 81.
- Seeger, R. C., and Oppenheim, J. J. (1970a). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **30**, 693.
- Seeger, R. C., and Oppenheim, J. J. (1970b). *J. Exp. Med.* **132**, 44.
- Sela, M. (1969). *Science* **166**, 1365.
- Sela, M., Schechter, B., Schechter, I., and Borek, F. (1967). *Cold Spring Harbor Symp. Quant. Biol.* **32**, 537.
- Shin, H. S., Smith, M. R., and Wood, W. B. (1969). *J. Exp. Med.* **130**, 1229.
- Shortman, K., and Palmer, J. (1971). *Cell. Immunol.* **2**, 399.
- Shortman, K., Diener, E., Russell, P., and Armstrong, W. D. (1970). *J. Exp. Med.* **131**, 461.
- Siegel, I. (1970). *J. Immunol.* **105**, 879.
- Spiegelberg, H. L., and Weigle, W. O. (1967). *J. Immunol.* **98**, 1020.
- Spiegelberg, H. L., Miescher, P. A., and Benacerraf, B. (1963). *J. Immunol.* **90**, 751.
- Spitznagel, J. K., and Allison, A. C. (1970a). *J. Immunol.* **104**, 119.
- Spitznagel, J. K., and Allison, A. C. (1970b). *J. Immunol.* **104**, 128.
- Steinberg, S. U., Munro, J. A., Fleming, W. A., French, V. I., Stark, J. M., and White, R. G. (1970). *Immunology* **18**, 635.
- Stern, K., Spencer, K., and Farquhar, M. (1955). *Proc. Soc. Exp. Biol. Med.* **89**, 126.
- Sulitzeanu, D., Kleinman, R., Benezra, D., and Gery, I. (1971). *Nature (London)* **229**, 254.
- Szakal, A. K., and Hanna, M. G., Jr. (1967). *Exp. Mol. Pathol.* **8**, 75.
- Talmage, D. W., Radovich, J., and Hemmingsen, H. (1969). *J. Allergy* **43**, 323.
- Theis, G. A., and Thorbecke, G. J. (1970). *J. Exp. Med.* **131**, 970.
- Thiery, J. P. (1962). *J. Microsc. (Paris)* **1**, 275.
- Tsan, M., and Berlin, R. D. (1971). *J. Exp. Med.* **134**, 1016.
- Uhr, J. W. (1965). *Proc. Nat. Acad. Sci. U. S.* **54**, 1599.
- Uhr, J. W., and Weissman, G. (1965). *J. Immunol.* **94**, 544.
- Uhr, J. W., and Weissman, G. (1968). *J. Reticuloendothel. Soc.* **5**, 243.
- Unanue, E. R. (1968a). Unpublished results.
- Unanue, E. R. (1968b). *Nature (London)* **218**, 36.
- Unanue, E. R. (1969). *J. Immunol.* **102**, 893.
- Unanue, E. R. (1970). *J. Immunol.* **105**, 1339.
- Unanue, E. R. (1972). *J. Immunol.* (in press).
- Unanue, E. R., and Askonas, B. A. (1968a). *J. Exp. Med.* **127**, 915.
- Unanue, E. R., and Askonas, B. A. (1968b). *Immunology* **15**, 287.
- Unanue, E. R., and Cerottini, J.-C. (1970). *J. Exp. Med.* **131**, 711.
- Unanue, E. R., and Cerottini, J.-C. (1971). In "Developmental Aspects of Antibody Formation and Structure" (J. Sterzl and I. Riha, eds.), 2nd ed., Vol. 2, p. 521. Academic Press, New York.

- Unanue, E. R., and Feldman, J. D. (1971). *Cell. Immunol.* **2**, 269.
- Unanue, E. R., Askonas, B. A., and Allison, A. C. (1969a). *J. Immunol.* **103**, 71.
- Unanue, E. R., Cerottini, J.-C., and Bedford, M. (1969b). *Nature (London)* **222**, 1193.
- Unanue, E. R., Schmidtke, J., Cruchaud, A., and Grey, H. M. (1972). *Proc. Int. Symp. Immunopathol.*, 6th, 1971 (in press).
- Valentine, M. D., Bloch, K. J., and Austen, K. F. (1967). *J. Immunol.* **99**, 98.
- Vaughan, R. B. (1965). *Immunology* **8**, 245.
- Vaughan, R. B., and Boyden, S. V. (1964). *Immunology* **7**, 118.
- Virolainen, M., Lahti, A., and Hayry, P. (1972). *Advan. Exp. Biol. Med.* (in press).
- Volkman, A. (1966). *J. Exp. Med.* **124**, 241.
- Volkman, A., and Gowans, J. L. (1965a). *Brit. J. Exp. Pathol.* **46**, 50.
- Volkman, A., and Gowans, J. L. (1965b). *Brit. J. Exp. Pathol.* **46**, 62.
- Walsh, T. E., and Smith, C. A. (1951). *J. Immunol.* **66**, 303.
- Wardlaw, A. C., and Howard, J. G. (1959). *Brit. J. Exp. Pathol.* **40**, 113.
- Warner, N. L., and Byrt, P. (1970). *Nature (London)* **226**, 942.
- Weissman, G., and Dukor, P. (1970). *Advan. Immunol.* **12**, 283-331.
- Weissman, G., Dukor, P., and Zurier, R. B. (1971a). *Nature (London)* **231**, 131.
- Weissman, G., Zurier, R. B., Spieler, P. J., and Goldstein, I. M. (1971b). *J. Exp. Med.* **134**, No. 3, Part 2, 1495.
- White, R. G. (1963). *Ciba Found. Study Group [Pap.]* **16**, 6.
- White, R. G., Coons, A. H., and Connolly, J. M. (1955). *J. Exp. Med.* **102**, 73.
- White, R. G., French, I., and Stark, J. M. (1967). In "Germinal Centers in Immune Responses" (H. Cottier *et al.*, eds.), p. 131. Springer-Verlag, Berlin and New York.
- Wiener, E., and Levanon, D. (1968). *Lab. Invest.* **19**, 584.
- Williams, G. M. (1966a). *Immunology* **11**, 467.
- Williams, G. M. (1966b). *Immunology* **11**, 475.
- Williams, G. M., and Ada, G. L. (1967). *Immunology* **13**, 249.
- Wood, W. B., Jr. (1951). *Harvey Lect.* **47**, 72.
- Wood, W. B., Jr., Smith, M. R., and Watson, B. (1946). *J. Exp. Med.* **84**, 387.
- Zatz, M. M., and Lance, E. M. (1971). *J. Exp. Med.* **134**, 224.
- Zilversmit, D. B., Boyd, G. A., and Brucer, M. (1952). *J. Lab. Clin. Med.* **40**, 255.