AN IMMUNOFLUORESCENT STUDY OF THE IDENTITY OF CELLS REACTING WITH ANTI-LYMPHOCYTIC SERA

C. R. TAYLOR

Department of Pathology, Radcliffe Infirmary, Oxford

PLATES XCVIII–CVI

IN a study of the developing lymphoid system of the human foetus the problem of identification of lymphocytes becomes increasingly difficult as cells occur singly, or in small clusters, rather than in organised tissue populations.

It was decided to investigate the possibility of labelling lymphocytes in tissue sections so as to render them more easily identifiable.

The fluorescent antibody technique has been used successfully in visually marking a variety of antigens (Glynn and Holborow, 1959; Moller, 1961; St Marie, 1962; Mellors and Korngold, 1963). Attempts were made to utilise this technique to label lymphocytes in foetal tissues, although I recognised that certain lymphocyte antigens might not mature until relatively late in development (θ-antigen appears in mouse thymocytes about day 16 of foetal life, according to Raff, 1971).

Moller had used immunofluorescent methods to label living lymphocytes in suspension, the labelled cells giving a positive " ring reaction ". Variations of this technique have been used by Johansson and Klein (1970), Pernis, Forni and Amante (1970), Raff (1970, 1971), Raff, Sternberg and Taylor (1970), and Wilson and Nossal (1971) in demonstrating the presence of immunoglobulin on the surface of a population of lymphocytes from various species. It was also possible to demonstrate in mice that anti-θ antibodies could be used to label a proportion of lymphocytes (T lymphocytes, Raff, 1970, 1971).

In 1968 Denman and Frenkel were able to show that anti-lymphocytic serum (ALS) administered *in vivo* was specifically bound to lymphoid cells. This specific binding has been quantitated (Phondke, Gokhale and Sundaram, 1971) and shown to be proportional to the immunosuppressive potency of the ALS used (Thomas, Mosedale and Zola, 1971).

Attempts were therefore made to label lymphocytes with ALS by a fluorescent sandwich technique, at first on separated lymphocytes, later on tissue sections.

MATERIALS AND METHODS

Anti-lymphocytic sera

RALS 1 (rabbit anti-human-lymphocyte serum 1). Peripheral blood lymphocytes were separated from two patients with chronic lymphatic leukaemia, and injected into a rabbit. The priming injection (10^8 lymphocytes from patient A) was administered intramuscularly with complete Freund's adjuvant (Difco, Detroit, Michigan) and the response was boosted at 4 wk by intravenous injection of 40×10^6 lymphocytes (from patient B). Blood was taken 1 wk later and allowed to clot at 37°C; the serum was separated and stored at −20°C in small volumes. Serum was heat-inactivated at 56°C for 30 min. before use.

HALS (horse anti-human lymphocyte serum). A second serum raised in the horse against normal human peripheral blood lymphocytes after the method of Woiwood *et al.* (1970) was obtained from Wellcome Laboratories, Beckenham, Kent.

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RALS 2. A further serum was raised in the rabbit in an attempt to repeat the observations with RALS 1. The antigen lymphocytes were all derived from a single chronic lymphatic leukaemic patient (C. H.). Immunisation was entirely by the intravenous route. The priming dose was 50×10^6 cells, with a booster dose at 4 wk of 50×10^6 cells. These represent relatively small immunising doses.

Lymphocytes were separated from defibrinated blood on a Triosil-Ficoll gradient (10 ml of 34 per cent. Triosil, 24 ml of 9 per cent. Ficoll), and washed with phosphate-buffered saline (PBS) before injection.

Immuno-electrophoresis and Ouchterlony diffusion of RALS 1 against whole human serum and prepared human IgG, IgA, and IgM revealed no component antibodies against human serum proteins.

Fluorescein-conjugated antisera

Fluorescein-conjugated swine anti-rabbit-immunoglobulin (SWAR/F), goat anti-rabbit-immunoglobulin (GAR/F) and rabbit anti-horse-immunoglobulin (RAH/F) were obtained from Nordic Pharmaceuticals (Tilburg, Netherlands).

The conjugated antisera were absorbed with human liver, or incubated with whole human serum, or both, to reduce non-specific background staining (Nilsson, 1967), and were used at a dilution of 1 in 10.

Absorption techniques. 0·1 ml volumes of ALS were absorbed with 50×10^6 lymphocytes or polymorphs, or freshly ground human liver, obtained at necropsy, by incubation for 1 hr at 37°C; supernatant was obtained by centrifugation. Absorbed ALS was used at a dilution of 1 in 10 for fluorescent studies.

Attempts to elute the absorbed ALS from the lymphocytes and use this for fluorescent labelling were unsuccessful.

Preparations examined for specific fluorescent staining

(1) Peripheral blood lymphocytes from normals and chronic lymphatic leukaemic patients were separated on a Triosil-Ficoll gradient, deposited on a glass slide and alcohol-fixed. ALS was added for 30 min. at room temperature, the slides were washed for 60 min. in PBS, and exposed to the appropriate conjugate for 30 min. Slides were again thoroughly washed in PBS.

(2) Living lymphocytes (obtained as above) in suspension were exposed to ALS and the appropriate conjugate, after the method of Moller (1961) and Raff (1970).

(3) Fresh lymph-nodes were taken from surgical specimens and fixed in cold alcohol, for processing by the method of St Marie. Sections were cut at 10 μm, taken through xylol at 4°C, rehydrated through 100 per cent. alcohol and washed in PBS, *p*H 7·3. The ALS, at dilutions from 1 in 20 to 1 in 80, was added for 30 min. and the sections were washed in changes of PBS for 90 min., before the addition of the fluorescein conjugate for 30 min. Sections were again washed and mounted in 80 per cent. buffered glycerol for examination.

(4) Fresh blood films were fixed in absolute alcohol and exposed to ALS and conjugate as described.

(5) Foetuses obtained from therapeutic terminations were examined at necropsy and their tissues were fixed in cold alcohol, and processed and stained with ALS as described for lymph-node. The tissues taken were: thymus, liver, spleen, appendix, small and large bowel and marrow smears.

Preparations were examined for fluorescence with a Leitz Ortholux microscope with one of two alternative light sources: (*a*) an Osram HB 200 high pressure mercury lamp with BG 12 (3 mm) and BG 38 (glass) excitation filters, and a 510 (glass) suppression filter; or (*b*) Philips Quartz Iodine Al/215 lamp in a Leitz housing with a Balzer interference filter and a 510 (glass) suppression filter. Photomicrographs were taken with the above systems on High Speed Ectochrome (daylight ASA 160) or Gaf Anscochrome (daylight ASA 500).

The control systems used are summarised in table I. Moreover, each section was to some extent its own control in the negative tissue components surrounding the positive cells.

Histological staining

Sections were cut and stained to examine the cell populations in lymph-node and foetal tissues.

In addition sections that had been subjected to fluorescence studies and repeated washings were eluted with citrate-phosphate buffer *p*H 4 and stained with haematoxylin and eosin (HE)

TABLE I

Experimental and control systems for immunofluorescence tests

System	Section or film treated		Degree of specific fluorescence in	
	first with	secondly with	positive cells‡	background
Experimental	RALS 1 or RALS 2	SWAR/F or GAR/F	++++§	+
	HALS	RAH/F	++++	+
Control 1	PBS	PBS	−	−‖
Control 2	PBS	SWAR/F or GAR/F or RAH/F	−	± or +
Control 3	NRS*	SWAR/F or GAR/F	−	+
	NHS†	RAH/F	−	+
Control 4	RALS 1 or RALS 2	RAH/F	−	+
	HALS	GAR/F	−	+
Control 5	RALS or HALS absorbed with:	GAR/F when derivatives of RALS were used first;		
	(*a*) lymphocytes		−	+
	(*b*) polymorphs		−	±
	(*c*) liver	RAH/F when derivatives of HALS were used first	+++	± or +
	(*d*) whole serum		++++	±
	(e) human IgG, IgA, IgM		+++	±

* NRS = Normal rabbit serum; † NHS = normal horse serum; other abbreviations are explained under *Materials and methods*.

‡ The brightly positive cells of foetal thymus, and the polymorphs of lymph-nodes.

§ ±, +, +++ and ++++ refer to increasing degrees of "specific" fluorescence, i.e., apple-green fluorescence.

‖ Blue autofluorescence only, easily distinguishable from "specific" fluorescence.

and with methyl green-pyronin and by the periodic acid-Schiff (PAS) and a modified chloro-acetate esterase method (Yam, Li and Crosby, 1971). Areas of sections that had been photographed under fluorescence were located and the microscope field was compared with the positive cells on the photomicrographs. It was therefore possible to locate and study individual positive cells with certainty. The method suffered from the variably poor cell morphology, the result of several hours soaking in buffer and antibody solutions.

Cytotoxicity testing

Peripheral blood lymphocytes were separated by Triosil-Ficoll gradient and adjusted to 2×10^6 per ml. 1 μl of lymphocyte suspension was added to an equal volume of RALS 1 and incubated at 37°C for 20 min.; 2 μl of fresh rabbit serum (complement) was added and incubation continued for a further 30 min. Cell death was assessed by phase-contrast microscopy.

Results

The results of the cytotoxicity testing are summarised in table II, which shows that RALS 1 gave a high titre of activity against lymphocytes from normal persons and a higher titre still against lymphocytes from patients with chronic lymphatic leukaemia.

Table II

Cytotoxicity of rabbit anti-human-lymphocyte serum (RALS 1) to peripheral blood lymphocytes separated on a Triosil-Ficoll gradient

Test lymphocytes	Mean percentage kill of test lymphocyte suspension treated with an equal volume of RALS 1 diluted 1 in						
	2	4	8	16	32	64	128
Normal	100	100	100	100	99	75	0
From three cases of chronic lymphatic leukaemia	100	100	100	100	100	99	25

Fluorescence studies

Lymphocyte film

When treated with RALS 1, lymphocytes from the blood of normal persons showed positive cytoplasmic staining (fig. 1*a*) that was not obviously localised to the cell membrane. Fluorescence was scored as ++++ in 60 per cent. of cells; the remainder stained with variable intensity. In six cases of chronic lymphatic leukaemia the percentage of strongly positive cells ranged from 50 to 95 per cent. None of the lymphocytes was entirely negative.

RALS 2 and HALS produced similar results on the lymphocyte films.

Living lymphocytes in suspension

When treated with RALS 1, 50–70 per cent. of lymphocytes from normal persons showed positive staining (ring positive cells, fig. 1*b*) clearly localised to the cell-surface membranes. The remaining cells were entirely negative, or showed only an occasional positive granule. In the chronic lymphatic leukaemias the proportion of positive cells ranged from 50 to 90 per cent. The finding of negatively staining lymphocytes in suspension is in contrast with the observation made on fixed lymphocyte films, and may be a reflection of the different methods of exposing lymphocytes to ALS. Membrane staining would be difficult to

IDENTITY OF CELLS REACTING WITH ANTI-LYMPHOCYTIC SERA

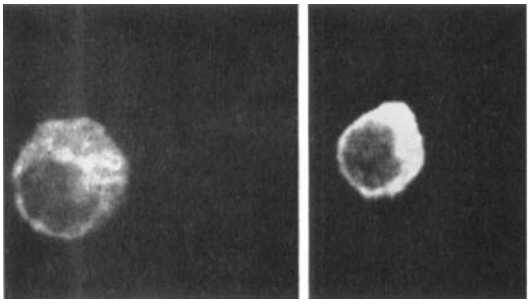

FIG. 1*a*.—Medium and small lymphocyte in blood film; bright staining of cytoplasm is not equivalent of surface membrane "ring" stain seen in lymphocytes in suspension. ×400.

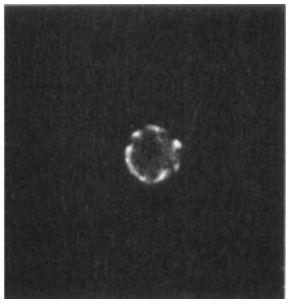

FIG. 1*b*.—Ring staining; small lymphocyte in suspension. ×400.

FIG. 2.—Many brightly positive cells in sinuses of reactive lymph-node. There are only occasional cells in the medullary cord towards the top of the figure. ×100.

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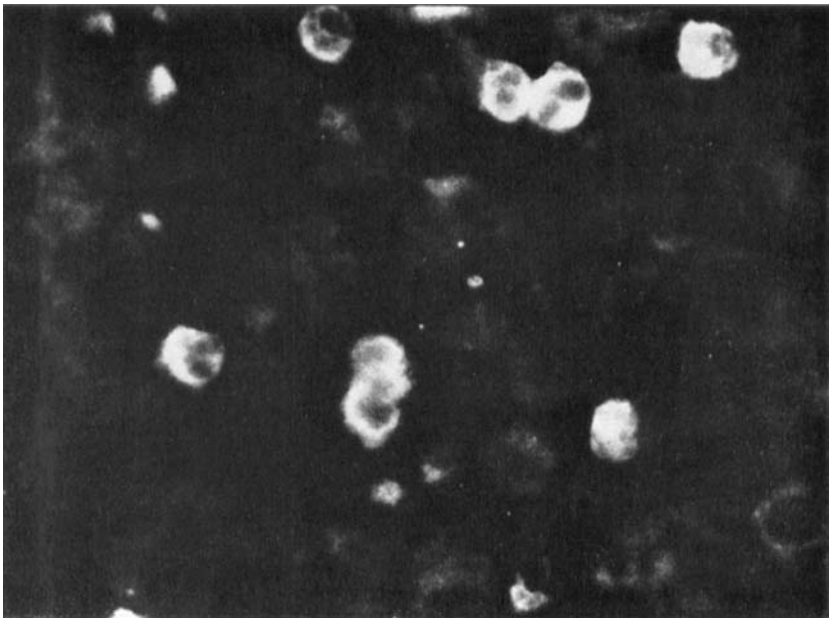

FIG. 3.—Lymph-node sinus; polymorphonuclear form can be discerned in some cells. ×400.

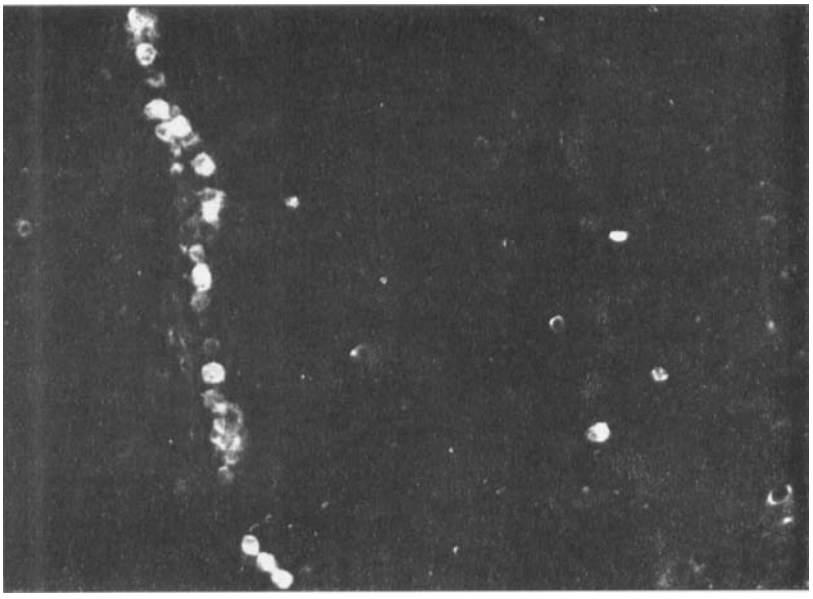

FIG. 4.—Reactive lymph-node: capillary contains many positive cells. Occasional positive cells are seen in the substance of the node. ×100.

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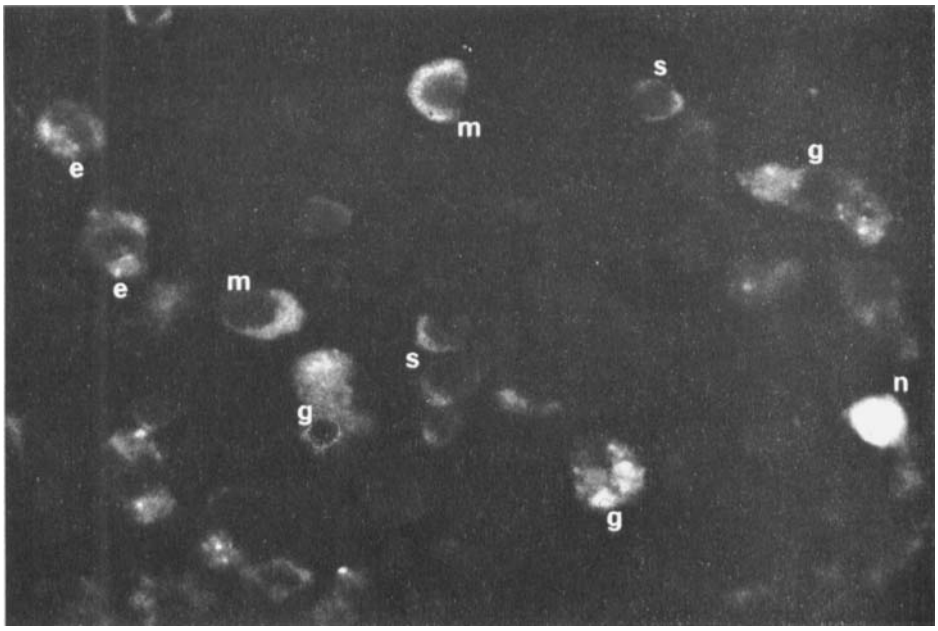

FIG. 5.—Reactive lymph-node: n(++++) neutrophil, s(++) small lymphocytes, m(++) larger mononuclears, g(++++) granules in histiocytes. e Eosinophils showing autofluorescence only (clearly distinguishable from green positive cells). ×400.

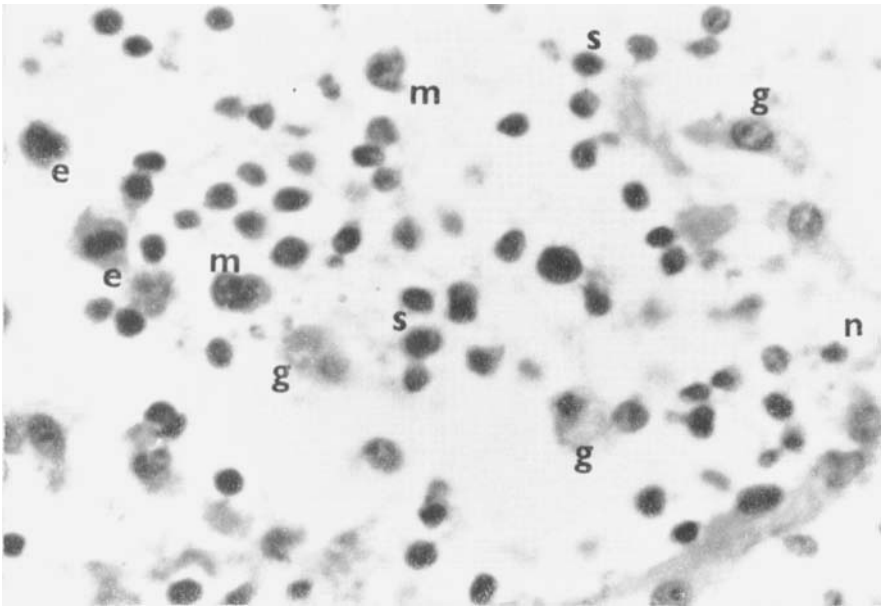

FIG. 6.—Same field as in fig. 5, eluted and stained with haematoxylin and eosin (HE). Legend as for fig. 5.

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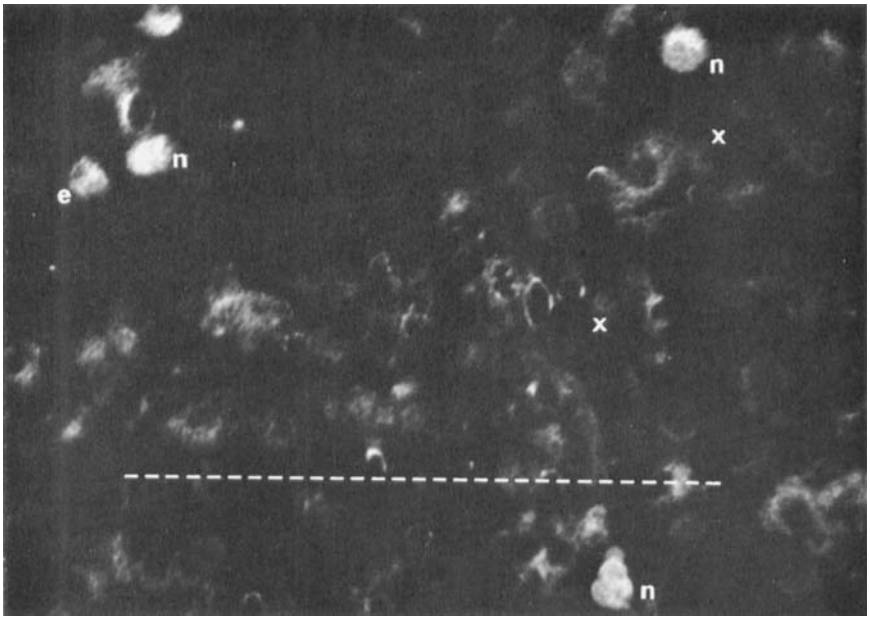

FIG. 7.—Reactive lymph-node: the blue autofluorescence of eosinophils (e) was clearly distinguished from brightly positive (green) neutrophils (n). Negative neutrophils also present (x). Interrupted line marks limits of field of fig. 8. ×400.

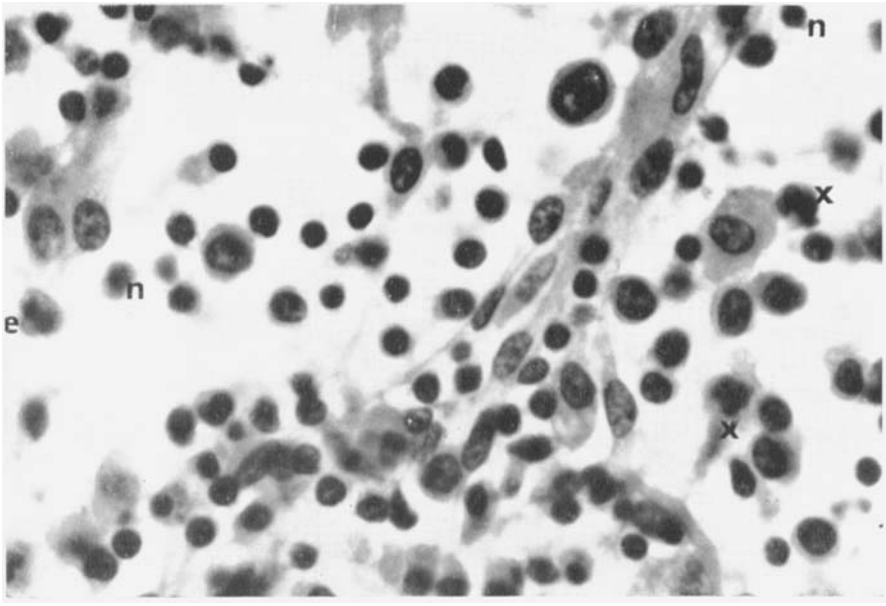

FIG. 8.—Part of field of fig. 7, eluted and stained with HE. Cell morphology has suffered, but cells are recognisable with microscope as in legend for fig. 7.

FIG. 9 is on plate CIV.

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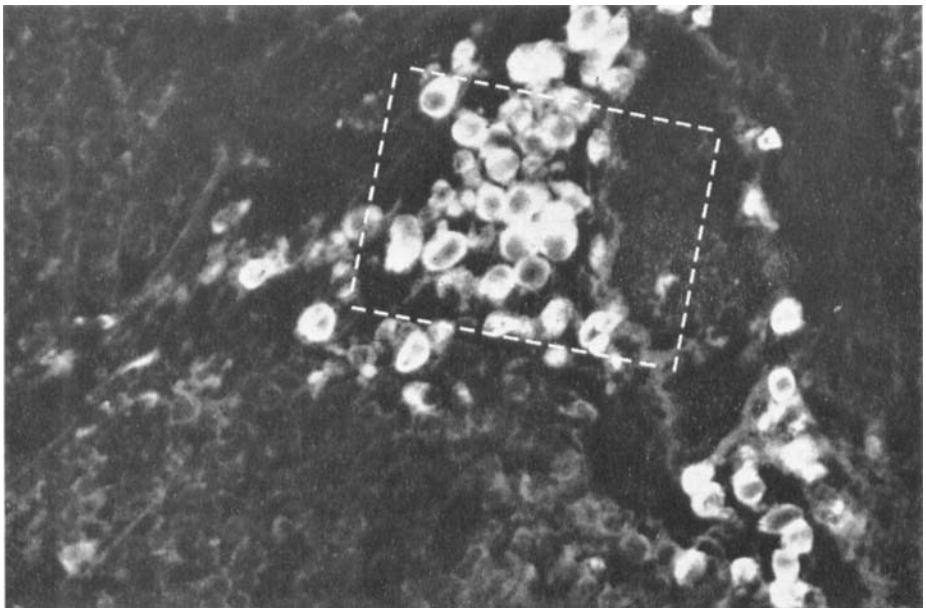

FIG. 10.—17-cm foetus: thymus with brightly positive cells in loose connective tissue surrounding a vessel. ×400.

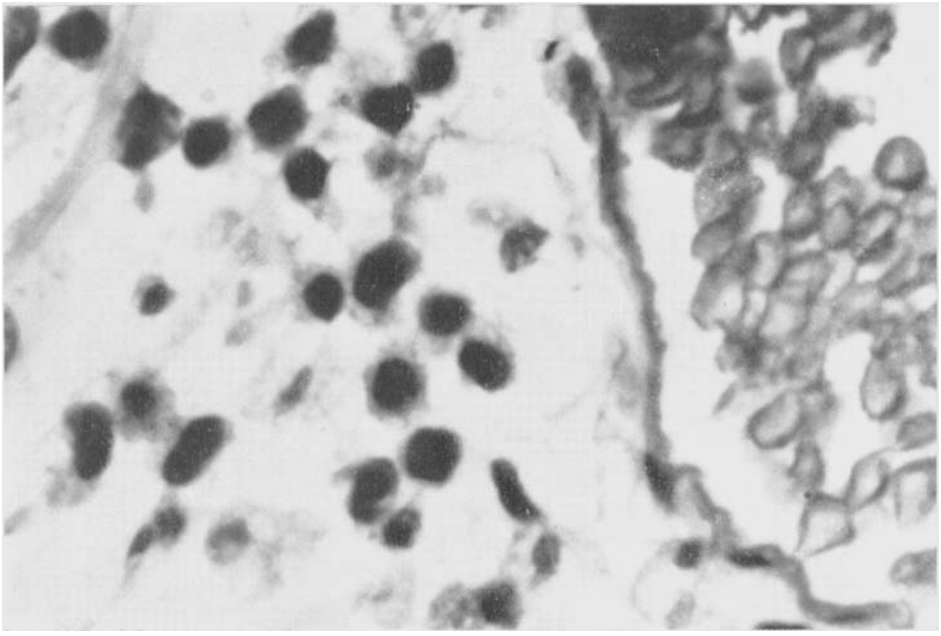

FIG. 11.—Part of field of fig. 10 as delineated, after elution and staining with HE. Section has suffered some distortion.

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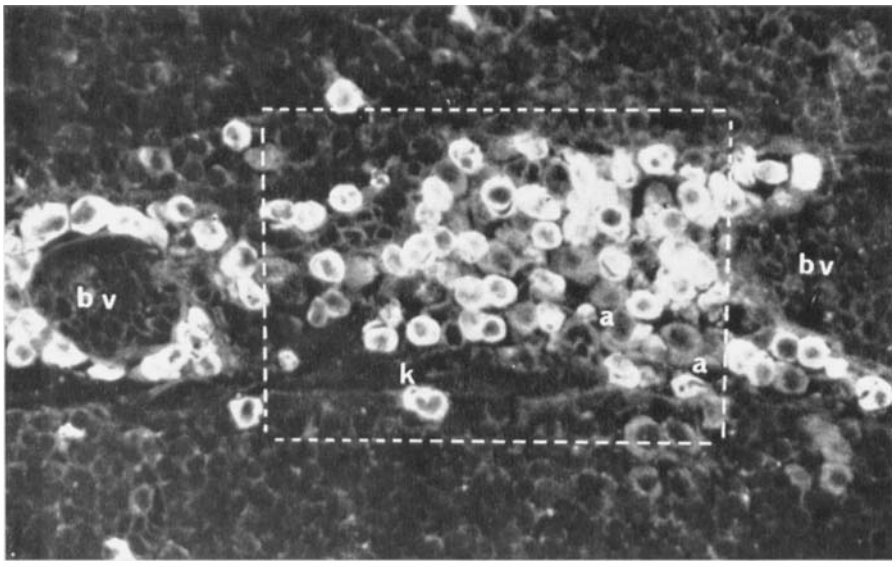

FIG. 12.—18-cm foetus: thymus. Autofluorescent cells (a) appear pale grey. Occasional positive cells have kidney-shaped nuclei (k). bv = Blood vessel. ×400.

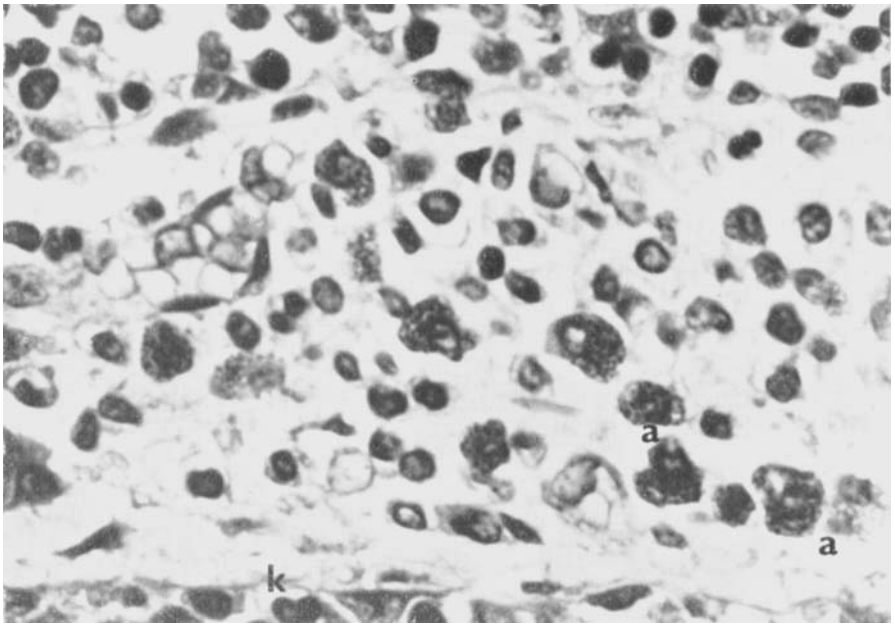

FIG. 13.—Part of field of fig. 12 as delineated. Legend as for fig. 12. Autofluorescent cells contain numerous eosinophilic granules. Some of the brightly positive cells are easily identified.

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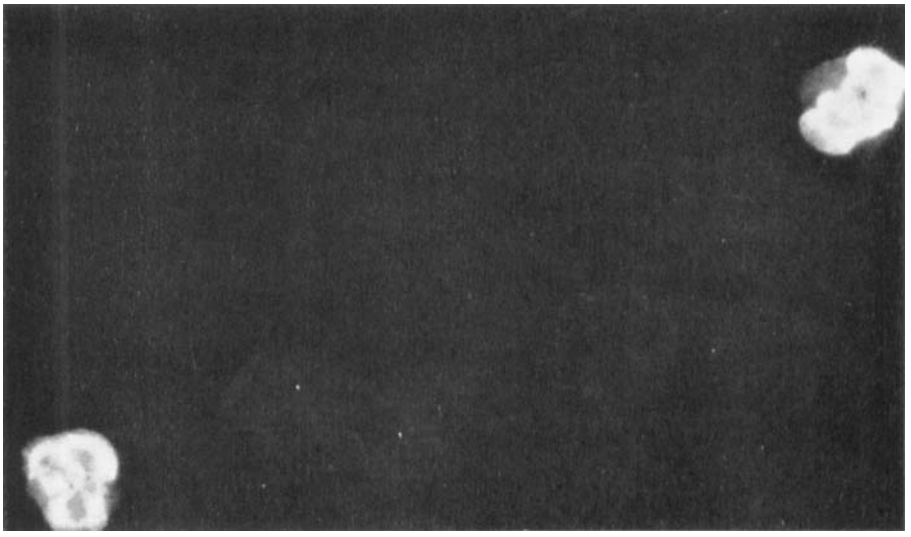

FIG. 9.—Blood film; bright nuclear staining of two neutrophils. Background of negative red cells. ×400.

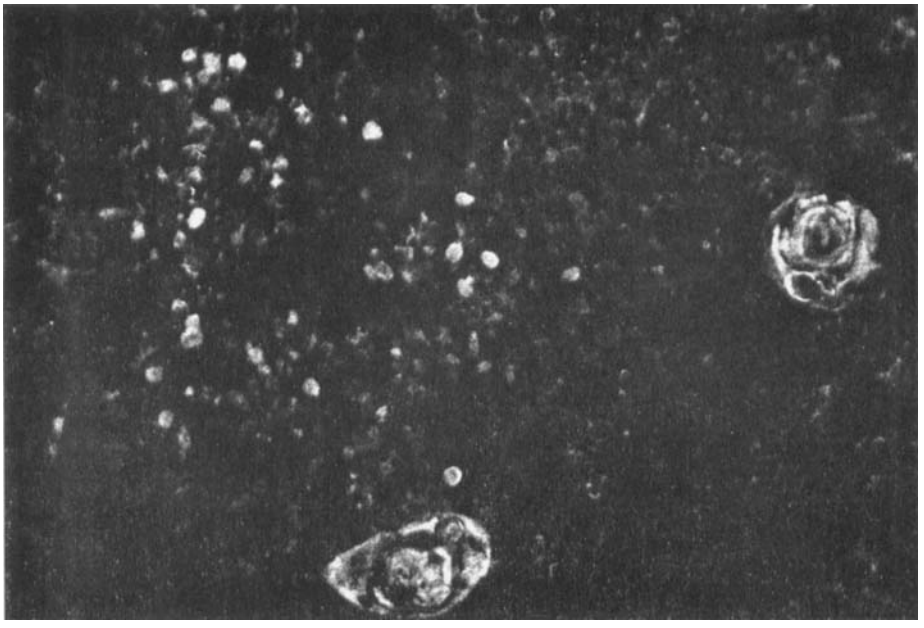

FIG. 14.—20-cm foetus. Positive cells in thymic medulla. Two brightly fluorescent Hassall's corpuscles. ×100.

IDENTITY OF CELLS REACTING WITH ANTI-LYMPHOCYTIC SERA

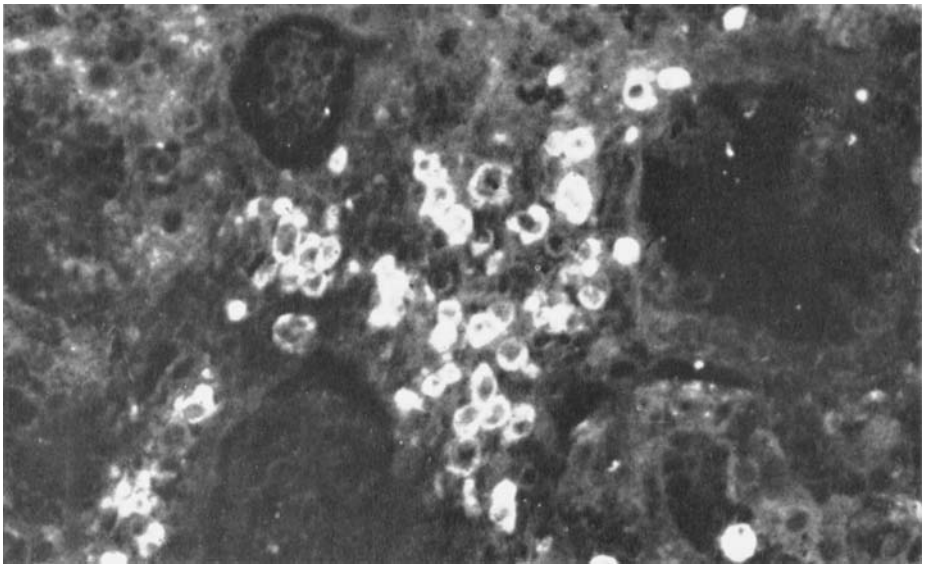

FIG. 15.—17-cm foetus: liver; positive cells in portal tract area. ×400.

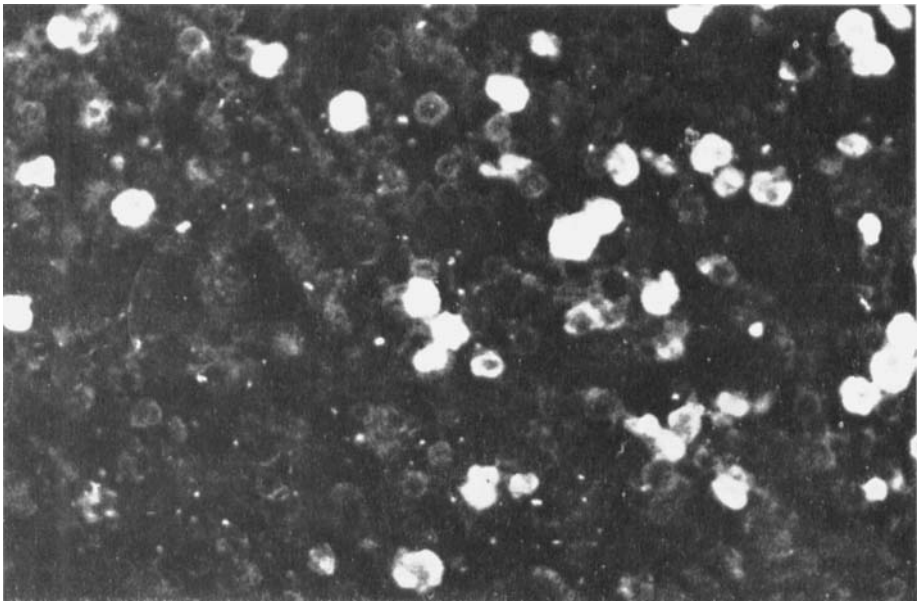

FIG. 16.—Stillborn foetus: spleen. Many positive cells present at this stage of development (though as stillbirths these are not representative of normal development). ×400.

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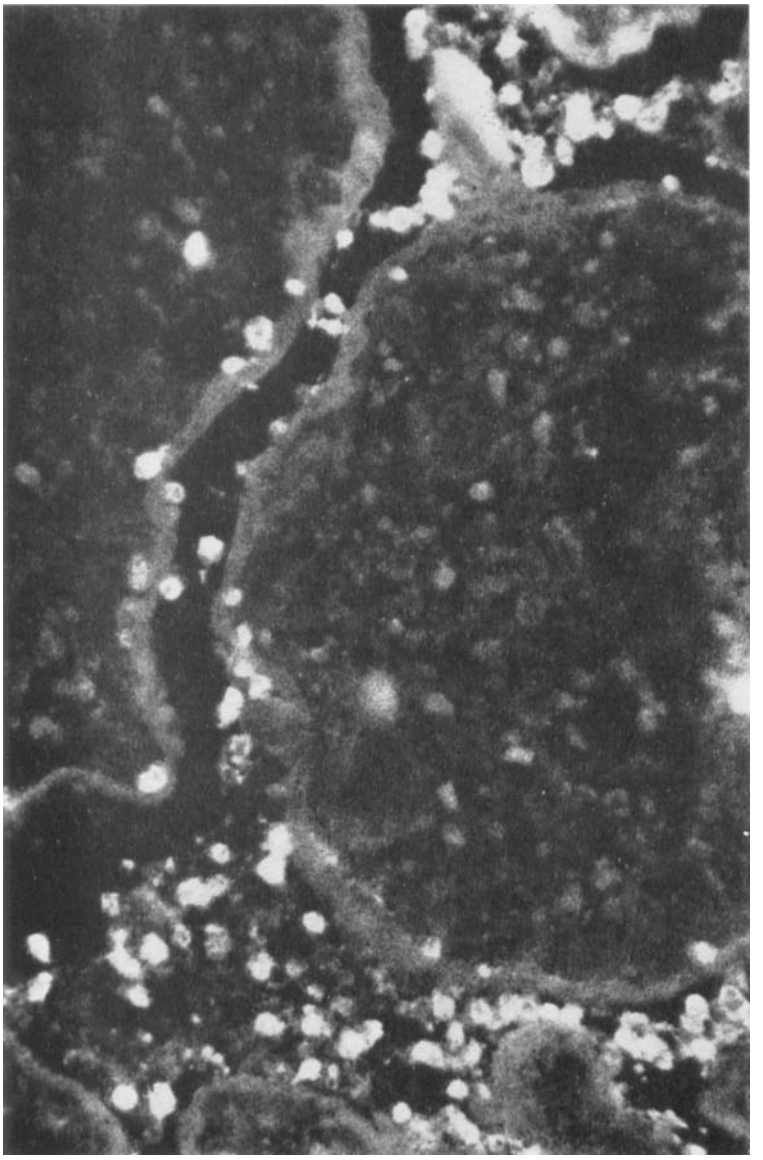

FIG. 17.—Placenta of 17-cm foetus. Positive cells in intervillous spaces. × 100.

demonstrate on fixed filmed lymphocytes, and living lymphocytes in suspension will exclude the antisera and prevent any cytoplasmic staining.

Lymph-nodes

It was felt that when the technique was applied to sections any specific labelling might be obscured by autofluorescence, non-specific protein binding and the presence of antibodies in the ALS directed against non-lymphocyte or histocompatibility antigens.

With the three different anti-lymphocytic sera used a number of very strongly positive cells were seen, comprising 1–5 per cent. of the total cells present. With RALS 1 these cells were scored as $++++$, with a background of $+$ to $\pm$, and were located mainly in the sinuses of the node (fig. 2). Initially morphology was difficult to define, but as the technique was improved it became apparent that at least a proportion of these positive cells were neutrophils (fig. 3). Occasionally they could be seen within the capillaries of the node (fig. 4). At higher dilutions of RALS 1 staining clearly outlined the nucleus.

A number of other cells were scored $++$ and were again predominantly in the sinuses. Some of these had the morphology of small lymphocytes; others were larger mononuclear cells (figs. 5 and 6). A minority of histiocytes (reticular cells) contained fluorescence-positive granules (figs. 5 and 6). The vast majority of lymphocytes and macrophages in the node showed no fluorescence, this finding being in contrast with the positive staining of lymphocytes in films. Eosinophils were consistently negative, and on detailed inspection a number of negatively staining neutrophils were seen (figs. 7 and 8).

HALS provided similar results, but appeared to have a lower titre of specific antibody. As the results with RALS 1 were unexpected, RALS 2 was prepared as described, and produced results almost identical with those given by RALS 1.

The features described were seen in a reactive lymph-node from a case of Crohn's disease, and also in other reactive nodes, and in histologically normal nodes, where the number of brightly positive cells was, however, reduced.

Blood films

Fresh fixed blood films were used to investigate the remarkable positive staining of neutrophils. Findings are reported in table III. The neutrophil nuclear staining with RALS 1 diluted 1 in 40 (fig. 9) disappeared on dilution, and on absorption with liver extract (table IV), and the nucleus was then clearly outlined as seen in a lymph-node. Bright nuclear staining was not seen in lymph-node sections, possibly because of some alteration of antigenicity during processing.

Eosinophils and basophils were entirely negative. It was not possible to define a distinct negative population of lymphocytes or neutrophils, though there was variation in intensity of fluorescence from cell to cell. This generally is in agreement with the lymphocyte fluorescence seen in films of separated lymphocytes, where there were no entirely negative cells. However, the

lymphocytes observed in St Marie sections were largely negative and the small number showing positive staining did so with moderate intensity. In addition

TABLE III

Specific staining of cells in blood films by fluorescent anti-lymphocyte sera

Serum	Dilution 1 in	Degree of specific " staining " by this dilution of serum of						
		neutrophil		lympho-cytes	mono-cytes	eosino-phils	platelets	red blood cells
		nuclei	cyto-plasm					
RALS 1	40	+++++	++++	++++	+++	−	++	−
	200	−	+++*	+++†	++	−	+	−
HALS	40	−	+++*	+++†	++	−	+	−
	200	−	+++*	+++†	++	−	++	−

* Especialy perinuclear.
† Variable brightness, but all lymphocytes showed some degree of fluorescence, as did those in films of separated lymphocytes.

TABLE IV

Effect of absorption with various materials on the ability of a rabbit-anti-human-lymphocyte serum (RALS 1) to " stain " certain cells

Serum absorbed with	Dilution of serum or its derivative in	Degree to which the serum of its derivative " stained "			
		blood film neutrophils and polymorphs	lymph-node neutrophils	perivascular cells of foetal thymus	background
Nil	30	++++*	++++	++++	± or +
Nil	200	++++	+++	+++	+
Liver†	...	+++	++	++	±
Lymphocytes	...	−	−	−	±
Foetal thymocytes	...	++	...	±	±
Polymorphs	...	−	−	−	±
Human serum	...	++++*	++++	+++	±

Results with serum RALS 2 were similar.
* Staining of polymorph nuclei +++++, positive to a dilution of 1 in 80 with serum RALS 1, and to a dilution of 1 in 8 with serum RALS 2.
† The liver preparations used for absorption contained a variable number of lymphocytes and polymorphs.

occasional negative neutrophils were seen in sections, but not in films, again probably a reflection of the different methods of preparation.

Lymphocyte staining was no longer apparent with RALS 1 at 1 in 500, and neutrophil staining was lost at 1 in 1600.

RALS 2 was in all respects similar to RALS 1, except that strong nuclear staining was seen only at low dilutions of RALS 2 (to 1 in 8). HALS showed no nuclear staining.

Foetal tissues

The striking feature with all three antisera was of brightly positive cells (++++) in a distribution that was in the early foetus exclusively perivascular (figs. 10 and 12), but later became both more prominent and more diffuse in distribution in lymphoid tissues, particularly the thymus. These positive cells were large mononuclears, with oval or kidney-shaped nuclei, and eosinophilic cytoplasm, sometimes vacuolated. Others had more dense nuclei and less

TABLE V

The frequency of brightly positive cells in foetal organs and tissues

Foetuses*		Approximate percentage of all cells showing specific staining in				
Crown-rump length (cm)	ovulation age (wk)	thymus	spleen	liver	gut	marrow
11–14	16–19	4–6	2–3	4–6	2–3	4–6
15–17†	19–22	6–9	4–6	6–9	4–6	8–12
18–21	22–25	8–12	6–9	6–9	4–6	8–12
Born dead‡	32–34	10–15	10–15	8–12	...	...

* Each group contains three or four foetuses. † See fig. 15; ‡ See fig. 16.

cytoplasm (figs. 11 and 13). Cell preservation was not good after sections had been processed for fluorescence, nevertheless the conclusion was that positive cells were not of uniform morphology, and did not constitute a single recognisable cell-type. All the brightly positive cells in the thymus were pyronin-negative, and chloro-acetate esterase-negative. Some showed weak PAS positivity.

The accompanying table (table V) represents the frequency and organ distribution of these ++++ cells in foetuses. It is notable that in some of the mature foetuses a minority of the positive cells were neutrophils.

In addition, within the thymic medulla and cortex were a number of cells (*c.* 10 per cent.) staining ++ in a background of − to ±, with the morphology of medium and large lymphocytes.

Hassall's corpuscles were present occasionally in the smallest foetuses studied (11 cm), and showed variable fluorescence to a score of ++++ (fig. 14), in which event HE staining commonly showed nuclear debris within the corpuscle.

In the placenta (examined from some 15–17-cm foetuses) many positive cells were seen in the maternal blood spaces. The majority of these ++++ cells were clearly neutrophils, and they were particularly prominent in glucose-induced terminations (fig. 17).

Absorption studies

Absorption studies showed that all labelling activity of the sera was entirely removed by lymphocytes, and equally by polymorphs. Foetal thymocytes were less effective, even when sera were twice absorbed. Absorption with large volumes of mashed human liver produced little diminution in specific labelling. The results are summarised in table IV, and tend to confirm the specificity of the ALS to lymphocytes and polymorphs.

Discussion

Medawar (1967) has stated that ALS must consist of a mixture of antibodies against cellular antigens. The spectrum of antibodies produced will depend on the number of antigens introduced and therefore on the purity of the lymphocyte preparation used for immunisation.

Even pure lymphocyte preparations will present a variety of antigens for immunisation. Surface antigens include histocompatibility antigens (in mice, Davis and Silverman, 1968), and any existing lymphocyte-specific antigen. In addition there are the cytoplasmic and cell-organelle components, together with nuclear material. This could readily explain the induction of antibodies labelling lymphocytes, but is less able to account for the extraordinarily high titre to neutrophils. That the ALS used here has been shown to bind specifically with lymphocytes and neutrophils does not necessarily imply any biological activity, such as induction of lymphopenia, or alteration of cell function. However, some of the early studies with ALS indicated activity against polymorphs, as detected by cytotoxicity or the induction of neutropenia.

In 1898 Metchnikoff noted that an antiserum raised against lymph-node cells agglutinated and killed polymorphonuclear leucocytes. Chew, Stephens and Lawrence (1936) reviewed the attempts at preparing anti-leucocyte antisera and noted that their own preparation, using inflammatory exudate cells as antigen, suppressed lymphocytes to a small degree, neutrophils to a very large degree, and eosinophils and basophils not at all.

With the in-vivo work of Cruickshank (1941) and the repeated demonstration of marked lymphopenia after administration of antiserum against lymph-node cells, the possibility of " anti-lymphocyte " sera affecting cells other than lymphocytes was largely ignored. The conclusion that an antiserum against lymphocytes should react with lymphocytes was comfortably drawn, and with logic in support, was not further questioned until recent years.

Evidence of possible activity of ALS against macrophages has been presented by Barth *et al.* (1969), Foerster *et al.* (1969), Huber, Michlmayr and Fudenberg (1969), Sheagren *et al.* (1969), Di Luzio and Pisano (1970), Maclaurin and Humm (1970), Marsman, van der Hart and van Loghem (1970), and Hughes *et al.* (1971). There are also reports to the contrary (Mackaness and Hill, 1969, Chare and Boak, 1970); and Vreeken, van Aken and Eijsvoogel (1969) suggested that some of the effects of reticulo-endothelial blockade by ALS might be due to anti-platelet activity. Evidence of activity against epithelial cells was also presented (Grob and Inderbitzin, 1969).

That anti-lymphocyte sera react with neutrophils has been less widely noted. Taub and Lance (1968) noted that, in mice, ALS produced a fall in lymphocyte and granulocyte counts at 6 hr after injection, but made no further reference to the granulocytopenic effect. Turk, Willoughby and Stevens (1968) showed that both ALS and anti-thymocyte serum would suppress the acute inflammatory response. This suppressive action, however, remained after the antisera were absorbed with polymorphs, and they concluded that the anti-inflammatory effect observed was not due to any cytotoxic effect on neutrophils.

Further, antisera directed against neutrophils have been described, but no specific effect against lymphocytes noted (Jankovic and Arsenijevic, 1959; Quie and Hirsch, 1964). Also Faber *et al.* (1964) described an antinuclear factor in Felty's syndrome specific to granulocytes.

In 1967 Lawrence, Craddock and Campbell showed that an anti-neutrophil serum (ANS) raised against guinea-pig neutrophils would, when administered intraperitoneally, produce a profound depletion of segmented neutrophils both in marrow and peripheral blood. They later extended this work (Lawrence, Barnett and Craddock, 1968) to show that an anti-thymocyte serum produced a similar depression of segmented neutrophils, together with marked lymphopenia. This in-vivo effect against neutrophils was not seen with ALS (raised against lymph-node lymphocytes), but the ALS did show a weak agglutination titre with neutrophils. Fluorescence studies were not conclusive, but sandwich techniques showed that both ANS and anti-thymocyte serum, but not ALS, had an affinity for Hassall's corpuscles. Lawrence *et al.*, 1968 also noted that a small number of myeloid cells (less than 1 per cent.) were present in thymus, and the conclusion was that these represented an extremely potent antigen, the immunising agent for the ANS activity in anti-thymocyte serum. The present investigation suggests another possibility.

The immunising lymphocytes for RALS 2 were observed to be contaminated with less than one neutrophil per 1000 cells, and the immunising dose was only 50×10^6 cells. It is possible that this small number of neutrophils induced the formation of the highly potent antiserum RALS 2, with activity similar to that described for RALS 1 and HALS. An alternative hypothesis is that both lymphocytes and neutrophils bear a common antigen that has been the immunogen for the different ALS described here. This explanation is furthered by the observation that all labelling activity could be removed by absorption with neutrophils or peripheral blood lymphocytes. That absorption was only partial with thymocytes from a 15-cm foetus could be explained by postulating that specific lymphocyte antigens had not developed in the 15-cm foetus, or that the relevant antigen is not present on thymocytes. The thymocytes of the 16-day foetal mouse bear θ-antigen (Raff, 1971) and it might be expected that any corresponding antigen in man would appear before the 15-cm stage.

Any common L and N (lymphocyte and neutrophil) antigen may be present at a higher density on neutrophils than on lymphocytes, where it is readily demonstrable by fluorescence methods only on fixed films of lymphocytes. That only a proportion of lymphocytes in suspension were positive whilst all lymphocytes in filmed preparations were positive, may reflect differences in method of

preparation. Any labelling of sectioned lymphocytes is less apparent and this may be the result of xylol–alcohol processing of tissues. Modified techniques are required that will allow direct comparison of lymphocytes in suspension with filmed lymphocytes and with lymphocytes in tissue sections, after the elimination of technical artefacts that have made interpretation difficult.

Even accepting the hypothesis of a common L and N antigen the nature of the brightly positive cells in the foetus is uncertain, and a detailed morphological analysis of the perivascular cells in the thymus will be necessary before any definite conclusion can be drawn. The positive cells do not seem to correspond to any single recognised morphological type, though they do have some features in common with primitive myeloid cells. Foci of granulocytopoiesis have been observed in the rat thymus (Sin and St Marie, 1965), and this would seem to warrant further investigation.

The demonstration of marked binding of ALS to neutrophils in sectioned lymph-nodes was unexpected, and compounds, rather than disperses, the problem of identifying cells of the lymphoid series. However, the possibility of identifying populations of cells in sections when they are not otherwise distinguishable by morphological means would seem to merit further study. Also, although each anti-lymphocytic serum described here is clearly composed of a spectrum of antibodies and not a single immunoglobulin species, the evidence of binding to cells other than lymphocytes (especially neutrophils) should be considered for its therapeutic implications, and possible biological activity against non-lymphoid cells.

Finally, it was not possible to demonstrate any clear distinction between RALS 1 and 2, raised against chronic lymphatic leukaemia lymphocytes, and HALS, raised against peripheral blood lymphocytes from normals, except that the titre of activity as measured by this study was much lower in HALS than in RALS 1 and 2. Studies of other anti-lymphocytic sera, prepared against normal and leukaemic lymphocytes, for activity against neutrophils and other cells will be of interest.

Summary

Anti-lymphocytic sera raised against peripheral blood lymphocytes from chronic lymphatic leukaemia patients and from normal individuals, have been applied to tissue sections with the aim of specifically labelling lymphoid cells by immunofluorescence techniques. Lymphocytes showed varying binding of ALS with different methods of preparation. Neutrophils showed marked binding of ALS as demonstrated by bright fluorescence in lymph-node sections. A number of brightly fluorescent cells were observed in foetal tissues, but the nature of these has not been established.

It is suggested that lymphocytes and neutrophils possess a common antigen, and that this has been an immunogen in the three anti-lymphocytic sera examined.

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