THE PHYSIOLOGY OF THE HUMAN RETICULAR CELL

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SUMMARY

When culturing cell suspensions from human lymph node and tonsil two types of cell were isolated. One was a typical macrophage while the other was a multi-branched, dendritic cell with a characteristic nuclear morphology. These cell types were also isolated from lung, spleen, thymus and peritoneal fluid. These large cells differed from fibroblasts in certain cytochemical reactions, in some ultrastructural aspects and also in their behaviour in culture.

The dendritic cell, which is called the reticular cell in this work, has a large nucleus with one or more prominent nucleoli. The branched cytoplasm shows pyroninophilic striations and rapidly accumulates lipid droplets. Electron microscopy showed that the cells contained dilated sacs filled with finely granular material. The membranes bounding these sacs were studded with ribosomes. Although these findings are consistent with a secretory function for the reticular cells, only occasional cells had an amorphous material around their membranes. This material could never be shown to have the periodicity characteristic of collagen. The cultured reticular cells grew over each other and failed to show contact inhibition, a characteristic feature of fibroblasts.

I have repeated the experiments of the earlier workers using vital dyes such as trypan blue and neutral red, and have confirmed the existence of two types of phagocytic cells. The reactivity of reticular cells towards a range of particles has been investigated and compared with that of macrophages from the mouse peritoneum and fibroblasts/

fibroblasts from cultures of human skin. It was found that the reticular cells ingested colloidal particles and yeast. Lymphocytes added to cultures of these cells adhered to the processes. The macrophages avidly ingested all the particles used, while the fibroblasts ingested none within three hours. However a few of the fibroblasts ingested yeast when the incubation period was extended to three days. Those experiments showed that the reticular cells had a range of activity intermediate between that of true macrophages and fibroblasts. I have also shown, using cytochemical techniques, that the reticular cells contain a range of hydrolytic enzymes similar to that of macrophages.

It is well known that antibody promotes the uptake of particles by macrophages. In this <u>in vitro</u> system, however, it was found that the reticular cells did not ingest antibody-coated erythrocytes, although it could be shown that iron-containing material accumulated in them if the coated erythrocytes were added to cultures containing both types of cell. Other workers have shown that antigens localise on a dendritic web in lymphoid tissue about the time at which 7s antibody production starts, and that further localisation of antigen is probably due to the formation of antigen-antibody complexes at the surfaces of the reticular cells. R.G. White suggested that these antigen-retaining cells are macrophages containing ingested antigen which have migrated to this area and settled down there and assumed a dendritic form. Another possible mechanism is that the macrophages with their ingested antigen migrated to this site and then transferred the antigenic material to a second cell type. It

was/

was therefore of interest to study the handling of antigens by cultures containing both types of cell, that is, reticular cells and macrophages in the same culture.

Particles such as Clostridium septicum and soluble material in the form of antigen-antibody complexes tagged with either fluorescein or I^{125} were used for the studies on cell interactions. The antigen or the complex was allowed to remain in contact with the monolayer for a time sufficient to allow the macrophages to ingest the material, and the non-ingested material was washed off. After incubating these cultures for various times the material originally found in the macrophages could be detected in the reticular cells. At the beginning of the experiment the antigens were not found within the reticular cells, but could be demonstrated in or on the cells at later times. Continuous and time lapse cinematography showed that bridge formation between macrophages and reticular cells occurred frequently. Material could therefore be transferred from macrophages to reticular cells via these bridges. It has also been shown that, although the reticular cells contain a range of hydrolytic enzymes, they do not degrade yeast at the same rate as do macrophages.

The conclusions drawn from these experiments are:-

1. Reticular cells are a distinct cell type with a characteristic morphology.
2. They concentrate vital dyes and are therefore cells of the reticulo-endothelial system as defined by Aschoff.
3. They are selectively phagocytic. Their phagocytic activity is independent of serologically-detectable antibody.
4. These cells, although containing degradative enzymes do not appear/

appear to have a phagosome-lysosome system for the disposal of ingested material, since they do not degrade the yeast at the same rate as do macrophages.

5. The reticular cells are capable of receiving material or information by intercellular transfer, as shown by time lapse studies.

These findings suggest that a possible *in vivo* rôle for the cells would be the retention of antigen but it has not yet been proven that the *in vivo* antigen-retaining cell described by White (1963) and Nossal (1964) is the reticular cell which has been characterised *in vitro*.

ABBREVIATIONS

R.E.S. - Reticulo-endothelial System

R.N.A. - Ribonucleic acid

U.V. - Ultra violet

E.M. - Electron microscope

P.B.R. - Prussian blue reaction

P.A.S. - Periodic acid Schiff

R.B.C. - Red blood cell

M.G.P. - Methyl green pyronin

CHAPTER I

INTRODUCTION

Since the description of phagocytosis by Metchnikoff (1884) and others in the last decades of the nineteenth century, much experimental work has been done to try to identify and classify the phagocytic cells and to determine their distribution, origin and functional importance. The early workers used benzidine dyes as described by Goldmann (1909) and by Aschoff (1924) and his pupil Kiyono (1920). Their work united all the phagocytic cells into one system which was defined by the ability to store ingested dye which had been administered to the intact animal. In these experiments it was noted that not all cells stained to the same extent and that the degree of staining was determined at least partly by the particle size and the concentration of the substance used. Efforts were made to separate the phagocytic cells into different classes. Cunningham, Sabin and Doan (1923-24) distinguished two types of phagocytic cell in the rabbit spleen. They called one type the clasmatocyte and the other the monocyte. The clasmatocytes were identified as follows: "They were usually large cells 15-30 micra in diameter. These cells always reacted to neutral red, the aggregations of the stain varied greatly in size and color (light red to deep maroon) and were scattered irregularly throughout the cell." The monocytes were identified with those of the peripheral blood and described thus: "These cells were almost always uniform in size, about 15 micra in diameter and their horseshoe or kidney shaped nuclei were large in comparison to the amount of cytoplasm. . . . Opposite the hof of the nucleus there was a clear central spot surrounded by a rosette of/

of small, radiating aggregations of neutral red, the most peripheral of which were considerably larger than the others." These studies were done on preparations made from spleen punctures from living animals and the authors concluded that "however difficult it may be to establish criteria for two distinct types of phagocytic cells in fixed tissues, in living material definite and satisfactory criteria can be established for separating these cells into two distinct types." This same group of workers then extended this division of phagocytic cells to include those found in the connective tissues:- "Thus we consider that the weight of evidence points to two separate strains of phagocytic cells of the connective tissues: clasmatocytes, which are of endothelial origin, and come into the blood stream only occasionally and abnormally, and monocytes, which are a constant type of blood cell, arising largely in the spleen, but also a specific of the diffuse connective tissues, where they both arise and function." Later this group demonstrated that a few clasmatocytes were to be found in normal blood.

The use of vital and supra-vital stains on living preparations was extended to histological material by Cappell (1929) in his extensive studies on the distribution of these dyes in the whole animal. He used not only vital dyes such as trypan blue but also particulate material such as carbon and noted that the demarcation of the phagocytic cell system was dose dependent. "If the dye be used in too high a concentration the vacuoles and granules of the segregation apparatus of the fibrocytes appear to become larger and more/

more numerous, and thus the sharp distinction which can usually be made between fibrocytes and histiocytes may no longer be so clearly defined and difficulty may be experienced in deciding to which group a given cell belongs." In his descriptions of the types of cells which take up these dyes (in the spleen) he noted that "the Malpighian bodies remain almost completely free from the vital stain; careful search with the oil immersion lens will however, reveal scanty very fine granules in the reticular cells of the lymphoid follicles. . . . This phenomenon strengthens the opinion that the reticulo-endothelial elements, while possessing certain properties in common which justify their being grouped together into a 'system', are yet strikingly selective and specialised in their activities in the various organs." The presence of only a few granules in the cells of the Malpighian body was to some extent dependent on the choice of animal since in guinea pigs occasionally these structures showed marked staining whilst in rabbits this never occurred.

The lymph glands were also found by Cappell to stain with trypan blue or with issamine blue and he describes, as well as true macrophages, the following: "The reticular elements of the sinuses and those supporting the lymphocytes in their meshwork of fibrils are also selectively stained, and the cytoplasmic processes which extend along the reticulum fibrils are filled with dye particles." He found that the reticular cells of the lymphoid follicles were capable of ingesting a small amount of particulate matter, e.g. carbon, within one hour of an intravenous injection, and noted "in the/

the pulp spaces in all situations the carbon or iron is contained in many free phagocytic cells in addition to the fixed reticular cells." The reticular cells, which had finer granules of carbon or iron, did not appear to be so mobile. These cells were especially prominent in the omentum where they were called mesothelial cells and were said to be similar to the cells lining the serous sacs.

Evidence for the separation of the two types of cells described above from fibroblasts was given by Evans and Scott (1920). These workers observed that there were distinct differences in the way in which the two cell types stored the dyes. The macrophages stored the dye in large droplets which were usually arranged in clusters, whereas the fibroblasts contained scattered small granules.

In studies on regeneration many workers have noted a spindle-shaped cell which they have hesitated to call a fibroblast (Cappeli, 1929; Bloom and Taliaferro, 1938; Booz, 1959). Perhaps the best instance of this is from Stuart (1962) describing splenic regeneration: "Perhaps the most important cell in regeneration is the spindle mesenchymal cell which unequivocally dominates the repair of Malpighian bodies. It cannot with propriety be called a fibroblast since fibrous tissue is never formed even when it is present in large numbers." Bloom and Taliaferro (1938) noted that similar cells, which they thought of as reticular cells, formed sheaths around the periarterial lymphatic tissue of regenerating canary spleens. These cells were frequently phagocytic. These workers were also of the opinion that transformation or modulation of macrophages/

macrophages to spindle or reticular cells was a common occurrence after injury to the avian spleen, and that cells at the spindle or fibroblast-like stage were phagocytic, as were the true reticular cells. They also believed that reticular cells could give rise to fibroblasts which produce collagen.

At the present time it is generally accepted that the reticular or reticulum cell is a large mononuclear cell which is intimately associated with the lattice of fibres of the reticulo-endothelial system, especially in the lymph nodes, spleen and tonsil. In spite of many detailed studies of in vivo material there are still conflicting views as to the origin and function of these cells. I believe that this cell is part of the RES but that its functions and morphology make it quite distinct from the macrophage and that it is the second type of phagocytic cell described in the work reviewed above.

Since the advent of the electron microscope the morphology of the reticulum cell has been more clearly defined. Light microscope studies had given only a very vague description which could apply to many primitive cell types e.g. that given by Cruikshank, Dodds and Gardner (1968), "It is of moderate or large size, oval, polygonal or elongated. The nucleus is oval or reniform and has an open chromatin network with conspicuous nucleolus." The early E.M. studies were by Fresen (1960) and Weiss (1958). These were extended by Roberts and Latta (1964) who studied the red pulp of the spleen of the rabbit. According to their report it is possible to divide/

divide reticulum or reticular cells into three main types, although intermediate forms were found. The first form was the most primitive, with a smooth nuclear membrane, homogeneous nucleoplasm and prominent nucleoli. The cytoplasm contained moderate numbers of spherical mitochondria, few RNA particles and inclusion bodies. Rough endoplasmic reticulum varied with the state of the cell, being more prominent when inclusions were present. When the sections were stained with uranyl acetate, the staining qualities of this cell were lighter than the two subsequent types. The second type was usually attached to the reticular fibres of the spleen, showed more electron dense or dark areas when stained with uranyl acetate than did type one and had more prominent endoplasmic reticulum. The third category of reticular cell was always attached to the lattice of fibres. It usually was spindle-shaped with cytoplasmic projections extending along the splenic basement membrane. The cytoplasm had large numbers of RNA granules, moderately well developed endoplasmic reticulum and ovoid mitochondria with poorly developed cristae which were often replaced by a homogenous matrix. This is similar to the descriptions given by Han (1961) and to the later one given by Swartzendruber (1966) for the reticular cells of the germinal centres of lymphoid tissues.

Kajikawa (1964), using the electron microscope, studied the lymph node, especially the formation of reticular fibres. He compared the structure of the reticular cells with that of fibroblasts in a healing wound. While the fibroblasts had large amounts of/

of well developed (rough-surfaced) endoplasmic reticulum in the form of cisternae which contained finely flocculent material, the reticular cells had smooth-surfaced endoplasmic reticulum, cytoplasmic inclusions and occasional lysosomes. The fibrils which frequently accumulated at the periphery of the fibroblastic cytoplasm, appeared to form by condensation of the flocculated material in the Golgi vesicles connected to the endoplasmic reticulum. The Golgi vesicles and the oval or round profiles of the endoplasmic reticulum of the reticular cells were apparently empty. Some rough-surfaced endoplasmic reticulum was found as isolated sacs and some of the smooth type had ribosomes on the outer surface.

The structure of the immature cells of the series was similar to that described by both Roberts and Latta, and Kajikawa who thought that this indicated that these cells had two possible lines of differentiation; one to fibroblasts and one to reticular cells. His evidence was not sufficient for him to form conclusions about the site of formation of the reticular fibres.

Studying the response of the rat peritoneum to implants of agar or egg white, Curran and Clark (1964) observed that the initial layer of macrophages assumed a fibroblastic appearance at the point most distant from the implant. These layers of cells, which closely resemble macrophages near the egg white, and fibroblasts at outer edge, became covered with layers of cells identical to the mesothelium or serosal cells which line the peritoneal sac. These cells/

cells are similar to endothelium except that they have large mitochondria and prominent endoplasmic reticulum. The mesothelial cells lining the peritoneum had previously been shown to be phagocytic by French and his colleagues in 1960.

The network of cells described by Cappell using vital dyes is very similar to that shown by silver impregnation methods (Marshall, 1946), and to that found by Ortega and Mellors (1957) when tracing gamma-globulins in lymph nodes using fluorescent tracers. Mellors and Brzosko (1962) reported that antigen-antibody complexes were localised in germinal centres especially when immune clearance could be demonstrated. They concluded that this phenomenon could be related to antibody production.

A web-like distribution of antigen was reported by White in 1963 and by Nossal and his colleagues in 1964. White used fluorescein-labelled antibody to determine the distribution of intravenously injected serum albumin in chickens, while Nossal's group used I^{131} and I^{125} labelled flagellar antigens given into the footpad of rats. Both experiments showed that the localisation of the antigen in the follicles became more prominent at or just before, the detectable onset of 7s antibody production. Electron microscope studies using autoradiography showed that most of the antigen in the follicles was associated with the surface of the cytoplasmic processes of reticular cells, (Szakal and Hanna, 1968).

Balfour and Humphrey (1967) showed that the localisation of antigen on the dendritic cells was antibody dependent. Using animals/

animals which were truly tolerant, i.e., showing no antibody production either by immunofluorescent methods or by a sensitive haemagglutination technique, they found no localisation in follicles. However in animals which were only partially tolerant or in those in which tolerance had been broken, follicular localisation occurred. They also found that staining (fluorescence) was arranged around post capillary venules in a manner similar to germinal centres. Using the technique of both autoradiography and fluorescence, the first for antigen and the second for antibody, they showed that the dendritic cells were histiocytes or macrophages.

At the same symposium, White (1967) reported similar findings and suggested that the antibody coating the dendritic cells complexed with the antigen, and then the lymphocytes, possibly with antibody on their surface, aggregated around this antigen. This is the earliest stage of germinal centre formation, which then enlarges to become a recognisable germinal centre by growth of individual cells and multiplication of the lymphoid elements.

Thus it can be seen that there is still confusion about the function of the reticular or reticulum cell which although it has been well described in vivo has proved difficult to isolate and study in vitro. There is also still some doubt as to its functional capabilities and about possible potential as a precursor of other cell types. Rhodin (1967) expresses his opinion that fibroblasts can transform into macrophages as follows: "It has been claimed that fibroblasts can transform into macrophages in inflammation, but it/

it is most likely that the fibroblast is normally the precursor of the macrophage. The reason for this assumption is based on the situation which exists in lymphoid organs like the spleen, the lymph nodes and the bone marrow. Here, special cells, reticular cells, have the ultrastructure and the function of macrophages. In addition they are the only cells available which can be assumed to produce the collagen fibrils as well as the reticular fibrils of these organs."

Other experimental work has implicated the macrophage in the induction of a primary response, at least to a particulate antigen, (Stuart and Davidson, 1964; Fishman and Adler, 1963; Askonas and Rhodes, 1965; Gallily and Feldman, 1967). It is not yet clear whether the cells involved in the primary response are the same as those of the dendritic web. This is because macrophages are pleomorphic and present techniques are not precise or sensitive enough to settle this point.

In reviewing older literature dealing with the tissue culture of haemopoietic or lymphopoietic tissue, one finds that explants were mainly used. Observations on phagocytosis were, in the main, secondary. However, even at that time, it was clear that two types of phagocytic cells were present, (Maximow, 1922; Carrel and Ebeling, 1922; Lewis, M.R., 1925; Bloom, 1927). Perhaps the most clear description was that given by W.H. Lewis and his colleagues (1921) who, when studying cultures from human lymph nodes, reported that after 3-4 days in culture the prominent cell type was endothelial with a predilection for clear areas on the coverslip. These cells could assume either the shape of a fibroblast or that of a macrophage.

A/

A few years later Sabin, Doan and Cunningham (1923-24) showed that there were two types of phagocytic cell present in peritoneal exudates in animals. They used the distribution of neutral red granules as their indicator of cell type as previously described. Later these workers (1928) showed that these large cells, the clasmatocytes found in the blood, were desquamated endothelial cells. They postulated that the function of this type of cell and that of macrophages differed with respect to physiological debris. They stated in passing, that giant cells formed from endothelial-type cells have scattered nuclei.

McJunkin (1926-27) repeated the work on cultures of spleen and showed that, given the appropriate cultural conditions, both types of phagocyte could form a reticular network with intercellular cytoplasmic connections.

Carrel and Ebeling (1926 a and b) showed that there were major differences between macrophages and fibroblasts. The characteristics which identify the macrophage in culture have been expanded and restated by Willmer (1965). He classified cells in culture into six main types, only two of which are relevant to this discussion - mechanocytes and amoebocytes - and they are described as follows:-

"Mechanocytes (fibroblasts), again from a wide variety of sources, all of which are, however, mesenchymal, emerge as a loose network of cells, in which the cells are polarised and partly contiguous. They may sometimes grow in the form of sheets, when derived from endothelia and mesothelia.

Amoebocytes, derived from monocytes in the blood, from the macrophages/

macrophages in practically every tissue and from the microglia, show much more random movement, and these cells remain isolated from each other. These also may however under some conditions produce loosely knit sheets of cells. They also have a tendency to aggregrate into giant cells."

Fibroblasts display the phenomenon, described by Abercrombie and Heaysman (1954), of contact inhibition, which is defined by Willmer thus: "The behaviour in question can be summed up by saying that when contact inhibition is operative, a cell will not use another cell as a substrate for its locomotion." This was seen as a cessation of movement in a given direction as soon as the membranes of the two cells came into close contact. When this occurred the membranes fused and all movement ceased. After some time 'ruffles' of cytoplasm appeared in another part of the cell and the cell moved off in that direction, provided there was cell-free space available. Carrel and Ebeling (1926a) found that fibroblasts never grew as isolated units but remained in contact with other cells, while macrophages, on the other hand, lived as independent units and died if they congregated in dense masses.

Fibroblasts are not phagocytic for bacteria, erythrocytes or effete cells, although they will ingest, presumably by pinocytosis, small amounts of carbon. Lines of fibroblasts which have been maintained in culture for many generations, e.g. L strain cells, are phagocytic for some particles (Epstein et al., 1964). However this appears to be a general property of 'eternal' cell lines since it has been shown that HeLa cells, epithelioid cells derived from human cervical/

cervical cancer, are also phagocytic for some particles (Gordon *et al.*, 1965).

Stuart (1967) isolated cells from the peritoneal cavity of humans and showed that there were two types of cell present. One population was similar to the macrophage obtained from mouse peritoneum and had similar functional properties. The other cells were much larger, morphologically similar to the reticulum cells seen in tissue sections, and did not ingest either antibody-coated erythrocytes or starch granules.

Cells with morphology and functions similar to these large cells found in the peritoneal cavity can be isolated from human tonsil, lung, thymus, spleen and lymph nodes.

This thesis deals with the *in vitro* properties of cells isolated from the lymphoid organs which resemble the reticular cells described *in vivo*. Because of the ease of obtaining them most of the studies were done on peritoneal cells. The use of *in vitro* techniques allowed functional studies to be done. These studies are important because morphology can be misleading. This was well expressed by Carrell and Ebeling (1926) who said: "The determination of the specific physiological properties of a cell is of evident importance, as the individuality of a cell depends on its functions far more than on its staining reactions."

As well as characterising the functional abilities of these cells, an attempt has been made to study their potential for development in response to transforming agents and to assess their capacity and methods for handling antigens.

CHAPTER II

MORPHOLOGY, CYTOCHEMISTRY AND CINEMATOGRAPHY OF RETICULAR CELLS

The cells under investigation were obtained from spleen, lymph nodes and ascitic fluid. The ascitic fluid came from patients treated by peritoneal dialysis for renal failure. Ascitic fluid was also obtained from a patient with malignant ascites caused by gastric carcinoma and from another patient suffering from thrombosis of the inferior vena cava.

The morphology of the reticular cells was studied after various periods in culture, and careful examination showed that the cells sometimes contained nuclear debris (Fig. 1). In one case the cultures had accidentally become infected with yeast and I noted that the reticular cells had phagocytosed the yeast cells. Since reticular cells do not ingest erythrocytes it seemed that this might be an example of selective phagocytic activity towards nucleated cells. This hypothesis has been tested by exposing reticular cells to dead or damaged leucocytes.

The question arises whether these cells ought to be included in the reticulo-endothelial system (RES). When one considers the morphological heterogeneity of the RES and the frequent association of macrophages with reticular cells it seems possible that they might be members of this functioning unit. Certainly it is important to determine whether these cells ought to be classified either as macrophages or as fibroblasts.

Willmer (1965) in his description of various cell types, said that the amoebocytes (macrophages) live without contact with one another, move randomly and show extensive pinocytosis. Mechanocytes on/

on the other hand show polar movement and this movement is inhibited when they make contact with one another. These contacts are extremely strong whereas these which occur between macrophages are loose and short lived.

Cytoplasmic 'bridging' was shown to occur between macrophages by Aronson (1963) and by Lindholm and Britton (1967). Therefore the movements of reticular cells have been compared with those of macrophages and lymphocytes. Intercellular connections between reticular cells and between macrophages have been studied.

MATERIALS AND METHODS

CELL CULTURES

In the case of peritoneal dialysis fluid, it was found that the early cycles of second and subsequent treatments gave the best cell yields. The fluids used as a source of cells were processed as described by Davidson (1967). The fluid was centrifuged at 200 g. in sterile pint blood bottles at 10°C for 20 minutes. The supernatant fluid was discarded, except in the case of ascitic fluid where a small portion was sterilised for use in the culture medium, in place of autologous serum. The sedimented cells were pooled and resuspended in tissue culture medium 199 with 10 i.u. heparin per ml. The suspension was recentrifuged, the supernatant discarded, and this sediment resuspended in a further volume of 199. The cell concentration was adjusted to 1.5 to 2 x 10^6 cells per ml. Serum or sterile ascitic fluid was then added to give a final concentration of/

of 10% or sometimes 5%.

The cells were grown in tubes (Pyrex 16 x 125 mm. rimless) with flying coverslips (Chance No. 1.19 x 6 mm.) and stoppered with white rubber bungs (Esco Rubber Ltd.). After seeding, the cultures were incubated at 37°C for 24 hours. Any adherent red cells were detached by gentle shaking and the medium was then changed. The cultures were incubated at 37°C for a further 24 hours and used for experiments at this time. This gave a mixed population of macrophages and reticular cells. When a culture containing predominantly reticular cells was required, the cultures were incubated for 4 days before use.

The large reticular cells could also be obtained from tonsils by disrupting the organ, allowing the cell suspension to settle onto coverslips for about 6 hours, changing the medium and then leaving the cells for a further 48 hours.

Lung and lymph nodes were obtained from the surgical theatre, minced, using scalpel blades, and incubated in 0.02% trypsin for 30 minutes, centrifuged at 200 g. for 20 minutes, resuspended in medium 199 and then cultures seeded, at approximately 5×10^6 cells per tube. These cultures were usually suitable for use after one or two weeks in culture.

Human spleen was obtained from patients undergoing surgical procedures. Part of the spleen (about 10 g.) was removed, the capsule dissected off, and the tissue minced into pieces 1-4mm^3. This finely divided tissue was gently homogenised in a loosely fitting/

fitting all-glass homogeniser containing medium 199. The suspension was filtered through a stainless steel mesh into a measuring cylinder, allowed to settle for 10 minutes at room temperature and then the number of cells in the supernatant was counted. Tubes and coverslips were seeded at 5-8 x 10^6 cells per tube in 10% pooled human serum. The medium was changed after 24 hours and the cells studied after a further 24 hours.

The length of time required for each culture to reach a state suitable for use was very variable. Those from more solid tissues such as lung and lymph node required longer than those from more easily disrupted tissues like spleen or tonsil. The cells from the former not only took longer to attach to glass but it also was more difficult to get rid of cell debris.

Subsequently it was found that satisfactory cultures could be obtained from reactive lymph nodes without using trypsin. These cultures could be used after 4-7 days.

Light microscopic studies were done on preparations stained by the following methods: Giemsa; haematoxylin and eosin; Heidenhain's haematoxylin; periodic acid Schiff (PAS) - haematoxylin; methyl-green and pyronin; Prussian blue (PBR) - neutral red; silver impregnations were carried out according to the techniques of Slidders, Fraser and Lendrum and Weil Davenport. The details of these methods are given in the appendix.

Time lapse cinematographic studies were done in order to ascertain if both cell types were present in the sample or if the reticular/

reticular cells arose from modification of the macrophages as a result of culture conditions. The equipment used was a Wild inverted microscope with a quartz iodine light source, to which was attached a Bolex cine-camera. An electrically operated shutter and a U.V. filter were inserted between the light source and the mirror. Films were made using x 20 or x 40 objectives with a x 10 eyepiece in the phototube. The most satisfactory interval between frames was found to be 3 seconds for the phase during which the cells were settling and 5 seconds for studying established cultures. The movements of the types of cells found in the sample were compared and contrasted by this method. The cultures were prepared in perspex culture chambers. These were 3 x 1 in. pieces of 1/8th in. perspex with a hole $\frac{1}{2}$ in. in diameter drilled through the centre. A coverslip was attached to the bottom by silicone grease, the chamber sterilized by U.V. light, and then the cell suspension added. The top coverslip was held in place by capillary attraction.

Electron microscope studies were done on the cultures of human peritoneal cells. The cultures were set up in baby bottles at 15×10^6 cells per bottle in 15 ml. medium 199 containing 10% human serum. The cultures were fixed after 2-4 days or after 10-14 days in culture. One sample was a control, i.e., a culture to which nothing had been added. The other culture was given 0.5 ml. of a heat - killed 1% suspension of yeast and incubated for a further 3 hours. In one set of experiments the cultures were washed in saline, fixed in osmium tetroxide for one hour then dehydrated through/

through graded alcohols and embedded in araldite. The araldite was polymerised at 56°C and the hot bottle plunged into an ice bath. This shattered the glass which detached from the araldite leaving the cells on the araldite. This method allowed the cells to be examined in the extended state. The other method used to prepare material involved scraping the cells off the walls of the culture vessels, centrifuging and then fixing them in gluteraldehyde for 24 hours. The samples were post-fixed in osmic acid, dehydrated and embedded in araldite. The detailed methods used are given in the appendix. Sections were cut on an L.K.B. ultramicrotome with glass knives, stained with lead citrate and examined on an electron microscope.

The cultures were examined, using histochemical methods for the following enzymes:-

acid phosphatase; esterase; leucine aminopeptidase; succinic dehydrogenase; lactic dehydrogenase; malic dehydrogenase; beta-glucuronidase; lipase and alkaline phosphatase. Detailed methods are given in the appendix.

RESULTS

Light microscope studies of the preparations of peritoneal cells made on flying coverslips showed that there were two morphologically distinct cell types present in the culture. One type was of variable shape, usually small with numerous short processes but the cells could be either round or spindle-shaped. These cells had a/

a reniform nucleus without nucleoli, and were considered to be typical macrophages (Figs. 2 and 3). The other type, called the reticular cell, had a large clear nucleus with two to four nucleoli (Fig. 1). Reticular cells are large cells and both bi- and tri-nuclear forms were found in most cultures. Functionally and enzymatically they were identical to the mononuclear forms. These multinucleate cells were also found in cultures from the lymphoid organs (see Figs.10 and 13).

The cells never aligned themselves parallel to one another as fibroblasts would have (Fig. 4). In these reticular cells the nucleoli and the striations often seen in the cytoplasm were pyroninophilic, suggesting that they contained large amounts of RNA (Fig. 5). After a few days in culture the reticular cells contained large numbers of fat globules in the cytoplasm (Fig. 6). Argyrophilic fibres, probably reticulin, could be demonstrated when the cells had been in culture for a few weeks (Figs. 7 and 8). If the initial suspension of peritoneal or spleen cells was contaminated with erythrocytes then PBR-positive material accumulated in the cytoplasm of the reticular cells (Fig. 9).

Reticular cells have also been isolated in the present study from lung, lymph node, spleen, thymus and tonsil. The cells obtained are illustrated in Figs.10-13. It is clear that these cells are pleomorphic although function tests confirm they are probably of the one type.

Electron microscope studies were done on cells from peritoneal fluid/

fluid after they had been in culture for 3, 10 or 14 days. The 3 day cultures were fixed and embedded as described in the materials and methods section of this chapter. Examination of the material showed that there were two types of cells present. One type of cell was the macrophage, the small cell with a reniform nucleus and no other striking features on stained preparations. The fine structure of the human macrophage is similar to that of the cell from the mouse peritoneum. They usually had an irregular and deeply indented surface, with many cytoplasmic processes, usually fairly short. Considerable quantities of endoplasmic reticulum were present, both granular and agranular. Within the cytoplasm were many smooth vesicles, containing material of varying electron density, and presumably representing the agranular reticulum. Elsewhere, commonly in the concavity of the kidney shaped nucleus, lay grouped vesicular and vacuolar profiles forming a typical Golgi apparatus. In any individual section a macrophage contained at least one granular body or lysosome and usually 4-6, but a few contained considerably more. The nucleus of the macrophage was commonly indented or bean-shaped and showed prominent nuclear pores. In the human cells studied here the macrophages frequently contained debris both of leucocytes and erythrocytes.

The other type of cell had an oval nucleus with one or more prominent nucleoli (Figs.14 and 15). The cytoplasm had few phagosomes or vacuoles but had many ribosomes, lipid droplets and mitochondria (Fig.16). These cells occasionally contained remnants of/

of degenerating cells (Fig.17), some of which were recognisable as lymphocytes.

The 10 day old culture had had the medium changed after 1, 3 and 7 days. The cells were processed in the same manner as the 3 day culture. Examination of these older cultures showed that the healthy cells were all reticular cells (Fig.18). Many of them showed cytoplasmic desmosome-like junctions with neighbouring cells (Fig.19). The mitochondria were numerous, pleomorphic and prominent (Fig.20). Pale, weakly osmiophilic lipid globules were also frequently seen. Many of the reticular cells contained dilated sacs filled with a finely granular material. These sacs were bound by a membrane studded with ribosomes, and polyribosomes were numerous in the cytoplasmic matrix (Fig.21). Figs.20 and 21 both from 10 day old cultures, illustrate the great variations in fine structure found within reticular cells. The nuclear structure was constant but the cytoplasmic organelles varied not only from cell to cell but also from one area of the cell to another. However, there were constant features in sufficient numbers to allow the comparison of the cells as variations of the one type.

An amorphous material was found around the cell membranes of cultures 14 days old (Fig.22). This was easily seen, since the cells had been fixed without removing them from the glass. Interdigitating cell processes were found in these cultures (Fig.23). The secreted material was in accord with the presence of the large numbers of ribosomes shown in Fig.21. As no studies using radio-

labelled amino acids were done there is no definitive evidence that this material was produced by the reticular cells but it is reasonable to conclude that it was.

Cytochemical studies showed that the cells from the human lymphoid tissues contained the same enzymes as do macrophages (Table I). There was no marked difference in the range or content of enzymes of the human macrophages and reticular cells. However, the reticular cells appeared to have increased amounts of degradative enzymes at the later stages of culture but as no quantitative studies were done this is not conclusive. Alkaline phosphatase could not be demonstrated by any technique at any time during these experiments. These qualitative observations suggested that degradative or lysosomal enzymes increased with length of time in culture. This is in accord with the quantitative studies carried out by Cohn and Benson (1965) on macrophages from the peritoneum of mice. The distribution of acid phosphatase and lactic dehydrogenase at 2 and 7 days is illustrated in Figs.24 to 27.

Phase contrast cinemicrophotographic studies showed that there were three main types of cell present in the peritoneal fluids. These were lymphocytes, macrophages and reticular cells. Each type of cell moves in a characteristic fashion. The lymphocytes are small, move relatively rapidly and in the film, dart in and around the reticular cells. Characteristically the "front" of the cell has a fringe of small pseudopodia behind which the nucleus ringed with cytoplasm is followed by the "uropod" or handle-like projection. When/

When the lymphocytes meet an obstacle the movement changes to a worm-like wriggling. The movement of lymphocytes is always purposeful unlike the random movement of macrophages. This difference was well expressed by MacCallum (1936). "Then there are lymphocytes which move humbly, like slugs crawling only a little way, with head to the ground. But also there are macrophages which reach out great arms, perhaps in two or more directions, and at the end of these arms there is a flourish of clear protoplasm with out-flung streamers that wave and search about for whatever can be seized, or else the whole advancing margin of the cell flows out and comes back like a wave, sucking in any particle that comes in its way."

Macrophages have small oval or kidney shaped nuclei. The extended cytoplasm contains phagosomes, lysosomes and mitochondria which all appear as phase dark granules. The cytoplasm is surrounded by a broad thin transparent membrane, the outer edge of which is continually in motion and appears as a refractile veil of hyaloplasm. Macrophages in culture are pleomorphic. Some are disc-shaped, some are stellate and yet others have a fringe of fine processes which end in a knob-like protrusion. Unless responding to a chemotactic force, macrophages tend to oscillate around a central point for long periods and then migrate randomly. The third type of cell in the peritoneal fluid cultures is very large, of the order of three times the size of the macrophage. These are the reticular cells which have one or up to three oval nuclei with well defined nuclear membranes and one or more prominent nucleoli. The cytoplasm/

cytoplasm is extensive with many granules and extends pseudopodia in two or three directions. The cells glide slowly across the field or ascillate gently around one point. When the cells move they leave behind a long strand of cytoplasm with a "blob" at the end very like the descriptions of clasmatocytes (Jacoby, 1965). The movement of these cells has been illustrated in the film which accompanies this thesis.

Lymphocytes dart around but do not make contact with reticular cells in the autologous situation. However macrophages form cytoplasmic bridges when they meet a reticular cell. Similar cytoplasm connections are made between reticular cells.

Pinocytosis could also be seen in reticular cells as could occasional vacuoles containing cell debris. Stained preparations showed that the cell debris was mainly nuclear material (Figs. 1 and 17). In the preparations for electron microscopy similar debris was also seen. However it appeared from these electron microscopic studies that there was not a true phagocytic vacuole but that the two membranes were close together and that the apparent vacuole seen on light microscopy was the degenerate, featureless cytoplasm of the ingested cell.

DISCUSSION

Willmer (1965) in his classification of cell-types in culture, separates mechanocytes (fibroblasts) from amoebocytes (macrophages). The fibroblasts are polarised and partly contiguous whilst macrophages/

macrophages remain isolated from each other. Jacoby (1965) stressed the pleomorphism of cultured mammalian macrophages thus: "The great polymorphism of mammalian macrophage cultures is striking. The two types of cell which are present need to be further studied. The large amoeboid bizarre forms, particularly, should be tested, in detail, as to their phagocytic and pinocytic properties. It was mentioned that these cells - in spite of, and contrary to their form - have often been called epithelioid cells: these, at any rate in man, are supposed to be non-phagocytic."

The reticular cells described here have long processes that cross the processes of other cells and they never at any time show contact inhibition, a phenomenon known to be an important feature of fibroblasts (Abercrombie, 1965). Our cells are larger than macrophages, their nuclear structure is quite different, and their movements in culture are slower and less erratic. The older cultures of reticular cells shared some features with both macrophages and fibroblasts in that they phagocytosed some particles but had extended cytoplasm like fibroblasts. In view of Jacoby's comments on the variable morphology of macrophages, further comparisons between reticular cells, macrophages and fibroblasts were necessary.

Intercellular bridges were found between reticular cells and between reticular cells and macrophages. Similar bridges between macrophages have been observed by Aronsen (1963), Lindholm and Britton (1967) and Maclaurin (1969). Occasionally the reticular cells formed tight junctions, a phenomen never observed with macrophages/

macrophages but known to occur with fibroblasts.

The histochemical studies showed that the reticular cells had a range of hydrolytic enzymes similar to those found in macrophages. The cells were alkaline phosphatase - negative and argyrophilic; this was in agreement with the findings of Lennert (1961), studying reticular cells in tissue sections. He also reported that fibroblasts gave positive reactions for alkaline phosphatase and failed to react with silver stains. These differences between reticular cells and fibroblast suggest that they are different cell types. Marshall and White (1950) also gave a careful description of reticular cells in vivo, in sections of the rabbit spleen. Although the observations on the reticular cells in vitro, are in agreement with theirs in many respects, they found, unlike Lennert, that the reticular cells were not argyrophilic. Another point of difference is that the reticular cell studied by Marshall and White did not have nucleoli. There is therefore some doubt whether the reticular cell isolated from humans is identical with that of Marshall and White.

Electron microscope studies have more clearly defined the structure of a reticular (or reticulum) cell. Early work on this was done by Fresen (1960) and Weiss (1958). Later Roberts and Latta (1964) studied the red pulp of the rabbit spleen. They divided the reticular cells into three main types but emphasised that intermediate forms were frequently seen. The first form was the most primitive, with a smooth nuclear membrane, homogeneous karyoplasm and prominent nucleoli. The cytoplasm contained moderate numbers of spherical mitochondria/

mitochondria, few ribosomes and some inclusion bodies. The general appearance was of a cell with little electron dense material, or as Roberts and Latta described it, "a light cell", with little fixed or definite form. The second type was "darker" than type I, had more prominent endoplasmic reticulum and was usually fixed, i.e., attached at some point to the splenic fibres. The third category was always fixed and usually spindle-shaped with cytoplasmic projections extending along the splenic basement membrane. The cytoplasm had large numbers of RNA granules, moderately well-developed endoplasmic reticulum and ovoid mitochondria with poorly developed cristae which were often replaced by a homogeneous matrix. This is similar to the descriptions given by Han (1961) and Swartzendruber (1966) for the reticular cells of the germinal centres of lymphoid tissue.

Kajikawa (1964) compared the structure of the reticular cells with that of fibroblasts in a healed wound. The fibroblasts had large amounts of well-developed rough-surfaced endoplasmic reticulum in the form of cisternae, which contained finely flocculent material; the reticular cells had smooth-surfaced endoplasmic reticulum, cytoplasmic inclusions and occasional lysosomes. In the fibroblasts the fibrils, which frequently accumulated at the periphery of the cytoplasm, appeared to form by condensation of the flocculated material in the Golgi vesicles connected to the endoplasmic reticulum. The Golgi vesicles and the oval or round profiles of the endoplasmic reticulum of the reticular cells were apparently empty. Some rough-surfaced endoplasmic reticulum was found as isolated sacs and some

of/

of the smooth type had ribosomes on the outer surface. The structure of the immature cells of the series was similar to that described by Roberts and Latta; Kajikawa thought that this indicated that these cells had two possible lines of differentiation: one to fibroblasts and one to reticular cells. The ultrastructure of cultured reticular cells agrees better with the description given for reticular cells occurring *in vivo* than with that for fibroblasts. The latter cell usually contains a prominent endoplasmic reticulum, which is minimal in cultured cells. Although Kajikawa mentions the presence of cytoplasmic inclusions, these and secondary lysosomes were rarely seen *in vitro*. This is surprising in view of abundant hydrolytic enzymes, and perhaps this cell does not have a vacuolar system for the discharge of lysosomes. The cultured cells show numerous sacs lined with ribosomes, which have not been commented on by workers who examined material from living tissues. It is probable that they are present in an exaggerated form during culture and they may correspond to the pyroninophilic striations seen with the light microscope.

In 3-week-old cultures, some cells showed signs of an extracellular secretion. This finding is more in keeping with their being mechanocytes than macrophages, although we consistently failed to identify either collagen fibrils or the abundant endoplasmic reticulum that is found in fibroblasts. However, we cannot exclude the possibility that reticular cells may transform to fibroblasts.

Macrophages in culture have a very variable morphology, and the possibility/

possibility that the reticular cells in the cultures were simply macrophages of a bizarre type had to be considered. Time lapse cinemicrophotography showed that both cell types were present and easily distinguished within 1 hour of obtaining the specimen, i.e., as soon as the cells had attached to glass. In addition, the two types of cell showed completely different methods of locomotion. The macrophages, typically, showed oscillation with occasional rapid, random movements across the fields while the reticular cells glided slowly across the field. The only time the reticular cells made rapid movements was when an intercellular connection was breaking. When two reticular cells come into contact they may do one of three things: (a) form an intercellular connection, (b) glide off in another direction, (c) glide slowly across one another. The last of these phenomena occurs relatively frequently even in cultures in which there is plenty of "cell free" space. The cells were usually seen to cross each other in such a way that the nuclei never came into close contact. This suggests that contact inhibition as shown by fibroblasts does not operate. Willmer (1965) summarised the conditions applying for contact inhibition as follows: "When the ruffled membrane on the leading edge of a moving fibroblast makes contact with another fibroblast, (1) it ceases its ruffling movements, and the associated pinocytosis; (2) it forms an adhesion to the other fibroblast so close that no line of demarcation between the two can be seen with the light microscope. . . . (3) if the ruffled membrane was expanding in size at the time of contact, the expansion/

expansion ceases, there may often be some contraction, momentarily drawing the cells together (Abercrombie and Heaysman, 1954); (4) the locomotory movement of the whole cell ceases; (5) another ruffled membrane appears or expands and draws the cell away in a new direction." The reticular cells have not been observed to behave in this way at any time.

These morphological and cytochemical studies suggest that the reticular cell is one of the cells of the reticulo-endothelial system but that it is intermediate in structure and movement pattern between macrophages which are cells of the reticulo-endothelial system, and fibroblasts which are not.

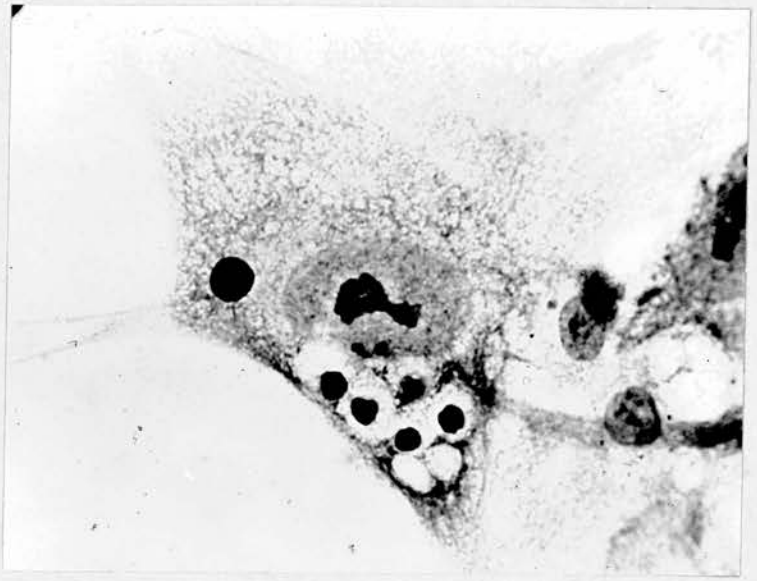

Fig. 1. Nuclear debris inside a reticular cell from the human peritoneum.

Giemsa x 800

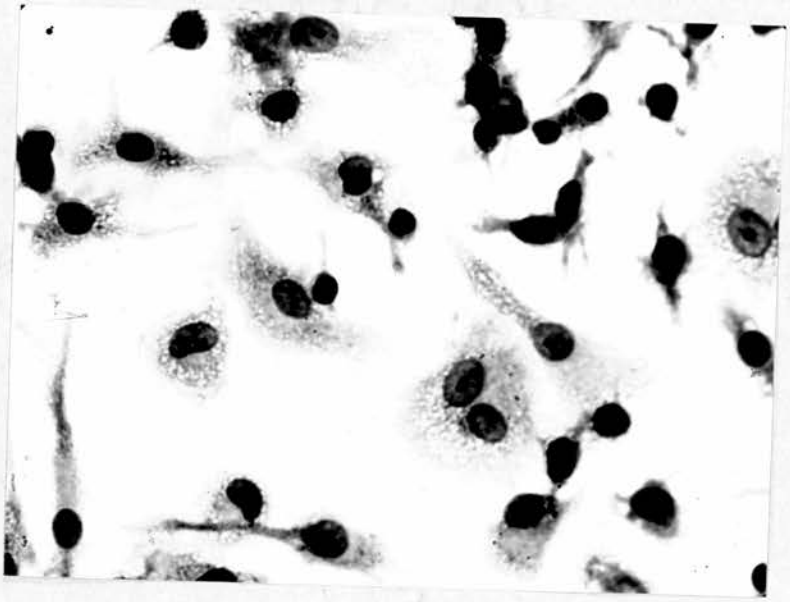

Fig. 2. From a 24 hour old culture of human peritoneal cells. Many of the cells are macrophages.

Giemsa x 525

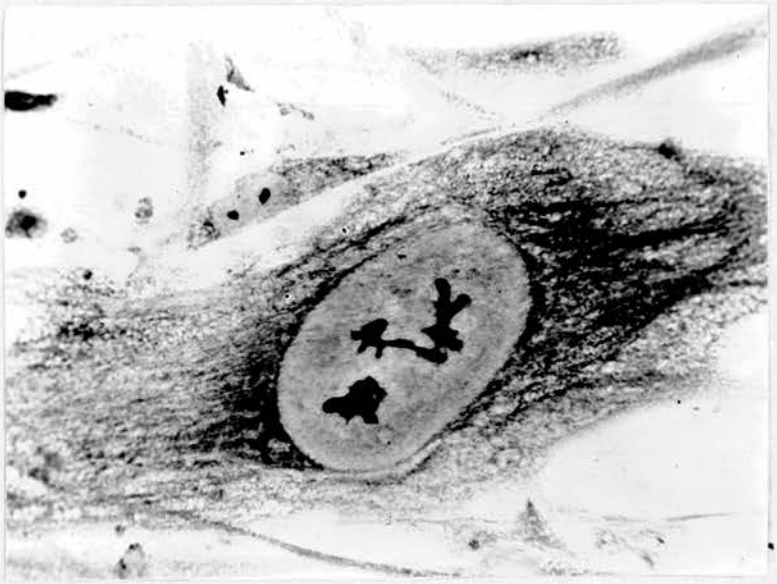

Fig.3. A reticular cell. Note the prominent nucleoli and the striations in the cytoplasm.

Giemsa x 700

Fig. 4. These are reticular cells from a culture of lung. Note the lack of contact inhibition. The cells cross each other randomly.

Giemsa x 275

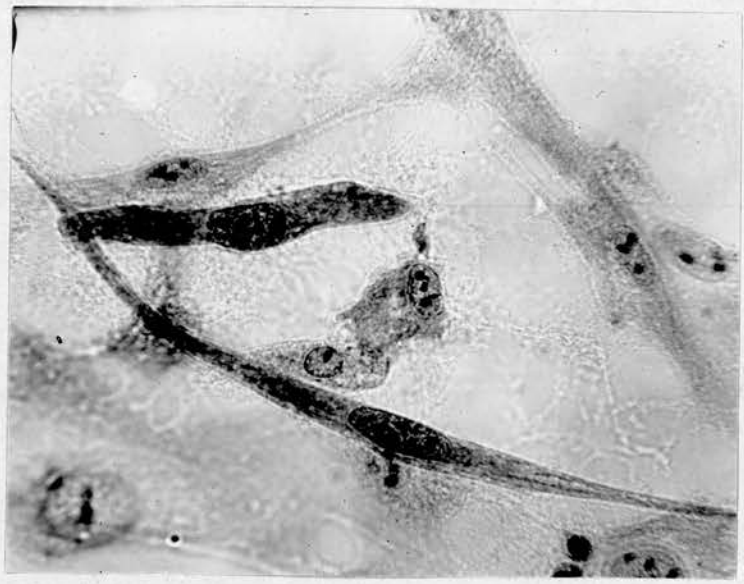

Fig.5. Reticular cells showing the prominent nucleoli. The cytoplasm appeared more granular than in Fig.2.

Methyl green pyronin x 700

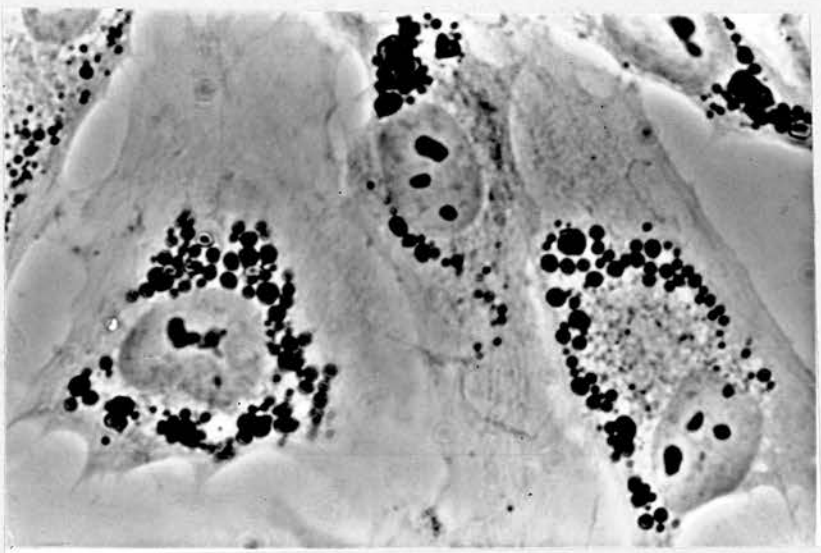

Fig. 6. Fat droplets in the cytoplasm of reticular cells after 4 days in culture.

Sudan IV - Haematoxylin x 400

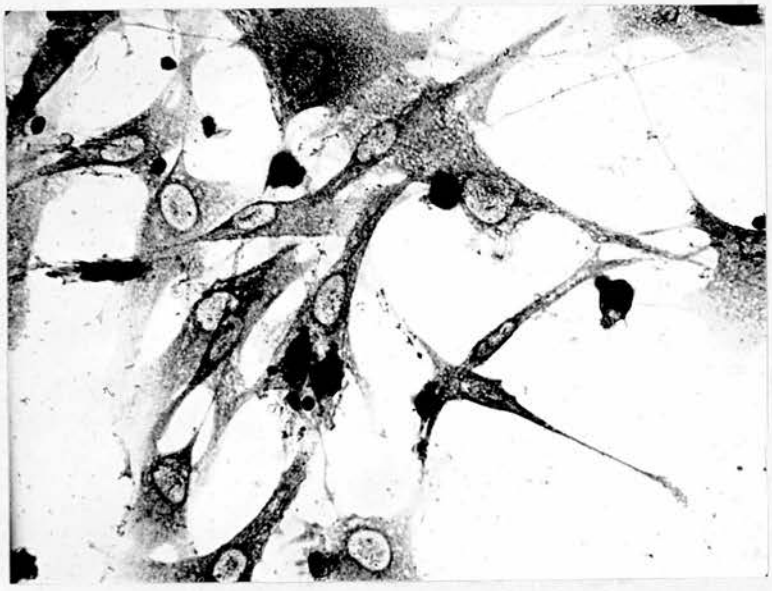

Fig. 7. A culture 10 days after isolation. Note the irregular beading on the processes, and the granularity of the cytoplasm.

Silver impregnation
Lendrum's method x 400

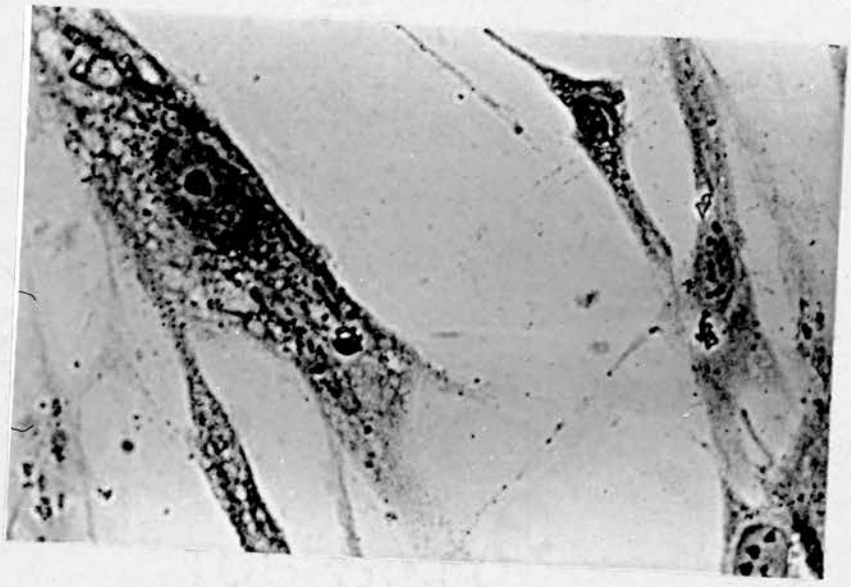

Fig. 8. A culture 7 days after isolation. Again the argyrophilic granules are prominent in the cytoplasm, the nucleoli also show silver deposition. The veils of cytoplasm linking the cells show little silver deposition.

Weil Davenport silver impregnation method

Phase x 200

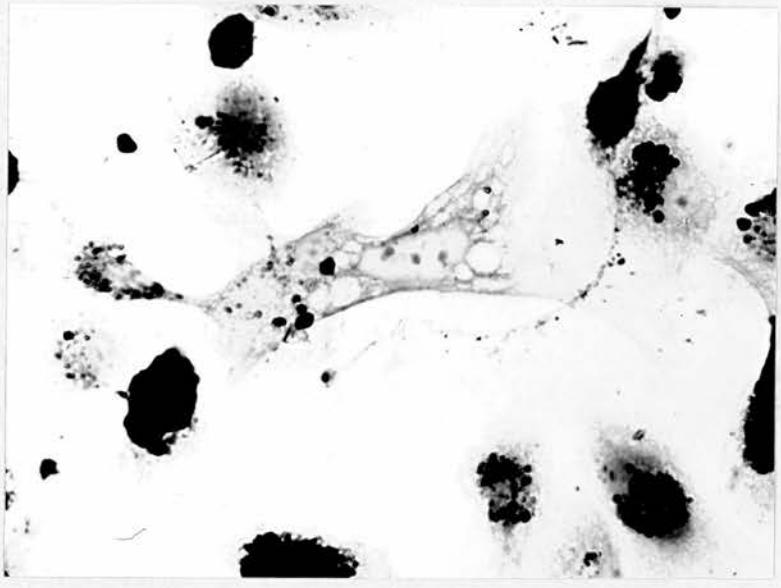

Fig. 9. Culture of human spleen from a case of haemolytic anaemia. Note the accumulation of iron (P.B.R.-positive material) in the macrophages and its apparent transfer to reticular cells via cytoplasmic connections.

P.B.R. Neutral Red x 450

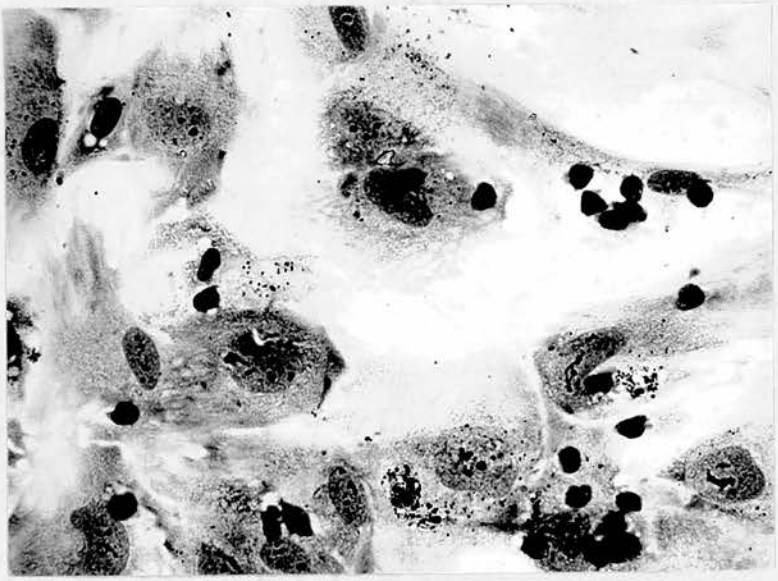

Fig.10. An 8 day old culture of cells from a human lymph node, showing carbon containing reticular cells.

Giemsa x 300

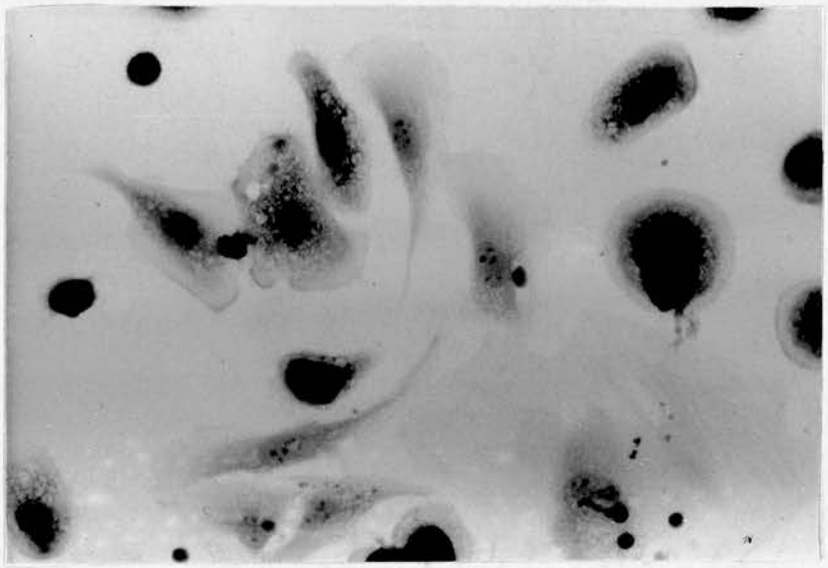

Fig.11. A culture of spleen 48 hours old. From a case of idiopathic thrombocytopenic purpura.

Giemsa x 200

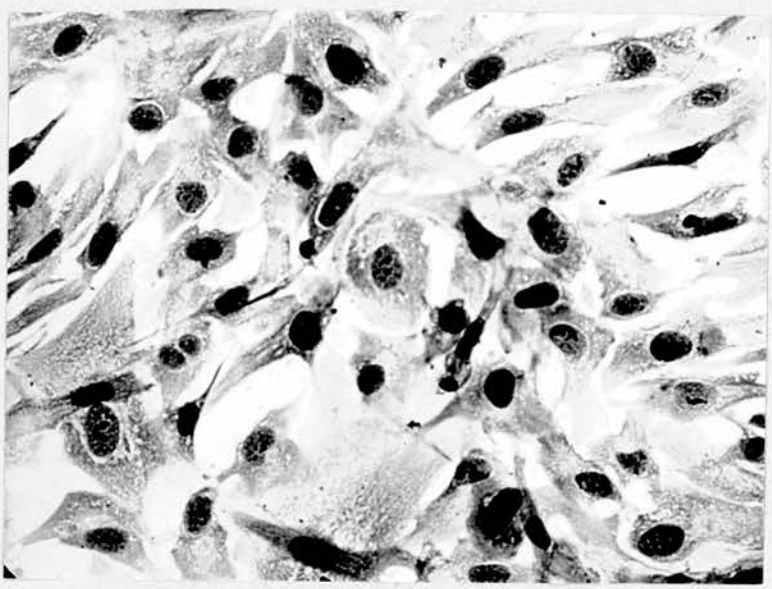

Fig.12. Reticular cells from a culture of lung.

Giemsa x 275

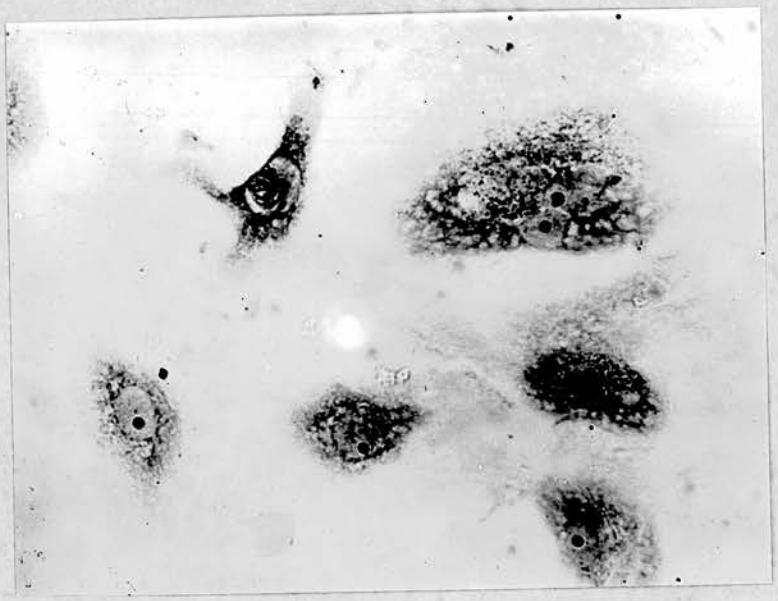

Fig.13. Cells from a culture of human thymus. Note pale-staining nucleus and prominent nucleoli.

Giemsa x 325

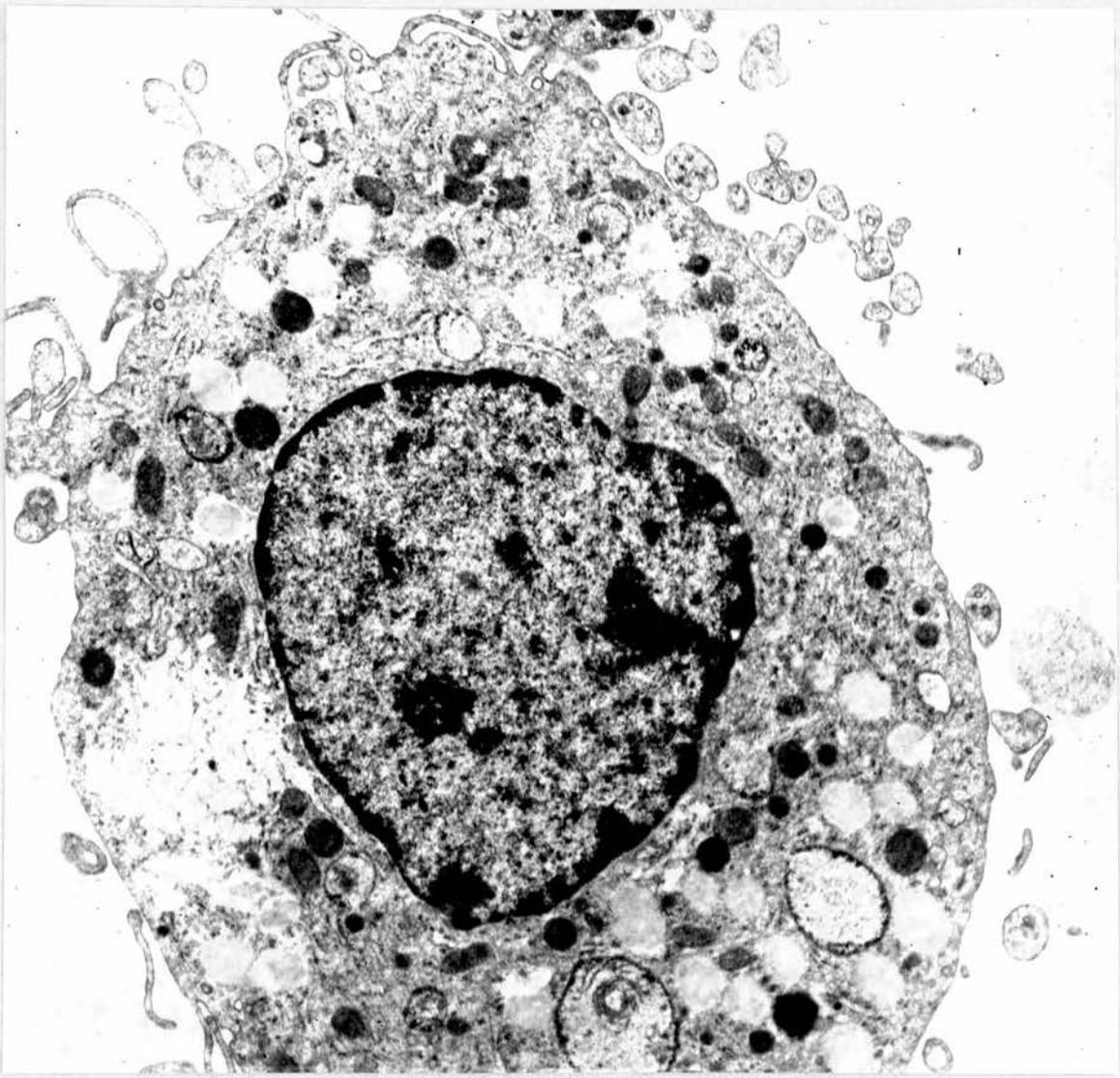

Fig.14. The general appearance of a reticular cell from a 3 day old culture.

EM x 8,000

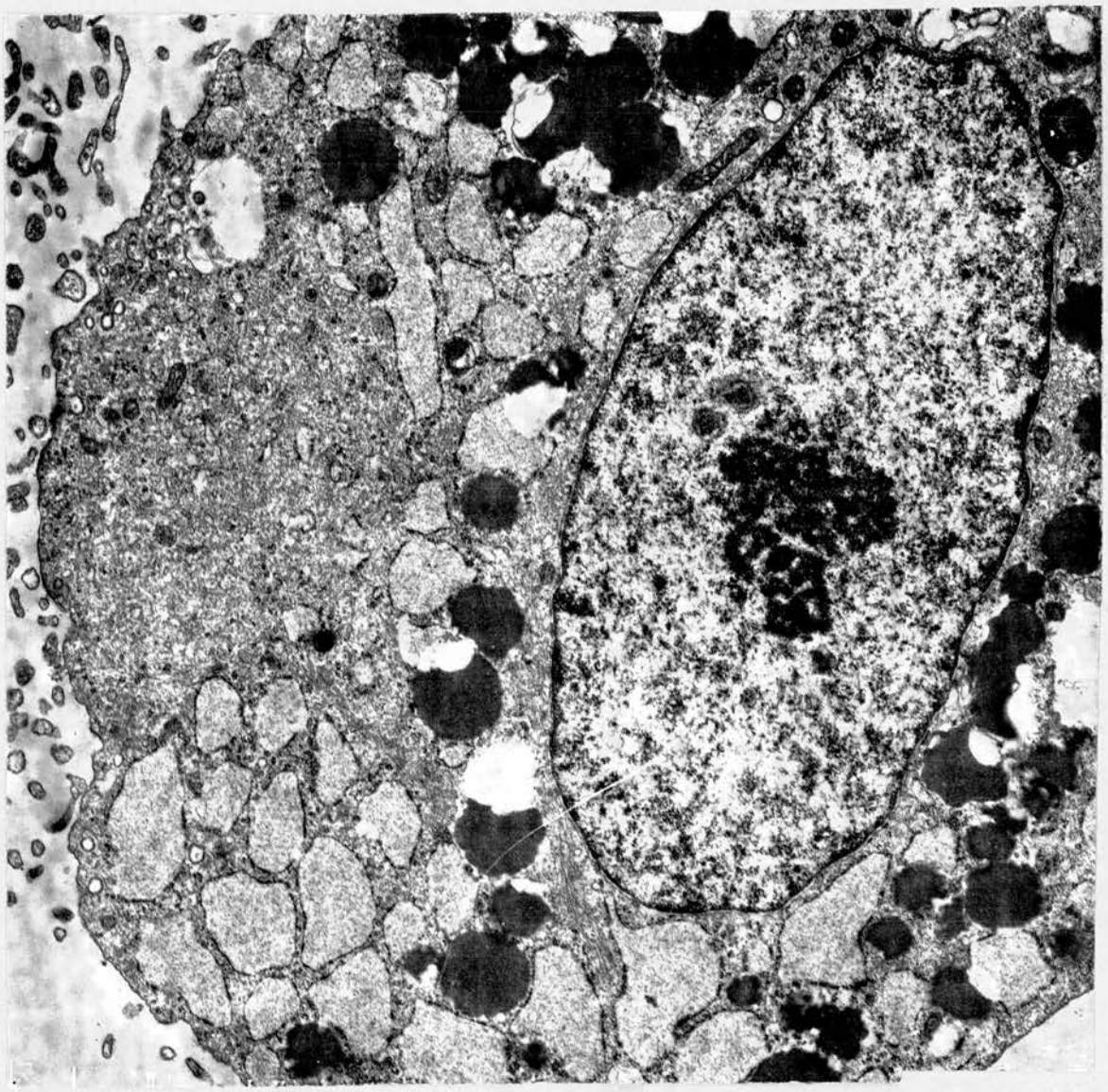

Fig.15. This shows the nucleus of a reticular cell with its prominent nucleolus. In the cytoplasm of the cell there are numerous lipid globules and dilated sacs containing flocculent material.

From a 3 day old culture. EM x 12,000

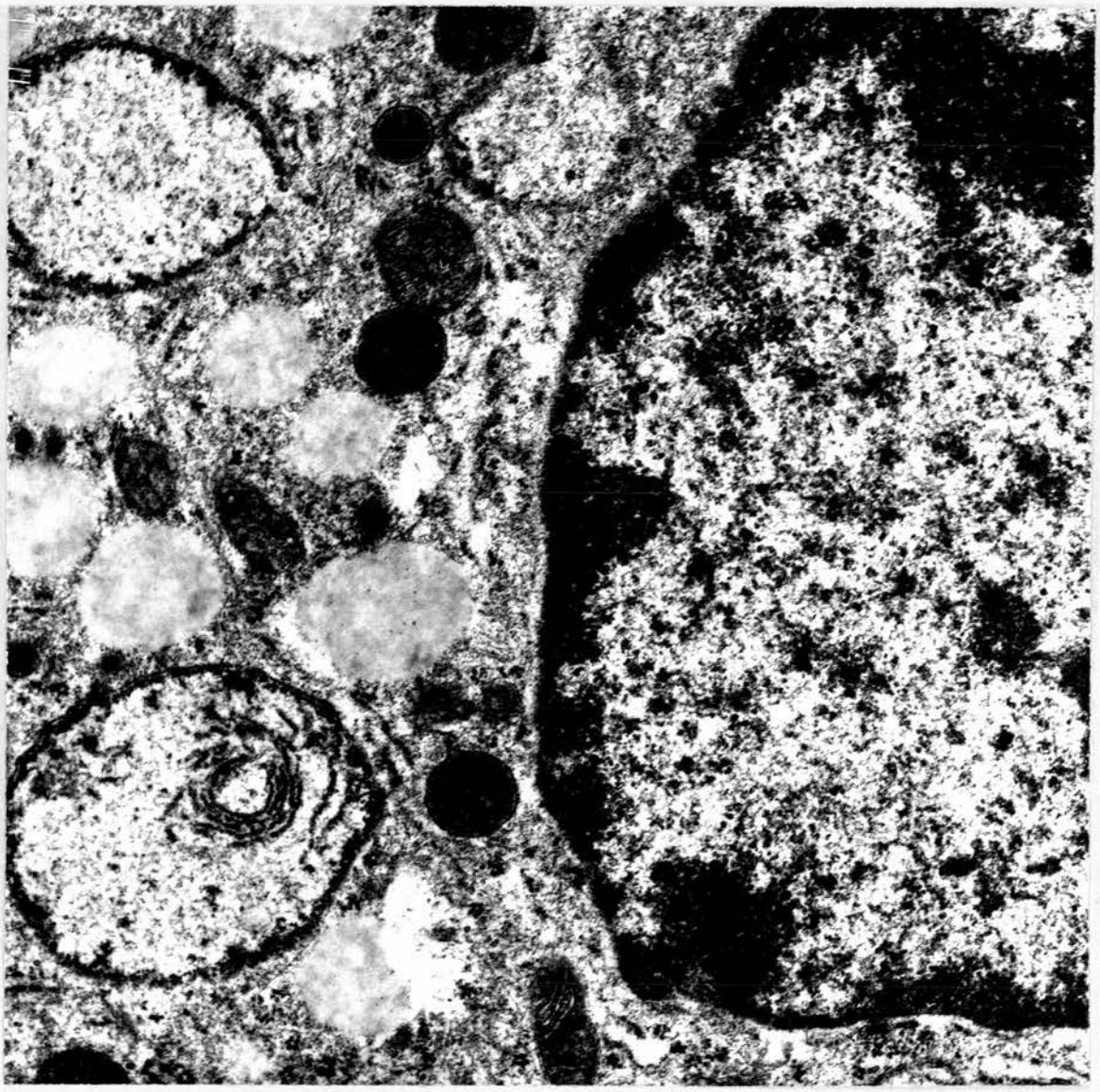

Fig.16. Part of the cytoplasm of a reticular cell showing mitochondria, lipophilic bodies and numerous prominent ribosomes especially associated with the membranes of the sacs.

From a 3 day old culture. EM x 32,000

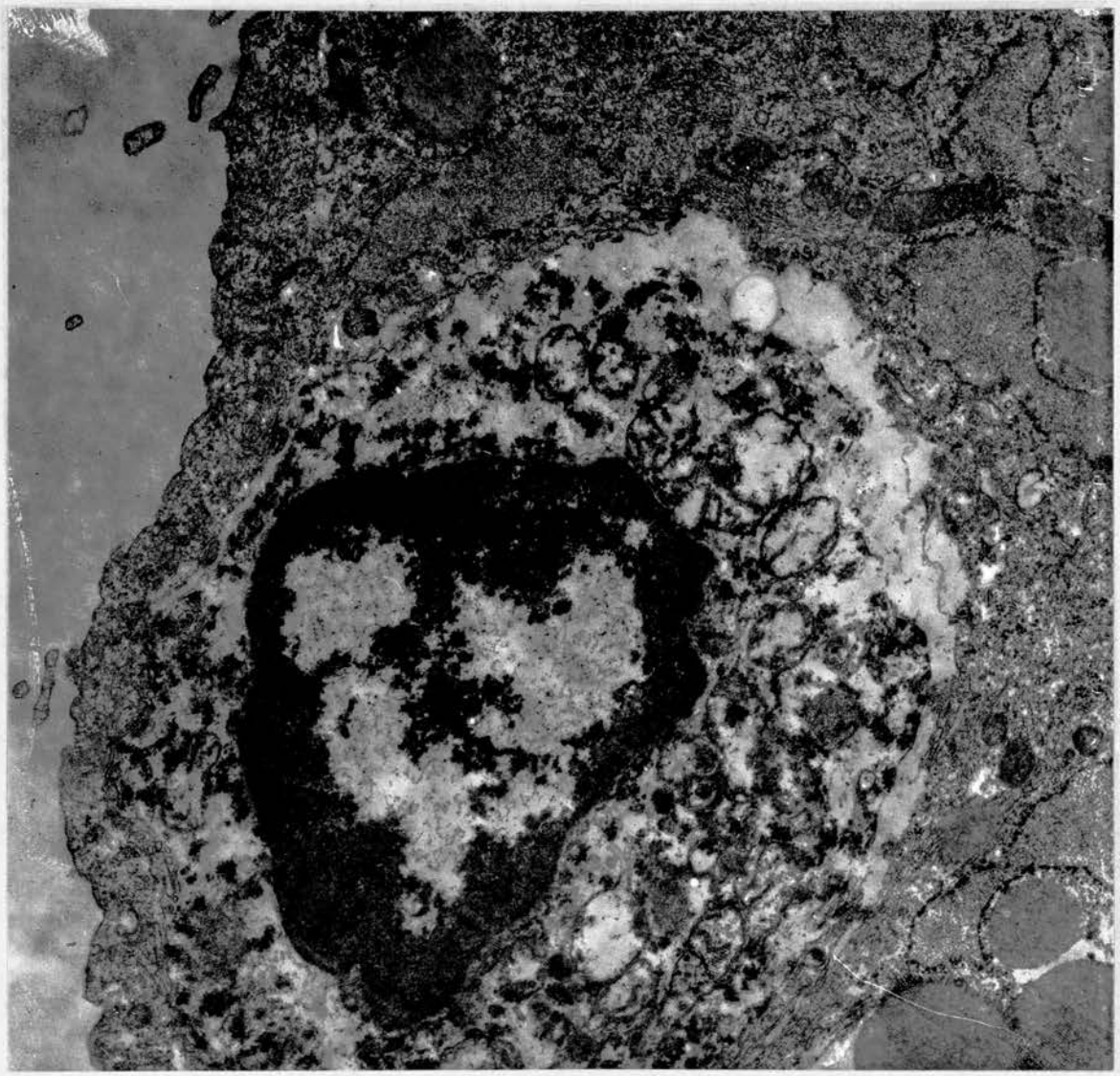

Fig.17. A degenerating leucocyte which has been ingested, intact, by a reticular cell.

From a 3 day old culture. EM x 20,000

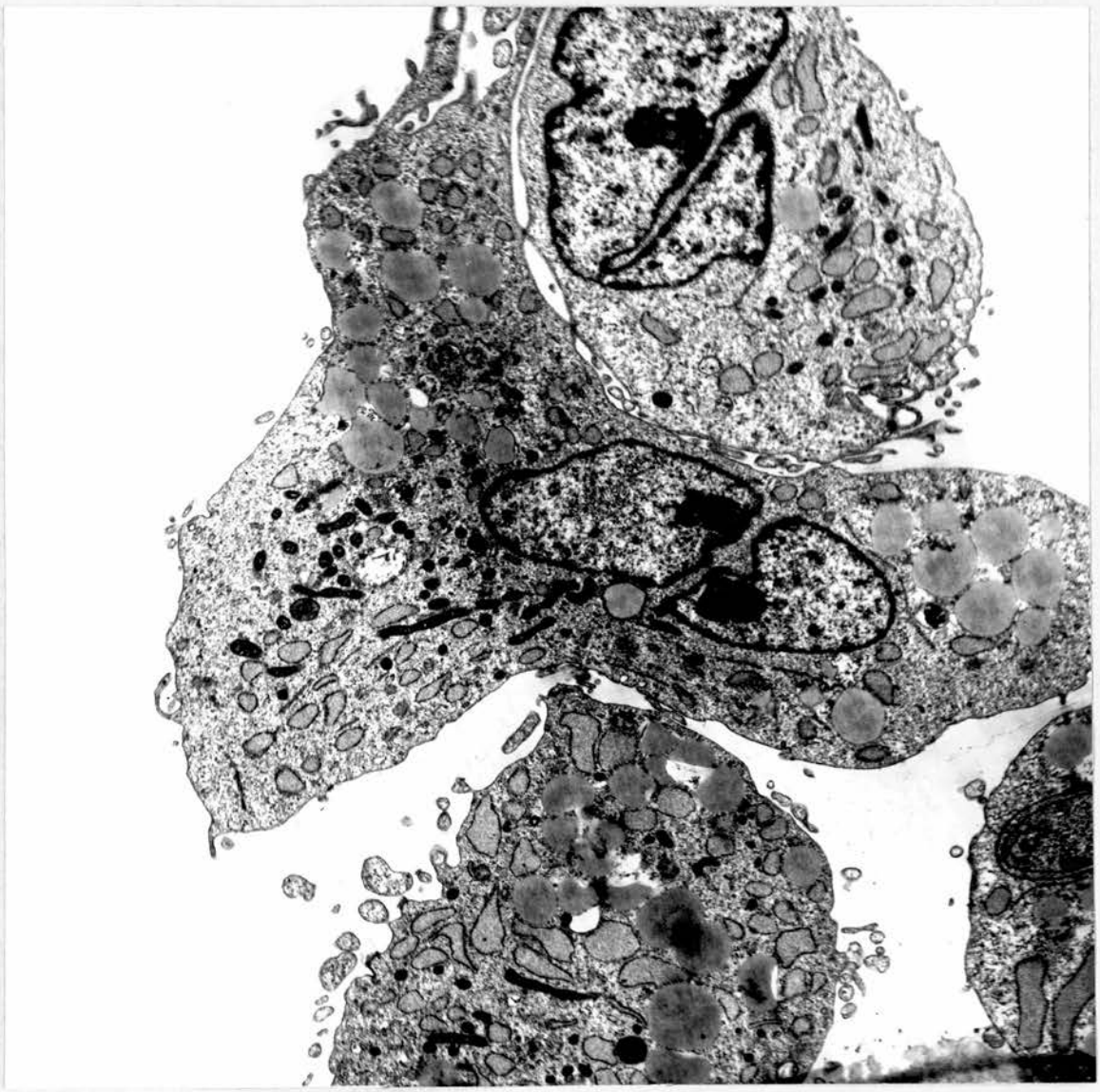

Fig.18. Three healthy reticular cells in close contact.

From a 10 day old culture. EM x 5,000

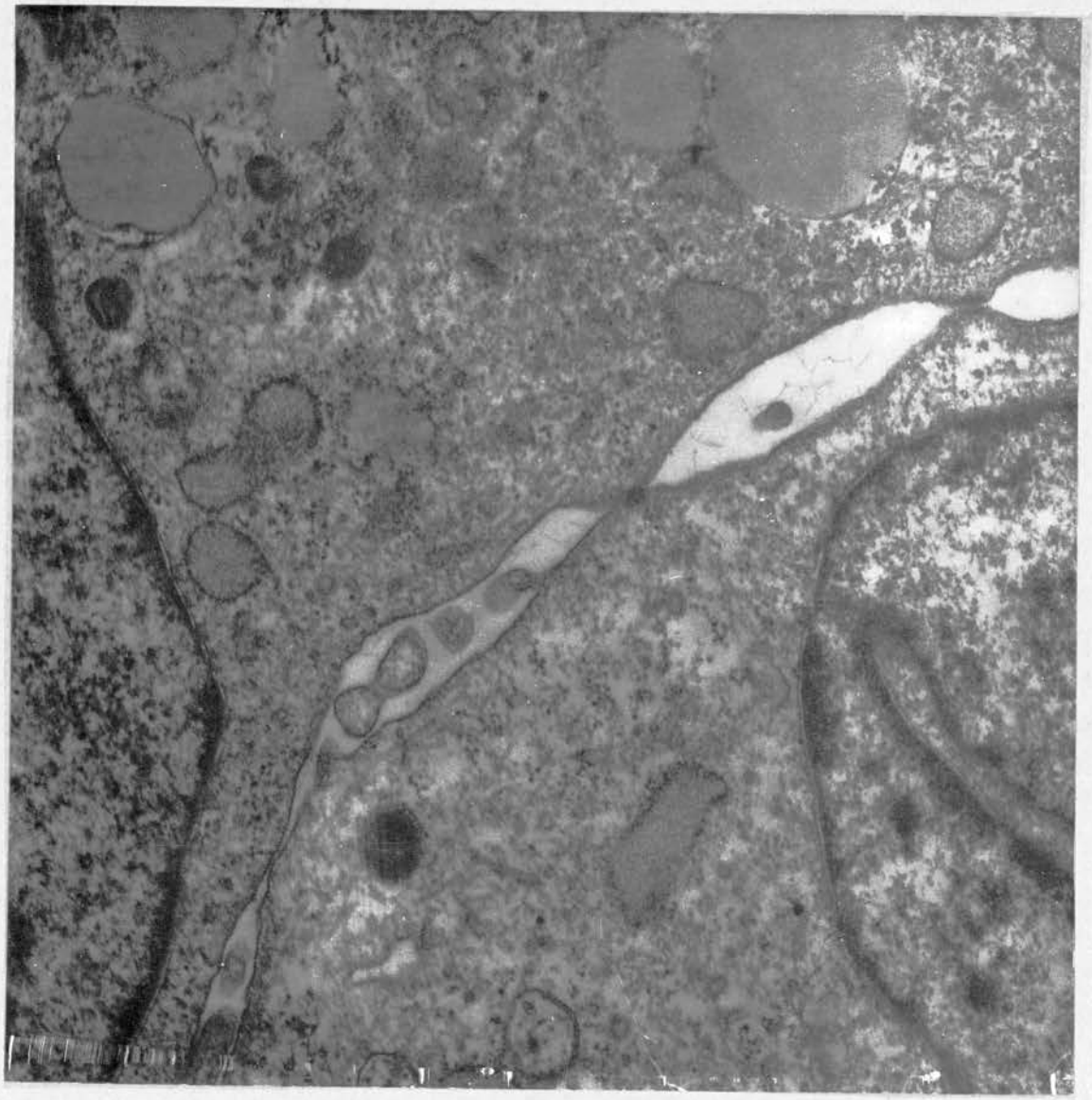

Fig.19. A high power electron micrograph of the desmosome-like intercellular junctions between reticular cells.

From a culture 10 days old. EM x 32,000

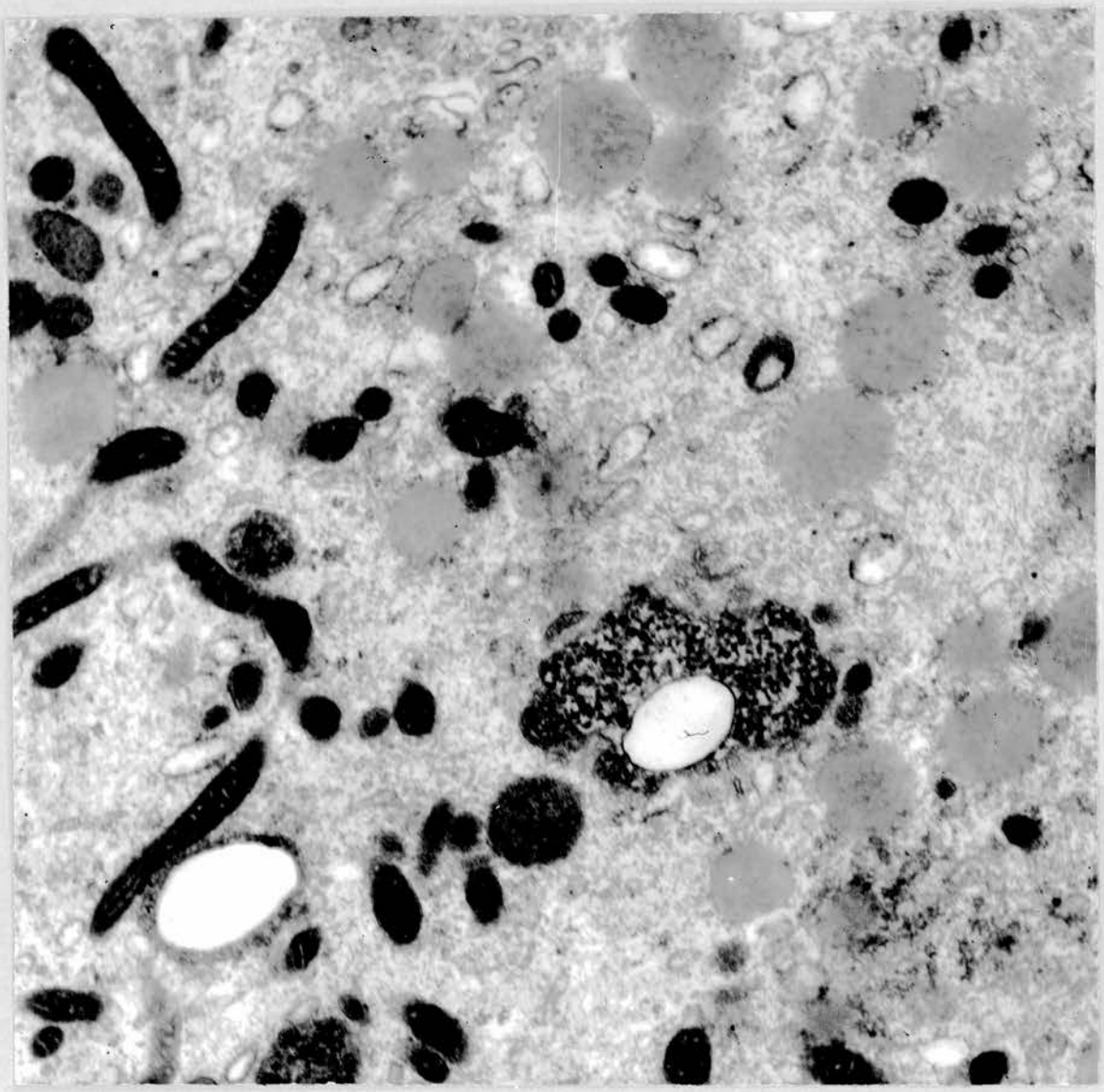

Fig.20. There are numerous pleomorphic mitochondria and pale-staining weakly osmiophilic lipid globules in this section of the cytoplasm of a reticular cell.

From a culture 10 days old. EM x 32,000

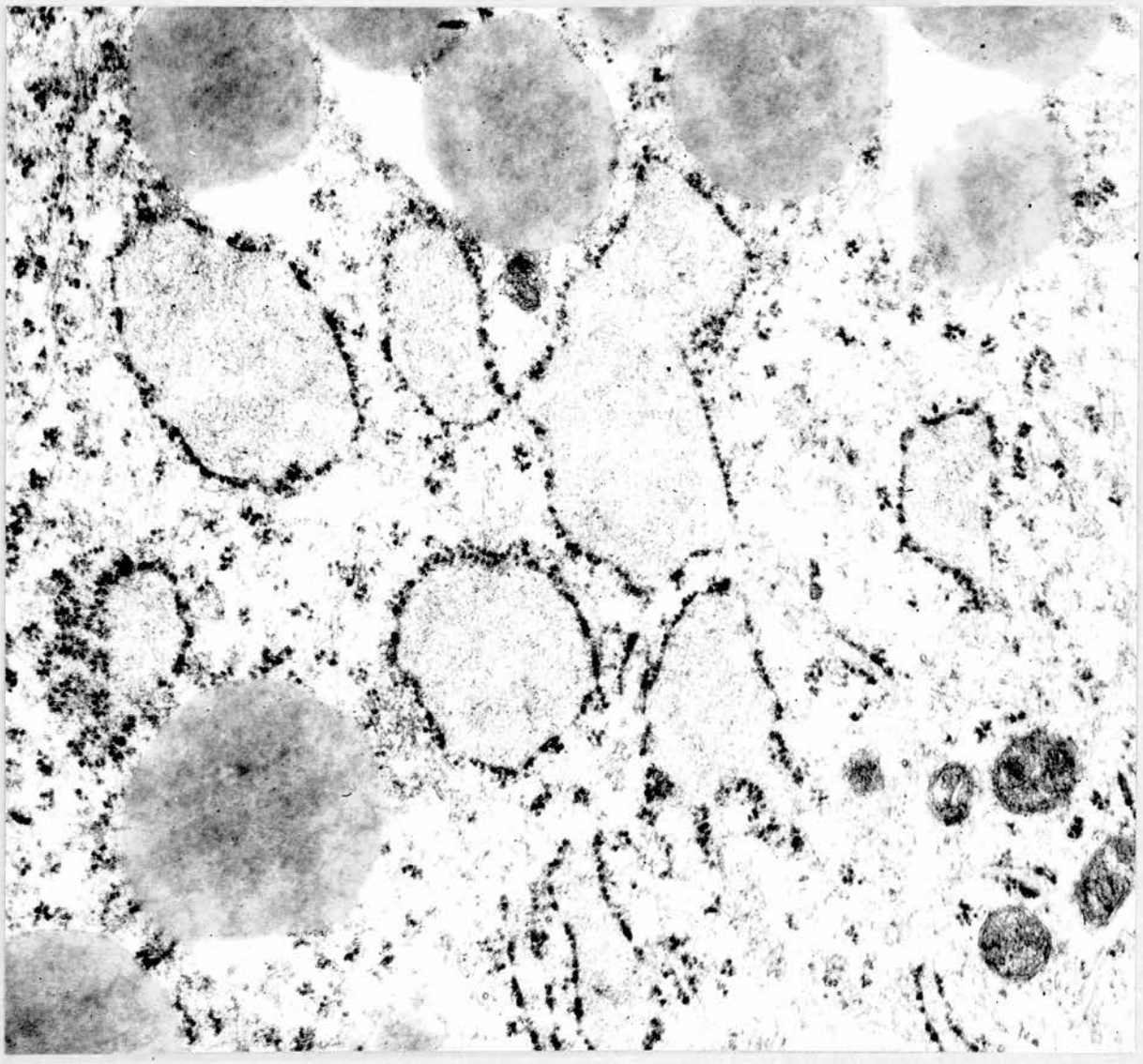

Fig.21. This shows numerous polyribosomes in the cytoplasmic matrix. The dilated sacs are filled with a finely granular material and bound by membranes studded with ribosomes.

From a culture 10 days old. EM x 40,000

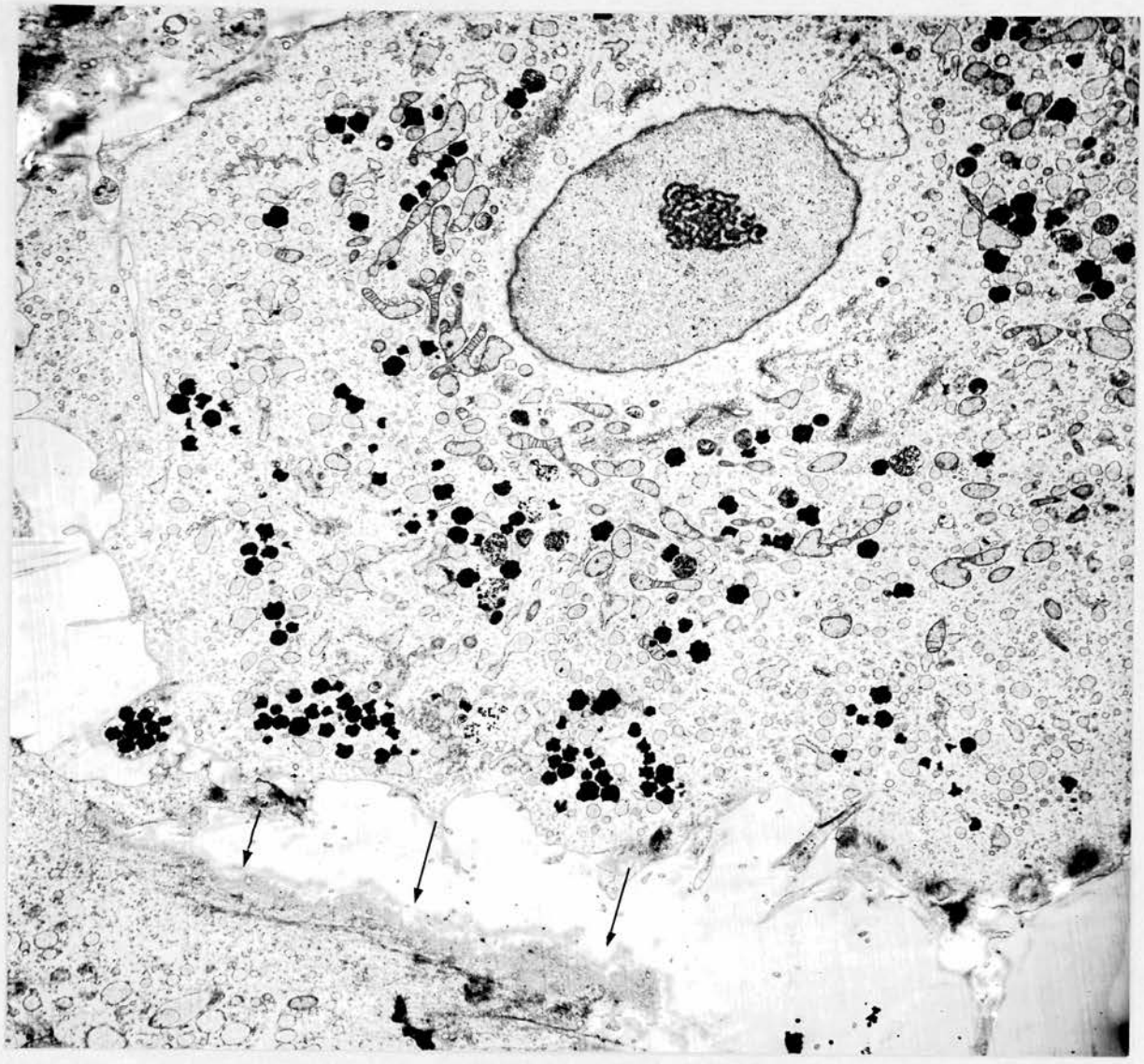

Fig.22. The reticular cell in the illustration has been in culture for 14 days. The nucleus still has the prominent nucleolus, the cytoplasm contains numerous pleomorphic mitochondria and there are a few phagosome-like structures. Note the amorphous, weakly osmiophilic material which appears to have been secreted by the cell at the bottom of the photograph (arrows).

EM x 3,000

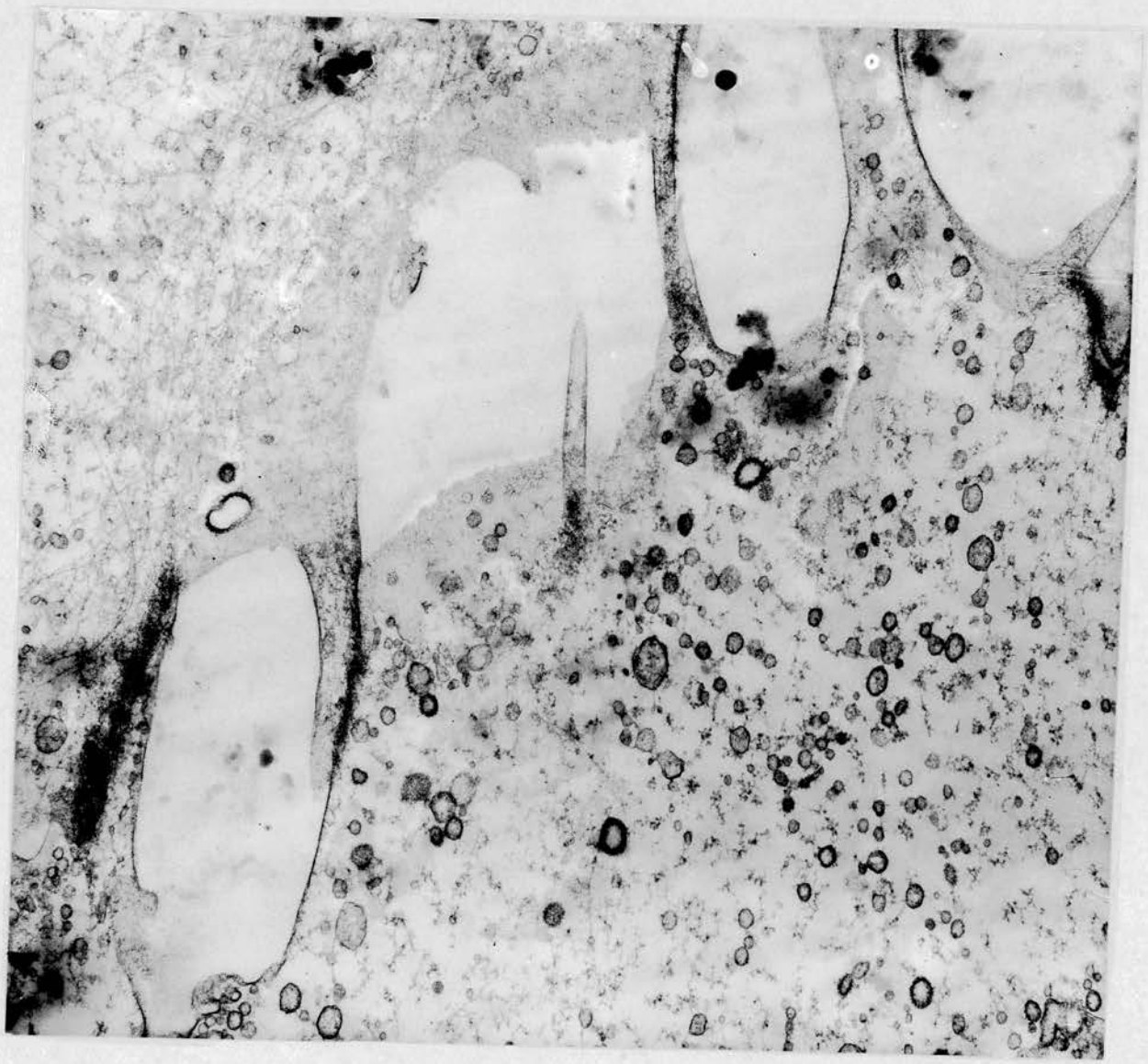

Fig.23. This illustrates the interdigitating cell processes typical of older cultures.

From a culture 14 days old. EM x 24,000

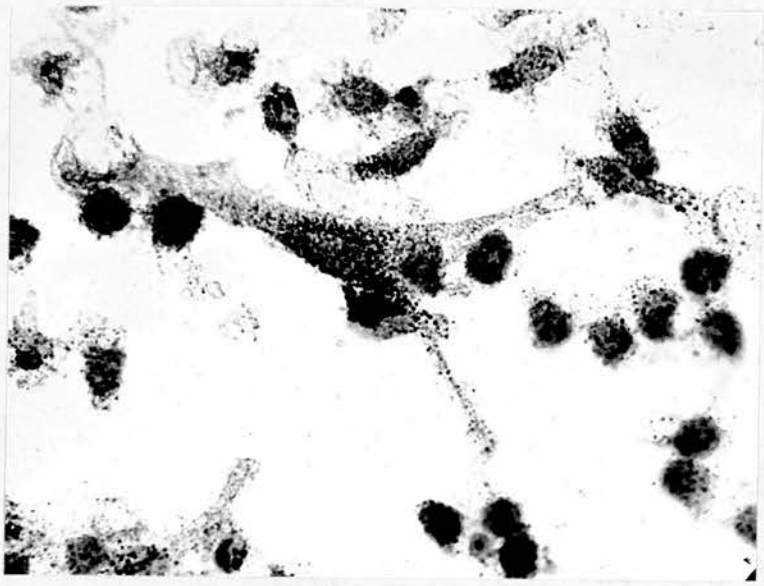

Fig.24. A 2 day old culture stained by Gomori's method for acid phosphatase.

x 500

Fig.25. A 7 day old culture stained by Gomori's method for acid phosphatase.

x 500

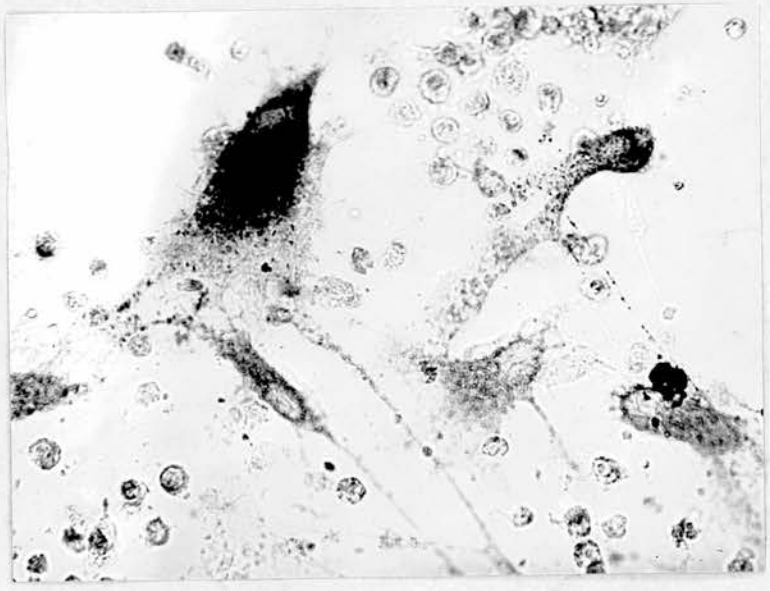

Fig.26. The content of lactic dehydrogenase in a 2 day old culture.

x 425

Fig.27. The content of lactic dehydrogenase in a 7 day old culture.

x 325

CHAPTER III

CLASSIFICATION OF RETICULAR CELLS IN TERMS OF THE RESPONSE TO VITAL DYES, PARTICLES AND NUCLEATED CELLS

INTRODUCTION

Allison and Young (1964) studied the distribution of various fluorescent materials, trypan blue and neutral red, when these were added to cultures of living cells. They showed that neutral red was taken up by lysosomes and that trypan blue was segregated into phagosomes only if the cells were viable. Dead cells showed diffuse non-granular staining with both of these dyes. Sabin and her co-workers (1924) showed that macrophages generally had a rosette of neutral red granules opposite the indentation of the nucleus.

An extensive study of the differences between macrophages and fibroblasts was carried out by Carrel and Ebeling (1926). They showed that fibroblasts had a few small granules which stained with neutral red, the numbers of which decreased as the culture increased in age and density. The mitochondria of fibroblasts were filamentous whereas those of macrophages tended to be rod-like. They also noted that macrophages showed more active movement than did fibroblasts. When fibroblasts were cultured in medium containing serum, these workers found that large numbers of fat globules accumulated in the cells.

In the studies on the morphology of the reticular cells I noted that reticular cells had ingested a yeast. Since the structure of the reticular cells was intermediate between that of macrophages and fibroblasts, experiments using vital dyes and a range of antigenic and non-antigenic particles were done to determine the range of phagocytic activity of the reticular cells and to compare this with that/

that of macrophages and fibroblasts.

It is known that macrophages can discriminate between healthy and injured cells. Effete red cells are selectively phagocytosed and effete neutrophil polymorphonuclear leucocytes are found within macrophages in smears from cases of peritonitis. In the human peritoneal fluids it has been shown that there is a second type of cell, the reticular cell in which nuclear debris is sometimes found. Investigations were therefore carried out to determine the interactions which occurred between reticular cells and autologous peripheral blood leucocytes and injured cells. Cells were injured by ageing and by anti-lymphocytic serum.

MATERIALS AND METHODS

a. Amoebocytes

Mouse macrophages were cultured as described by Davidson (1967). The mice were killed by rapid cervical dislocation, swabbed with alcohol and the skin and fur retracted to expose the peritoneal wall. The peritoneal cavity was then washed out with 3-4 ml. of tissue culture medium 199 with 10 i.u. heparin per ml. added. The number of cells in the suspension was counted, human serum added to a final concentration of 10% and tubes and coverslips set up at 1×10^6 cells per tube.

b. Epithelioid cells

This type was represented by the rabbit kidney cell. These were a continuous cell line supplied by Glaxo Laboratories Ltd.

The/

The cultures on flying coverslips were obtained from Dr. I. Smith, Virology Department, Edinburgh University. The activity of these cells was tested in both calf serum and human serum at a concentration of 10%.

c. Mechanocytes

Three sources of fibroblastic cells were used:-

(a) Monkey kidney, (b) baby hamster kidney, (c) human skin fibroblasts.

I am grateful to Dr. I. Smith of the Virology Department for supplying the monkey kidney and the baby hamster kidney cells and to Dr. A.D. Bain of the Royal Hospital for Sick Children, Edinburgh, for the human skin cells. All the cultures were supplied as flying coverslips cultures.

Monkey kidney: The cells were first sub-cultures from a commercially available primary culture. They are frequently contaminated by S.V.40; however no obvious signs of this were seen at the time these cultures were tested. The functional activity was tested in both 10% calf serum and in 10% human serum.

Baby hamster kidney: The cells were stored in liquid nitrogen, revived and used directly. The actual number of sub-cultures which they had undergone before storage was not known. Their activity was tested in 10% human serum only.

Human skin fibroblasts: These cells were the first sub-cultures from explants of skin from young children. The functional activity was tested in 10% human serum only.

Vital/

Vital dyes:

a. Nile blue sulphate; (a fat stain used by Lorrain-Smith): A stock solution of 0.5% was made up in distilled water, diluted 1/10 with medium 199 and 0.1 ml. added to each tube which was then incubated for 30 minutes at 37°C.

b. Neutral red: A 1/10 dilution of the routine staining solution (1% in distilled water) in medium 199 was made and 0.1 ml. of this was added to each culture before incubating the cells overnight at 37°C.

c. Trypan blue: The stock 2% suspension in distilled water was diluted 1/100 with 199, then 0.1 ml. added to each culture and then incubated for 60 minutes at 37°C.

These preparations treated with vital dyes were examined using phase contrast on a Leitz Orthomat microscope.

Nile blue sulphate is a true solution since the dye will pass through dialysis tubing. Neutral red, also a solution, has been shown by Allison and Young (1964) to stain the lysosomes. Trypan blue is a colloidal suspension which is ingested by pinocytosis into vacuoles or "phagosomes" (Allison and Young, 1964).

Inert Particles

a. Carbon was prepared by washing India ink three times in 0.85% saline then centrifuging it at 2000 g. for 30 minutes. A 1% stock solution was made in saline and autoclaved. The stock was diluted 1/100 in medium 199 before adding to the cultures which were then incubated/

incubated for either 3 hours or 3 days at 37°C before fixing and staining. The incubation times were standard for all particles.

b. Starch was prepared from Amaranthus cruentus (McMasterset al., 1955). The material was supplied by Dr. L. Burnett, New York University. 5 mg. starch was suspended in 1 ml. of 199 and 0.1 ml. was added to each tube.

Micro-Organisms

a. B.C.G.

The mycobacteria was obtained from material which had been prepared for human use but was out of date. One freeze dried ampoule contained 3.75 mg. wet weight of bacteria. The suspension was next diluted to 0.1 mg. bacteria per ml. and 0.1 ml. (i.e., 0.01 mg.) B.C.G. was added to each tube. These preparations were stained by the Fite-Faraco modification of the Ziehl-Neelsen stain, the detailed method for which is given in the appendix. The bacteria were non-viable.

b. Yeast

A 5 day culture of 0.1 g. of baker's yeast per 10 ml. of 10% sucrose solution was autoclaved, washed three times in physiological saline and made up to a 1% suspension. Each culture received 0.1 ml. of this suspension.

Erythrocytes

Human erythrocytes were obtained from a finger prick washed three times in 0.9% saline and made up to a 5% suspension. The RBC's were/

were coated with rabbit anti-human erythrocyte antibody, diluted approximately 1 in 200, for 30 minutes at 37°C, washed once and 0.1 ml. of a 5% suspension added to each tube. The rabbit antiserum was prepared in this laboratory. It caused lysis of erythrocytes to a titre of 1 in 64 in the presence of guinea pig complement, and agglutinated the RBC's to a titre of 1 in 400.

Preparation of Leucocytes

Leucocytes were obtained by dextran sedimentation from whole blood (obtained from the Blood Transfusion Service, R.I.E.) within 24 hours after collection. In one case, three days old blood was used since "altered" or damaged leucocytes were required. One volume of 6% dextran 250 in 0.9% saline was added to two volumes of blood containing either 10 units of heparin per ml. or acid-citrate-dextrose, and the mixture allowed to stand for 40-60 minutes. By this time the RBC's had sedimented leaving the leucocytes in suspension in the plasma. The supernatant was removed, centrifuged at 200 *g* for 10 minutes, the deposit gently resuspended in tissue culture medium 199 with 10 units heparin per ml., recentrifuged and the supernatant poured off. The cells were resuspended in 199 with heparin (approximately 1 ml.) counted and a sample added to the monolayer. In one case the leucocytes were incubated with a rabbit anti-leucocytic serum at a dilution of 1/32. This serum had both agglutinating and lytic activities. The leucocytes were incubated for 2 hours before being added to the cultures. A similar sample

was/

was incubated in normal rabbit serum at the same concentration as a control. In this case the incubation time was 2 hours but in all other cases it was 4 hours.

The number of cells added to each culture was between 3 and 10×10^6, usually $5\text{-}7 \times 10^6$. Numbers of cells within this range had no effect on the results.

Effects of antibody on the monolayer were assessed after 24 hours in culture in medium 199 containing 10% antibody-containing serum and comparing this with monolayers grown in 10% autologous serum.

Lymphoid and tumour cells

Cell suspensions of these types were added to the culture. They were prepared by grinding up the tissue in a hand homogeniser in medium 199 with heparin. The cells were counted and the concentration adjusted to 10×10^6 in 0.1 ml., the volume added to each culture.

Lymphoid tissue: This was obtained at operation for the removal of a lung tumour. Part of the node was processed for histological examination, which showed it to be free from tumour. A second source was a lymph node, obtained at post mortem, from a case of sarcoidosis.

Tumour tissue: The source was part of a lung tumour removed surgically. These cells were viable since part of the suspension was successfully established in culture in the laboratory.

RESULTS

In order to determine the class of cell to which the reticular cell belonged, cultures of cells of known type were established, and then treated with dyes and particles. The cells were mouse macrophages, kidney cells from rabbit, monkey and baby hamster, and human skin fibroblasts.

Supravital and Vital Dyes

The dyes used were Nile blue sulphate, neutral red and trypan blue. When the first two dyes were used, a few scattered granules were stained in the fibroblastic cells (in this case monkey kidney) and in the epithelioid cells (rabbit kidney). Trypan blue was not ingested by either of these cell lines. In contrast the mouse macrophage - the amoebocyte - concentrated large amounts of all three dyes which were concentrated in the form of granules in the perinuclear area. The reticular cells showed some uptake of all three dyes, all of them being taken up to a more marked extent than in the epithelioid or fibroblastic cells, but not to such a large extent as in the amoebocytes. The stained granules were scattered randomly throughout the cytoplasm of the reticular cells, not showing any tendency to aggregate in the perinuclear area. These results suggested that the reticular cells were cells with functional properties intermediate between these of macrophages and fibroblasts (Table III).

In order to investigate further the phagocytic properties of these/

these cells, experiments using large particles such as carbon, yeast starch and bacteria (B.C.G.) were performed. Monolayer cultures of each cell type were incubated with the particles for 3 hours and 3 days. Similar experiments were done using antibody-coated erythrocytes and aged leucocytes. The results of these experiments are summarised in Table II. The fibroblasts and epithelioid cells did not take up any of the particles after 3 hours' incubation but after 3 days both cell types showed a fine dusting of carbon and a small proportion of the fibroblastic cells had ingested some yeast. This was especially noticeable in the human skin cells. The antibody-coated erythrocytes and leucocytes were neither ingested by nor adhered to either fibroblasts or epithelioid cells. The mouse macrophages ingested all the particles within the first 3 hour incubation period. Again the reticular cells showed activity intermediate between that of macrophages and the other cell-types. The reticular cells ingested carbon and yeast within 3 hours (Fig.28) but none of the other particles at this time. Leucocytes however adhered to the processes of the reticular cells. After 3 days' incubation some starch had been ingested and some cells were found which contained B.C.G. Leucocytes were found both ingested by and adherent to the cytoplasm of the reticular cells.

I noted that, in the cultures incubated for 3 days, the macrophages tended to cluster around the reticular cells. This was very marked in the cultures to which carbon and B.C.G. were added (Figs.29 and 30). In most cases there appeared to be cytoplasmic connections/

connections between the macrophages and the reticular cells. The reticular cells had carbon after 3 hours but no reticular cells had B.C.G. in them after 3 hours. Closer examination of cultures after 3 days showed that individual bacteria could be found in cytoplasmic processes between macrophages and reticular cells. This suggested that B.C.G. entered the reticular cells by transfer rather than by direct ingestion. Phase contrast studies showed that this occurred but it is not certain that this is the only way in which B.C.G. entered the reticular cells.

In the cultures to which antibody-coated erythrocytes were added, it is clear that two cell populations are present (Fig.31). The macrophages ingested the red cells but as shown in the illustration not even small agglutinates lying near a reticular cell are ingested. Erythrocytes were never found inside the reticular cells, but when the initial suspension was heavily contaminated by erythrocytes, iron, as demonstrated by the Prussian blue reaction, accumulated in the cytoplasm (Fig.32).

The addition of starch particles also demonstrates two cell populations (Fig.33). The macrophages were most prominent during the first 4 days after isolation. The human macrophages were found to have a range of activity similar to that of mouse macrophages.

Dead Lymphoid Cells

The suspension of cells from the lymph node from the case of sarcoidosis/

sarcoidosis was incubated with the reticular cells for 2 hours then fixed and examined. Pyknotic lymphocyte nuclei were ingested at this time. No viable lymph node cells were noted, presumably as a result of autolytic changes in the body in the 24 hours which had elapsed between death and the post mortem.

Further experiments were done using a freshly resected lung containing a bronchogenic carcinoma. A regional lymph node and piece of tumour were dissected out and made into separate cell suspensions. The suspension of cells from the lymph node contained mainly lymphocytes and no tumour cells were seen. Subsequent histological examination of part of the lymph node confirmed the absence of tumour. These fresh lymphocytes were not ingested after 2 or 3 hours' contact. After 48 hours they had settled on top of the cultured reticular cells and appeared to be viable (Fig.34). This was thought to be a result of the established monolayer acting as a feeder layer, since Ginsberg (1965) showed that feeder layers prolonged the survival of lymphocytes in vitro. Although it is difficult to illustrate, careful focussing on the original preparation showed that the nuclei of the cells upon the cytoplasm of the reticular cells were in a different plane to those of the reticular cells and were surrounded by their own cytoplasm which was distinct from that of the reticular cells.

The cell suspension from the tumour mass gave rather different results. After 2 hours the cells had settled and were adherent to the reticular cells. After 24 hours some reticular cells contained large numbers of degenerate-looking nuclei, presumably damaged or dead/

dead tumour cells. After 48 hours, the reticular cells had digested much of the nuclear debris but some viable cells were seen in association with the reticular cells, which again appeared to be acting as a feeder layer (Fig.35). These cells were thought to be tumour cells, since the cytoplasm was larger than that of lymphocytes.

A suspension of human peripheral leucocytes was made by dextran sedimentation of 24 hour old ACD anticoagulated blood. The leucocytes were washed once and incubated with a rabbit antiserum to human peripheral blood leucocytes, before being added to the monolayers of reticular cells. This antiserum was made in this laboratory and had been shown to have both cytotoxic and agglutinating activity. The leucocytes incubated in normal rabbit serum or saline were not ingested by, nor did they adhere to the cultured cells. These control suspensions were not agglutinated on microscopic examination prior to adding them to the macrophages. The leucocytes incubated in the immune rabbit serum were agglutinated by that serum at the dilution used (1/32) and these cells were ingested by and adhered strongly to the cultured cells. The cell suspensions were washed once only before being added to the culture. Since the incubation time was only 2 hours it is unlikely that any free antibody could have had a marked effect on the reticular cells (Figs.36 and 37).

In the series of experiments to establish whether or not reticular cells interacted with autologous leucocytes, the reticular cells were obtained from four patients with nephritis and one with thrombosis of the inferior vena cava. In total, fourteen experiments/

experiments were done and thirteen of these showed no reaction: the one exception showed adherence of leucocytes to autologous reticular cells and some swelling of and damage to their nuclei after an incubation of 24 hours. All patients in this series had received blood transfusions. These results were reproducible; each patient was tested at least twice (Table IV).

Leucocytes, from 3 day old blood, were ingested by macrophages after 3 hours and adhered to the reticular cells. No interactions between leucocytes and other cell types were seen. The adherence to reticular cell processes will be further discussed in Chapter IV.

Staining Reactions

The long processes of the reticular cells showed beading when the cultures were stained by silver impregnation methods (Figs.7 and 8 in Chapter II). The fibroblastic cells showed this only where they were densely packed and single fibres were never stained by any of the silver stains.

It was shown by methyl green-pyronin staining that the reticular cells had large amounts of RNA in their cytoplasm. This appeared as pyroninophilic striations as described in Chapter II. They contained much more than did the mouse macrophages but about the same amount as the fibroblastic cells. This finding fits in with the electron microscopic structure of the two types of cell. The fibroblastic types have much more endoplasmic reticulum than do the macrophages.

When the cells of each type were stained using the periodic Schiff/

Schiff reaction, all of them showed some P.A.S. positive material in the cytoplasm. In the monkey kidney cells this was very intense and obscured the results of the experiments using starch. Interpretation of the results from the cultures of monkey kidney to which yeast had been added was possible since the yeast was more intensely P.A.S. positive than the cytoplasm. Sera containing antibodies to leucocytes caused aggregation of the monolayers (Fig.37a). This never occurred in autologous serum.

The conclusions drawn from these experiments are:-

1. The reticular cells do not fall distinctly into either the class of amoebocytes or that of mechanocytes (classification after Willmer, 1965).

2. The reticular cells are functionally more closely akin to the macrophages than they are to fibroblasts. The reticular cells ingest yeast and damaged leucocytes while fibroblasts never do. The reticular cell ingests carbon to a much greater degree than do fibroblasts. Lymphocytes adhere to the processes of reticular cells but never to those of fibroblasts.

3. The reticular cells are selectively phagocytic and ingest vital dyes therefore they must be included in the reticulo-endothelial system as defined by Aschoff.

4. Reticular cells do not ingest erythrocytes coated with a heterologous antiserum, whereas macrophages do. It seems that these cells fundamentally differ from macrophages in that they do not react with particles coated with antibody globulins.

DISCUSSION

Carrell and Ebeling (1926) stressed the importance of using functional as well as morphological criteria to classify cells thus: "The determination of the specific physiological properties of a cell is of evident importance, as the individuality of a cell depends on its functions far more than on its staining reactions." The use of functional tests is of paramount importance when it is realized that the composition of the medium has marked effects on the morphology of the cells. Willmer (1965) said: "Diagnosis of the class to which a cell belongs by the shape of the cell alone is thus precarious, if not impossible. Moreover, cells in all the classes so far mentioned (e.g. epitheliocytes, mechanocytes, amoebocytes, etcetera) are liable to have their shapes and movements modified to a greater or less extent by such external factors as have already been discussed and probably by many others besides, and in order to group cells into these classes with certainty, it is necessary to have much more information about them than can be gleaned from studying only their outward form."

The studies reported here confirmed the impression, gained from the morphology and cytochemistry, that human reticular cells are selectively phagocytic, again having activity intermediate between that of fibroblasts and macrophages. One other fact which emerged was that the phagocytic ability of the reticular cells was not fully expressed when the cells were in contact with the particles/

particles for only 3 hours. This is illustrated by my finding that starch and damaged peripheral blood leucocytes were taken up after 3 days' incubation to a much greater degree than after 3 hours. Some of the material found in the cytoplasm of the reticular cells at 3 days might have been transferred from macrophages since inter-cellular bridges containing carbon, B.C.G. and iron, have been seen. However there is no need to invoke macrophage mediated uptake of yeast since this particle was ingested within 3 hours. In the case of damaged leucocytes, nuclear debris was never seen in the "bridges" and by default it appears that effete leucocytes are directly phagocytosed. Trypan blue and neutral red are taken up directly by the reticular cells. The pattern of ingestion of carbon and vital dyes such as trypan blue was similar to that described by Cappell (1929) for reticular cells in both the Malpighian body and in the lymph node. As previously mentioned he noted that "the Malpighian bodies remain almost completely free from the vital stain; careful search with the oil immersion lens will however reveal scanty very fine granules in the reticular cells of the lymphoid follicles." In his report on lymph nodes Cappell said: "The reticular elements of the sinuses and those supporting the lymphocytes in their meshwork of fibrils are also selectively stained and the cytoplasmic processes which extend along the reticulum fibrils are filled with dye particles." The distribution of neutral red granules in the reticular cells was similar to that described by Cunningham et al., (1923-24) (for clasmatocytes) in these words, "These cells always reacted/

reacted to neutral red, the aggregations of the stain varied greatly in size and colour (light red to deep maroon) and were scattered irregularly throughout the cell."

It has been shown that the reticular cells are phagocytic and that they take up vital dyes and therefore are cells of the reticulo-endothelial system as defined by Aschoff (1924). The occurrence of intercellular bridges containing particles suggested that the reticular cells might store antigen. This could be of importance in the presentation of antigen to lymphocytes in the secondary phase of the immune response, since lymphocytes may adhere to the processes.

The interactions which occur between nucleated cells and reticular cells were studied in further detail and were found to be reproducible only if certain criteria were adhered to. These were that the monolayers had a sufficient number of cells, that both macrophages and reticular cells were present and that the number of cells added was regulated. Better results could probably be obtained in the experiments (using peripheral blood) if a suspension of pure lymphocytes had been used but it is impracticable in the autologous situation, since this requires larger blood samples from seriously ill patients. Despite the presence of polymorphonuclear leucocytes and platelets with their toxic granules and adhesive qualities respectively, reproducible negative results were obtained using autologous cells, except in one case which might have been due to the presence of virus since the patient at this time was recovering from hepatitis.

In/

In the experiments using aged peripheral blood leucocytes incubated for up to 3 hours, phagocytosis occurred to a limited degree and was more marked after 3 days. When a suspension of cells from the lymph node from a case of sarcoidosis was used the cells were ingested in a very short space of time (3 hours). This was thought to be due to autolytic changes which had taken place between the time the patient died and the time the post mortem took place.

When a suspension of tumour cells was added to monolayers there appeared to be two distinct stages of reaction. In the first stage pyknotic nuclei were ingested, and in the second stage, which took place after 48 hours, viable tumour cells were seen on top of the extended cytoplasm of the reticular cells. A similar sequence was seen with lymph node suspensions.

The "feeder layer" effect has been described by Ginsberg (1965) and has been shown by him to cause transformation of heterologous lymphocytes. If a similar effect could be shown in the homologous or autologous sensitised system, this would be evidence supporting White's view of the formation of germinal centres.

Phagocytosis was seen when peripheral blood leucocytes were pretreated with heterologous antileucocytic serum and also with homologous anti-serum. Adherence of the antibody-coated cells to the processes was also marked. Adherence was the commonest sign of interaction between monolayer cells and added leucocytes, especially with short (i.e., 3-4 hours') incubation times. In this reaction the long processes of the reticular cells play a conspicuous part and indeed/

indeed cultures without substantial numbers of these cells fail to give good reactions. White *et al.* (1967), in his discussion on the formation of germinal centres, suggests that the processes of dendritic cells are coated with antigen, thus enabling sensitised lymphocytes to adhere. This reasoning can be applied to the adherence phenomena noted in these experiments, although there is of course no evidence that the "dendritic cells" and the large cells observed in these cultures are the same. If the large peritoneal cell contains antibody of one kind or another on its processes and the antigen is on the surface of homologous leucocytes, then one can readily explain the adherence patterns noted in these cell culture reactions. The finding that dead or damaged leucocytes are ingested may reflect another line of specialisation *in vivo* i.e., to "tingible body" macrophages, as are found in the germinal centres.

The storage and handling of antigens and complexes is further studied in the next chapter.

TABLE II

Staining Reaction or Particles Added	Macrophages				Reticular Cells		Fibroblasts						Epithelioid	
	Mouse		Human		Human		Monkey kidney		Baby Hamster Kidney		Human skin		Rabbit kidney	
	3 hr	3 day	3 hr	3 day	3 hr	3 day	3 hr	3 day	3 hr	3 day	3 hr	3 day	3 hr	3 day
Antibody coated RBC	+	+	+	+	-	±	-	-	-	-	-	-	-	-
Lymphocytes stored at 4°C for 48 hours	+	+	±	±	adherence		-	-	-	-	-	-	-	-
Yeast	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Starch	+	+	+	+	±	±	No results*		-	-	-	-	-	-
Carbon	+	+	+	+	±	+	-	±	-	±	±	±	±	±
B.C.G.	+	+	+	+	-	±	-	-	-	-	-	-	-	-
P.A.S.	±	+	±	+	±	+	+	+	±	±	±	±	+	+
M.G.P. DNA		+	+		+		+		+		+		+	
M.G.P. RNA		±	±		+		+		+		+		±	
Silver impregnation	No silver on processes		Not done		Silver on processes		Silver seen		Grains only seen in dense areas				No silver grains	

* The cells were so strongly PAS positive that starch granules were not distinguishable.

TABLE III

Reactions between various types of cell and vital or supravital dyes

Cell Type	Nile Blue Sulphate	Neutral Red	Trypan Blue	Colloidal Carbon
Macrophages	Many stained granules	Many peri-nuclear granules	Many granules	Cytoplasm filled so that the nucleus was obscured
Reticular cells	Numerous scattered granules	Numerous scattered granules	Numerous scattered granules	Numerous discrete, large granules
Fibroblasts	Few granules	Few granules	Very few granules	Fine dusting: no granules
Epithelioid cells	Few granules	Few granules	No granules	No granules

TABLE IV

Absence of interaction between monolayers of human peritoneal cells and autologous leucocytes

Donor	N	McP	W	T	O
Diagnosis	Nephritis	Thrombosis of inferior vena cava	Nephritis	Nephritis	Nephritis
No. of Tests	2	4	2	4	2
Interaction (Present +, Absent -)	=	= = =	=	= = =	- +

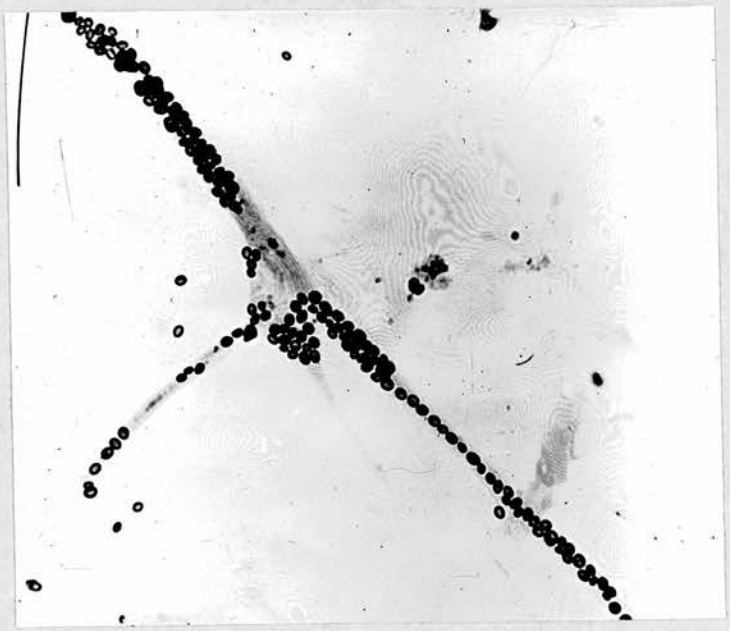

Fig.28. This illustrates the characteristic pattern of ingestion of yeast by reticular cells in culture. It recalls the "dendritic" pattern described by R.G. White.

PAS and Haematoxylin x 275

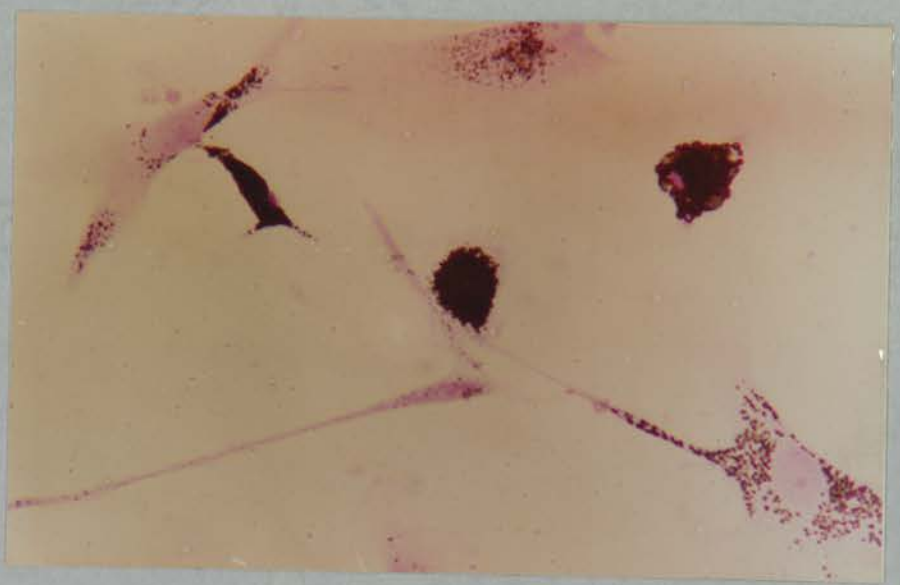

Fig.29. This illustrates the difference between macrophages and reticular cells in uptake of carbon after 3 hours' incubation. The macrophages appear as dense black masses while the reticular cells have numerous, discrete granules scattered throughout the extended cytoplasm.

Neutral Red x 600

Fig.30. This illustrates the uptake of B.C.G. by macrophages but not by reticular cells after only 3 hours' incubation. The macrophages appear as dense, dark masses with little detail visible.

Fite-Faraco x 700

Fig.31. This illustrates the uptake of antibody-coated erythrocytes by macrophages but not by reticular cells.

Giemsa x 450

Fig.32. This shows the accumulation of iron in the macrophages in a culture of human spleen from a case of haemolytic anaemia.

P.B.R. Neutral Red x 450

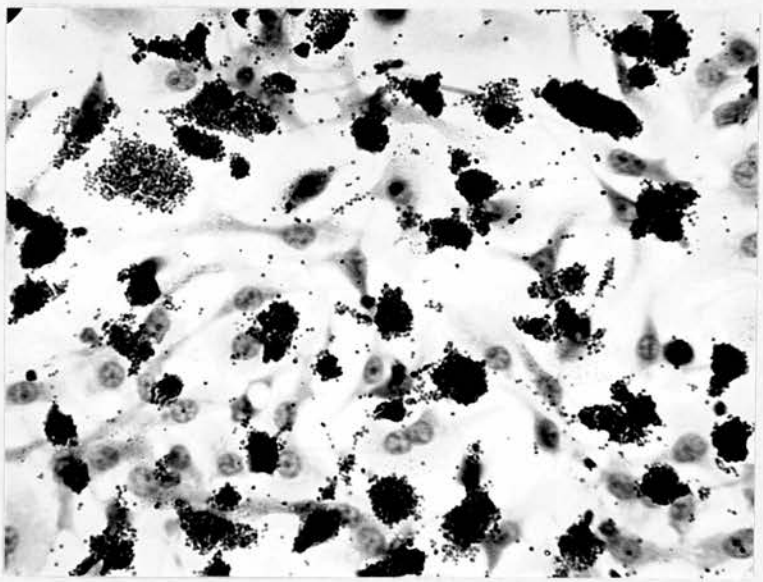

Fig.33. This shows that the reticular cells do not ingest starch to the same extent as do the macrophages in the culture. Compare this with the large numbers of yeasts in the reticular cell in Fig.28.

PAS and Haematoxylin x 350

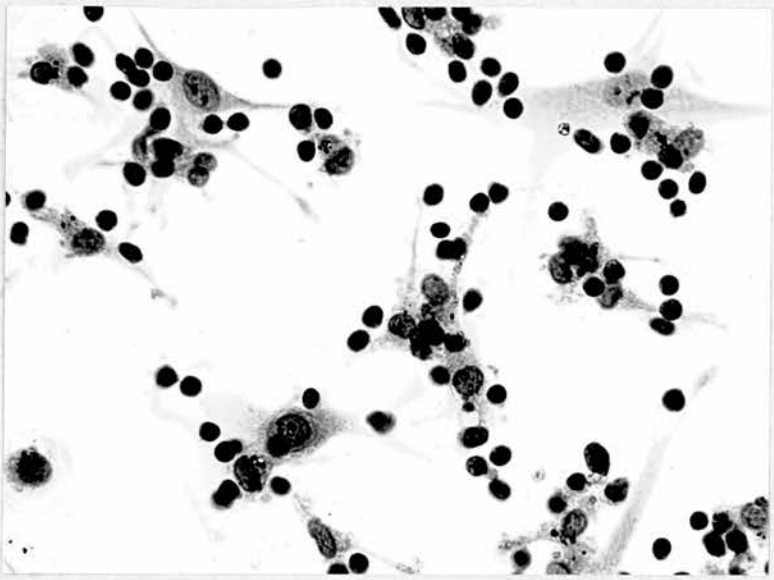

Fig.34. The lymphocytes added to this culture have settled on top of the reticular cells and appear to be still viable after 48 hours in culture. This is presumably due to a feeder layer effect.

Giemsa x 450

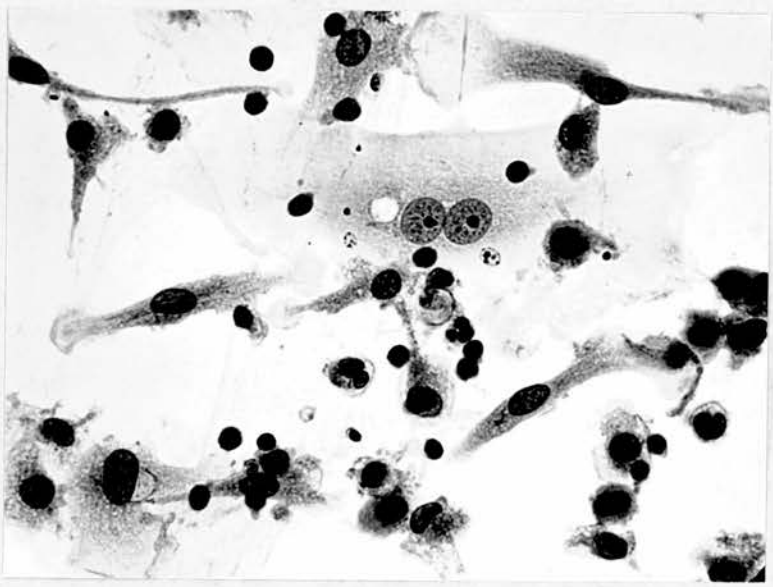

Fig.35. After 48 hours in culture some viable tumour cells were found in association with reticular cells. There are also pyknotic nuclei.

Giemsa x 450

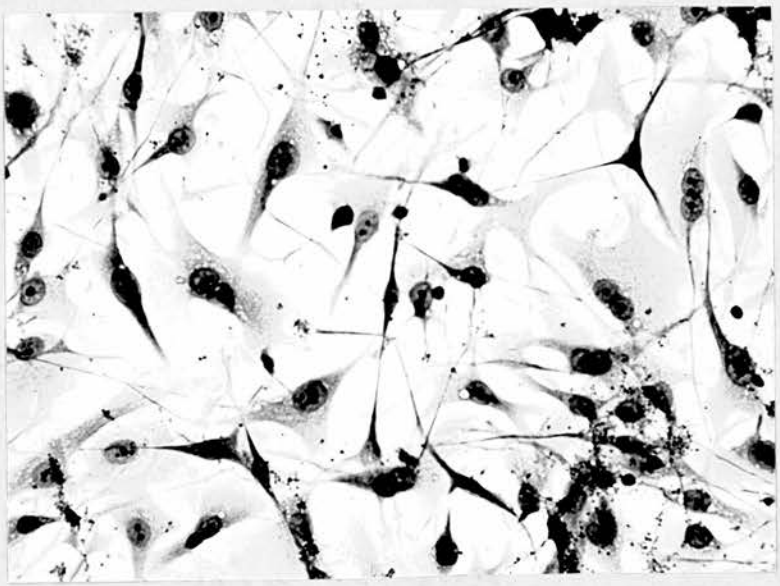

Fig.36. The appearances of a culture to which normal human peripheral blood lymphocytes were added, incubated for 2 hours and then fixed and stained. Note that there are very few lymphocytes adhering to the monolayer.

Giemsa x 250

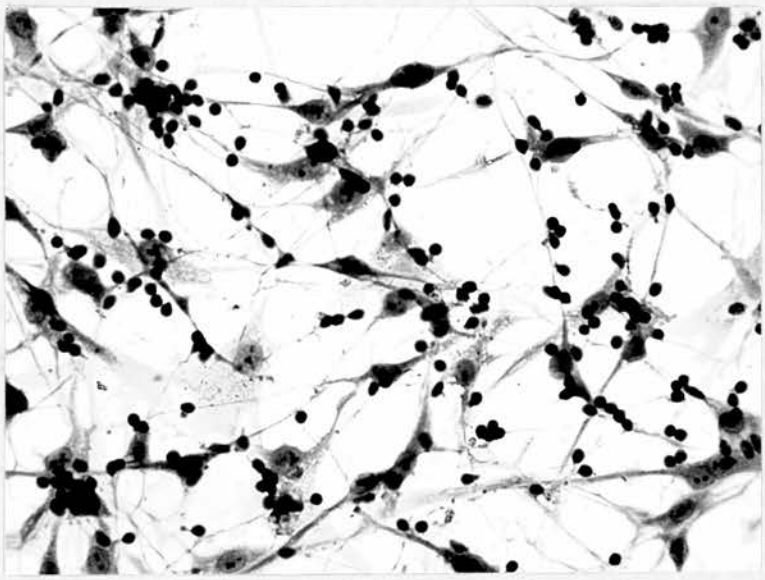

Fig.37. In contrast to the normal lymphocytes, those coated with antilymphocytic serum adhere strongly and in large numbers to the monolayer after 2 hours' incubation.

Giemsa x 250

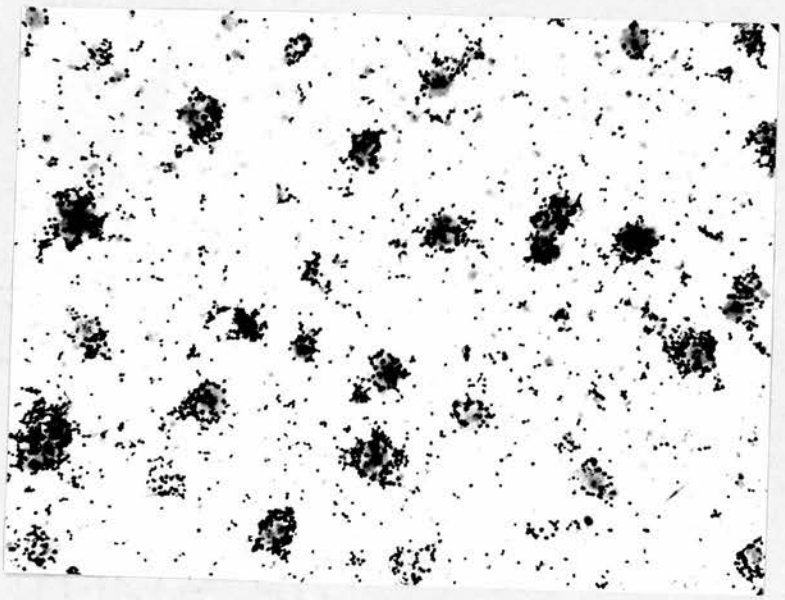

Fig.37a. This illustrates the effect of antilymphocytic serum on a monolayer containing both macrophages and reticular cells.

Giemsa x 55

CHAPTER IV

THE HANDLING OF ANTIGENS AND ANTIGEN-ANTIBODY COMPLEXES BY HUMAN PERITONEAL CELLS IN VITRO

INTRODUCTION

There has been much *in vivo* work which has shown that antigens are retained in the lymphoid tissues for relatively long periods of time (Nossal *et al.*, 1964; Campbell and Garvey, 1961; Szakal and Hanna, 1968). The distribution of the antigen, when assessed by microscopic examination for radio-label, is web-like, similar in appearance to the distribution of γ-globulins shown by Ortega and Mellors (1957). This lattice appearance also closely resembles the network of fibres demonstrated by Marshall and White (1950) using silver impregnation techniques. The cells which retain the antigen *in vivo* have variously been called dendritic cells, antigen retaining reticulum cells, or simply antigen retaining cells.

A close physical association between lymphocytes and dendritic cells has been noted *in vivo*. White *et al.* (1967) suggested that this was an early stage in germinal centre formation. Close association between lymphocytes and "large mobile cells" (Sharp, 1966) has been shown to occur in cultures of thymus. More recently Sharp (1971) has described the phase contrast appearance of this large motile cell. "Its shape tended to be elongated, with the long axis in the direction of movement; the advancing edge was usually marked by the presence of extremely active ruffled membranes. Behind this the main mass of cytoplasm contained the nucleus, which frequently had a kidney-shaped or indented outline and contained one or more nucleoli. . . The remainder of the cytoplasm was usually in the form of an extensive "tail", and it was to this, as well as to the more/

more "posterior" region of the main cytoplasmic mass, that lymphocytes adhered. . . The pattern of movement of the L.M.C., as seen in the time-lapse film was also quite characteristic. Surface activity was practically confined to the ruffled membranes at the leading edge, which seemed to draw the cell along. Movement tended to be in a straight line in contradistinction to the more haphazard motion of the lymphocytes and macrophages, in which changes of direction were more frequent." This description of the general appearance and movement of this thymic cell is very like that given in Chapter II for human reticular cells. My finding that the addition of antigenic particles (e.g., erythrocytes coated with heterologous antibody, yeast and B.C.G.) resulted in a redistribution of the macrophages from random dispersion across the coverslip to a close association with the reticular cells, led to a comparison of the handling antigens and antigen-antibody complexes by the two cell types present in cultures of human peritoneal fluids. Antigen-antibody complexes were included in the study because it has been reported that they localize to the dendritic cells in vivo (Ada and Lang, 1966). Reticular cells and macrophages have a similar complement of hydrolytic enzymes (Chapter II) and therefore it might be expected that the rate of degradation of ingested particles would be similar in both cell types. However this was not my impression and in this chapter some experiments have been carried out to compare the rates of degradation within these types of cell. I have also investigated the hypothesis that macrophages transfer material to/

to reticular cells. This has been done using Clostridium septicum which is not ingested by reticular cells but is ingested by small macrophages. The fluorescent tracer techniques were used to identify clostridial antigens.

The general plan of these experiments has been to add either antigen-antibody complexes or bacteria to the culture for a time sufficient to permit ingestion by macrophages. The cultures were then thoroughly washed and the distribution of complex or bacteria followed by removing coverslips at intervals of time. The ingested materials were identified by direct and indirect fluorescent tracer techniques and also by autoradiography.

Some experiments, using guinea pigs, were done in an attempt to show that the reticular cells could store antigen in vivo and continue this storage after isolation in vitro.

MATERIALS AND METHODS

Insoluble complexes were formed by mixing equal volumes of normal human serum with anti-human immunoglobulin serum conjugated with fluorescein isothiocyanate (Hoechst Pharamaceuticals Ltd.). The mixture was incubated for 2 hours at 37°C, then left overnight at 4°C. The precipitate formed was washed three times with sterile physiological saline made up to a 5% suspension and 0.2 ml. added to each culture.

Excess antigen was added to the precipitate, which then dissolved giving soluble complexes. The antiserum was purified goat/

goat gamma-globulin conjugated with the fluorochrome. Each culture received 0.2 ml. of the fluid containing soluble complexes.

The cultures treated with fluorescent complexes were examined with a Leitz Orthomat with an HBO 200 mercury vapour lamp, with phase lenses attached, in order that the state of preservation of the cultures could be assessed.

Radio-labelled complexes were prepared from human serum albumin, labelled with I^{125}, half life 60 days, (Radio Chemicals Centre, Amersham) mixed with an equal volume of rabbit anti-human serum albumin (Hoechst Pharmaceuticals Ltd.). The mixture was incubated for 2 hours at 37°C. No precipitate was formed and 0.2 ml., which was equivalent to 0.2 mg. albumin, was added to each culture. The detailed methods for preparing the autoradiographs are given in the appendix.

A heat-killed suspension of Clostridium septicum was obtained from Dr. J.G. Collee, Bacteriology Department, University of Edinburgh. The suspension was washed three times in physiological saline, made up to a suspension corresponding to Brown's capacity tube No.4, i.e., 2×10^9 bacteria per ml., and 0.1 ml. added to each tube. The cultures were incubated with this organism for 4 hours and then the medium changed. Coverslips were removed at various times after the addition of the bacteria, fixed in 90% ethanol for 1 minute and then dried. Fluorescein-labelled anti-Clostridium septicum serum (Burroughs Wellcome and Co.) was used to demonstrate the presence of the organism in the cultures using mercury vapour illumination on a

Leitz/

Leitz Orthomat with phase contrast lenses and condenser, as used for the fluorescein complexes.

An autoclaved, 1% suspension of washed baker's yeast, was prepared as described in Chapter II, and 0.1 ml. added to each culture. The cultures were incubated for 3 hours and then the medium changed. Coverslips were removed, fixed and stained at various times over a period of 20 days.

The preparations were stained P.A.S.-haematoxylin, or were examined after being treated by the fluorescent sandwich technique first described by Weller and Coons (1954) as detailed in Fluorescent Protein Tracing by R.C. Nairn (1969). The anti-yeast antibody was prepared by injecting rabbits with heat-killed yeast and collecting the antiserum a few weeks later. The fluorescent anti-rabbit serum was obtained from Hoechst Pharmaceuticals Ltd. Phase contrast studies of these preparations were also done. Selected coverslips from the last set of experiments were stained P.A.S. after records of the phase and fluorescent results had been made.

Electron microscopic examination of the reticular cells containing yeast was also done. The cultures, 14 days old, were incubated with yeast for 3 hours, then fixed and embedded in situ. One study was done on 3 day old macrophages incubated with starch for 3 hours. These cells were scraped off the bottle, fixed and embedded in suspension. The material was sectioned on an L.K.B. ultramicrotome and examined on an electron microscope. The cultures used in this series of experiments were from fluids from patients with/

with renal failure and from one patient with malignant ascites. The tumour cells were easily recognisable since they had round nuclei without nucleoli, were frequently multinucleate, and showed a diffuse autofluorescence. Macrophages and reticular cells never showed autofluorescence.

In vivo Experiments

Guinea pigs of both sexes weighing 200-400 g. were given four weekly I.P. injections of 1 ml. of human serum albumin (10 mg/ml.) and a fifth intraperitoneal injection of 20 mg. of albumin labelled with I^{125} (Radio Chemicals Centre, Amersham). The animals were killed 7 days after the last injection by giving $\frac{1}{2}$ ml. veterinary nembutal I.P. and exsanguinating the anaesthetised animal by cardiac puncture. The serum was tested by gel diffusion (Ouchterlony plates) for the presence of antibody to human serum albumin.

The spleen was removed aseptically and a small portion fixed in glutaraldehyde. Some of the blocks were processed for routine histology in paraffin wax and some were post-fixed in osmium tetroxide, dehydrated, embedded in araldite and thick araldite sections cut.

Dabs were made by lightly touching the cut surface of the spleen onto a clean microscope slide. The dabs were fixed in 90% ethanol for 3-5 minutes and air dried. The rest of the spleen was ground into a suspension using a hand homogeniser, as described for human spleen. The cells were centrifuged at mark 2 on an M.S.E. minor/

minor centrifuge, the supernatant discarded and 1 volume distilled water followed immediately by 1 volume 2x tissue culture medium 199 was added to lyse many of the erythrocytes in the suspension. The cells were then washed once in TC 199, resuspended and the cell concentration adjusted to approximately 2 x 10^6/ml. in medium containing 10% serum. Both guinea pig and pooled human serum were satisfactory. Tubes and coverslips were seeded with 1 ml. of the suspension and incubated at 37°C for 24 hours. When the non-adherent cells were gently washed off and fresh medium with serum added. The cultures were incubated for a further 48 hours and the first samples removed for autoradiography. Further samples were removed at days 5, 6, 7, 10, 13 and 15. Smears were made of the cell suspension which was used to seed the tubes. This was also processed for autoradiography.

The autoradiographic techniques are described in the appendix. The technique was similar for sections, dabs, smears and coverslips.

RESULTS

Antigen-Antibody Complexes

Soluble complexes, made by mixing normal human serum and fluorescent anti-human immunoglobulins were added to 3 day old cultures from a patient with malignant ascites. The coverslips were removed after 4, 8, 12, 24, 36, 48, 60, 72, 99 and 120 hours. The medium was changed after 4 hours and again after 3 days. The fluorescence of the vacuoles of the reticular cells at the early times/

times (Figs.38a and b, Figs.39a and b) became steadily fainter after the first medium change and had disappeared by 24 hours.

The nature of the material giving early fluorescence is not known but presumably is a pinocytosed complex which was rapidly degraded, perhaps by methods similar to that described for streptococcal M antigen-antibody complexes in macrophages (Gill and Cole, 1965). The macrophage contained ingested material which was seen as bright points of fluorescence as opposed to the diffuse type seen in the vacuoles of the reticular cells. After 24 hours the vacuolar fluorescence had gone from the reticular cells but a few bright points of fluorescent material were seen. This was most marked at 36 and 48 hours (Figs.40a and b, Figs.41a and b) but was found at 120 hours, the latest time studied.

It seemed probable that at least some of the material was found in the reticular cells as a result of these cells ingesting degenerating macrophages which had the complex in vacuoles. It was noticeable that macrophages with ingested material clustered around the reticular cells. This phenomenon was not observed in cultures which had not been treated with the complex. These control cultures showed no specific fluorescence.

Insoluble complexes were handled in a similar manner; the macrophages put out processes towards the reticular cells and numerous cellular connections were made. Eventually the reticular cells were found to contain fluorescent material. Again these static pictures did not show how the material moved from the macrophages/

macrophages into the reticular cells, and I failed to observe fluorescent material in the intercellular processes. The cytoplasmic connections (Fig.42) seen between the two cell types suggest that one way might be direct transfer, while the evidence that large cells will ingest nucleated cells suggests that ingestion of degenerate cells might be another method. No proof for either mechanism can be obtained from this type of study. The only way in which proof could be obtained would be to undertake time lapse cinematography using a suitable particle.

Radio-labelled complexes were treated in the same way as those which have just been described. They caused the macrophages to cluster around the reticular cells (Fig.43). Initially the radio-label was found in the macrophages and at this stage the cultures looked as if many of the macrophages had died and fallen off the coverslip. As the cultures "recovered", i.e., as the reticular cells increased in number, the labelled small cells which were left were found in close proximity to the reticular cells and in a few cases labelled material could be seen in the processes linking a small cell to a reticular cell (Fig.44).

The conclusions drawn from these experiments are:-

1. The addition of antigen-antibody complexes causes a marked redistribution of the macrophages in the culture.
2. Cytoplasmic bridges were formed between the two cell types and this may be a route for the transfer of antigens from macrophages to reticular cells.

3. /

3. Complexes appear in the reticular cells after the redistribution of cells has taken place, thus suggesting that transfer has taken place.

Handling of Clostridium septicum and yeast

Clostridium septicum was chosen because a specific antiserum to it was readily available. The washed bacteria were added to the cultures for 4 hours and then the medium was changed. The cultures were washed once at this stage to remove adherent bacteria. Preparations were fixed at various times after the initial incubation. At the early times the bacteria were seen as specific points of fluorescence in the macrophages (Figs.45a and b). Control cultures showed a diffuse fluorescence due to non-specific adsorption of the labelled protein. By day 2 the small macrophages were filled with amorphous fluorescent material, much brighter than the background, but not recognisable as bacteria. At this time some reticular cells had a few bacteria in their cytoplasm. By day 4 granular masses of fluorescent material with a definite shape but no visible nucleus were found in association with the reticular cells. On the 7th day some reticular cells had fluorescent granules (Figs.46a and b) and some had recognisable bacilli in their cytoplasm.

Yeast particles were readily ingested by both types of cell and it seemed worthwhile to compare their rate of degradation after phagocytosis by reticular cells or macrophages.

The first experiment was designed to find out if the two populations had different functions. Heat-killed yeast was added to/

to the cultures, left for 3 hours, and then the medium changed. The cultures were fixed and stained with P.A.S. at various times thereafter. This experiment lasted 20 days. The macrophages ingested the yeasts and digested them within 2 to 5 days (Fig.47). The cells then contained an amorphous mass of P.A.S. positive material not recognisable as yeasts. The reticular cells also ingested the yeasts but did not digest them rapidly. The yeasts inside the reticular cells retained their morphology, at least as judged by P.A.S. staining, for approximately 18 days. After 20 days many of the yeasts were beginning to look as if they were being digested; they had lost the intense purple colour that they originally had on P.A.S. staining (Fig.48). A characteristic finding at this time was loss of the central 'core' of P.A.S.-staining material.

On examination of these preparations it was not possible to see vacuoles around the yeasts as could be seen around the ingested nuclei of either dead lymphocytes or tumour cells. Electron microscopic studies showed that there was no space between the yeast (Fig.49) and the cytoplasm of the cell in which it was present (Figs.50 and 51). If the yeasts had been ingested into a phagocytic vacuole the limiting membrane must have been extremely closely applied to the yeast cell wall. Starch particles ingested by macrophages after 2 or 3 days in culture were in phagosomes (Fig.52).

The light microscopic study was repeated using fluorescence and phase contrast microscopy. These studies confirmed the finding reported above (Figs.53 and 54).

Phase/

Phase contrast studies, using time lapse cinematography, have shown that the macrophages and the reticular cells frequently have cytoplasmic connections. These occurred not only between cells of differing types but also between those of the same cell type. These connections were made and broken very rapidly. In some cases it appeared that small phase dark granules were passing from the macrophage to the reticular cells. When antigens such as B.C.G. or antibody-coated erythrocytes were added to the cultures which had the typical mixed population. the macrophages ingested the particles within 15 minutes, then migrated rapidly towards the reticular cells and the cytoplasmic bridges were formed between them. Mycobacteria have been observed to pass from one cell into the cytoplasm of another, but it was not possible to unequivocally identify the cells involved. Fixed preparations have shown B.C.G. in intercellular processes (Fig.55).

The conclusions drawn from these experiments are:-

1. Reticular cells and macrophages, although having the same range of enzymic activity, do not degrade yeasts in the same way. The rate of destruction is very much slower in the reticular cell than in the macrophage.
2. Cinematographic evidence has been obtained that "bridges" can be made and broken between reticular cells and macrophages.
3. Phase dark granules and mycobacteria have been observed in passage from macrophage to reticular cell.
4. Aggregation of macrophages containing particulate antigen around reticular cells was again confirmed.

In/

In the experiments using guinea pigs, all the animals injected with human serum albumin had produced antibody as shown by gel diffusion.

After exposing the sections, smears, dabs and coverslips for 8 weeks, they were developed and then stained with haematoxylin. The paraffin sections showed that the labelled antigen had localized mainly in the germinal centres with a little in the mantle zone. Examination of the araldite sections showed that the label was distributed in the typical "lacy pattern" described by Nossal (1964) and by White (1963). A similar pattern was found in the dabs, where the follicles were very prominent (Fig.56). Not all germinal centres showed the same degree of labelling, in either sections or dabs. In the smears a few cells (< 1%) were found to contain label. It was not possible to identify them further than the fact that they were mononuclear cells with more cytoplasm than the lymphocytes.

The cultures showed about 1% labelled cells. In these cultures it was difficult to identify the various cell types, and it was not technically feasible to test the phagocytic activity of the cultures used for autoradiography. In the majority of cases the label was found in cells of the type illustrated in Fig.57. These cells are of intermediate size and could be macrophages. However in some instances large cells with extensive cytoplasm were found which contained labelled material. The appearance of these large cells was strongly reminiscent of reticular cells (Fig.58).

The/

The difficulties encountered in this series of experiments were two-fold. Firstly, the reticular cells in the guinea pig cultures were not so readily distinguished morphologically as in the human. Secondly, the cytological detail which can be seen in autoradiographic preparations is never as clear as in normal material because of the difficulty of staining through the layer of emulsion.

This series of experiments showed that soluble antigen retained in the germinal centre 7 days after a secondary stimulation is retained by cells which can be cultured on glass and that it is not degraded by these cells in vitro for at least 14 days.

DISCUSSION

Since the finding that γ-globulin is distributed in a "lacy" pattern in germinal centres (Ortega and Mellors, 1957), much work has been done which shows that soluble antigen is distributed in a similar manner throughout the germinal centre. This distribution first occurs at about the time 7s antibody production starts (Nossal, 1964 and 1968; White, 1963 and 1967). In normal animals this can be simulated by using antigen-antibody complexes (Ada and Lang, 1966). These findings support the impression, gained from histological studies, that the formation of germinal centres is in some way associated with the secondary response to antigen (Conway, 1937; Ringertz and Adamson, 1950). Campbell and Garvey (1961) measuring the radio-activity of the whole organs, showed that a proportion of antigen was retained for long periods of time.

Evidence/

Evidence obtained by electron microscopy by Nossal's group and by Szakal and Hanna (1968) suggested that the antigen was associated with the membrane of the cells rather than with intra-cellular components. Inclusions in tingible body macrophages were sometimes labelled but not the surfaces of the cells. Inclusions were rarely seen in the cytoplasm of the reticular cells of the dendritic web.

The electron microscopic and conventional light microscopic studies on the localisation of antigen give at best a morphological definition of the type of cell but do not clearly define its functional characteristics. The main characteristics defined by these studies are:

1. The reticular cell is a "primitive cell", a finding apparently based on the absence of well developed cytoplasmic structures (Fresen, 1960; Kajikawa, 1964; Roberts and Latta, 1964).
2. The nucleus usually has at least one nucleolus.
3. The cell branches with cytoplasmic connections with other cells of the same type.
4. The cell stains lightly by silver impregnation methods but does not take up vital dyes under normal conditions *in vivo* (Marshall and White, 1950).
5. It is found in intimate contact with small lymphocytes (White *et al.*, 1967).
6. It retains antigen on its membranes for long periods of time (White, 1963; Nossal, 1964; Szakal and Hanna, 1968).

The reticular cell which has been studied in the experiments reported/

reported in this thesis has many of these characteristics. As defined by cultural methods it is a branched cell which shows movements typical of the amoebocyte rather than the mechanocyte class of cell. The nucleus has one or more well developed nuclei. The cytoplasm contains mitochondria but few other well developed organelles. The reticular cell stains with silver and takes up vital dyes. Lymphocytes adhere to the processes and the reticular cell has the full complement of degradative enzymes.

This evidence classified the reticular cell as a cell of the reticulo-endothelial system with properties very similar if not identical to those of the antigen-retaining cell *in vivo*.

In addition to characterising this cell *in vitro* I have reported the following findings.

1. This cell, although it has a full complement of enzymes did not degrade yeast in the same manner as did the macrophage. Electron microscopic studies showed that yeasts were not ingested into a vacuole but that nuclear or cell debris was. It has previously been shown that virus can exist free in the cytoplasm of HeLa cells (Epstein *et al.*, 1964) and that ferritin need not be segregated into vacuoles (Patterson *et al.*, 1965).
2. The reticular cells were found to be insensitive to gamma-globulin coated particles in that they were not ingested by the cells. Macrophages avidly ingested antibody-coated particles.

3./

3. Macrophages with ingested material e.g., antibody-coated erythrocytes or B.C.G., migrated rapidly towards the reticular cells in culture. After this had happened the reticular cells were found to contain the material. Whether the reticular cell obtained this material by ingesting the whole macrophage or by the macrophage transferring material across intracellular bridges is not yet known. Light microscopic and cinemicrophotographic studies suggest that transfer does occur. Antigenic material once inside the reticular cell could be transferred from cell to cell since cinematographic evidence has shown that cytoplasmic connections between reticular cells are common.

None of the experiments discussed above proved that the cell isolated from human lymphoid tissue was the antigen-retaining cell. In the experiments using guinea pigs I had hoped to prove that the antigen-retaining cell and the reticular cell were identical. I obtained further evidence to support this idea but it was not technically feasible to establish this beyond dispute.

However, it has been shown that there are at least two types of phagocytic cell present in the lymphoid tissue. These two cell types handle antigens in different ways, which is in accord with the <u>in vivo</u> findings.

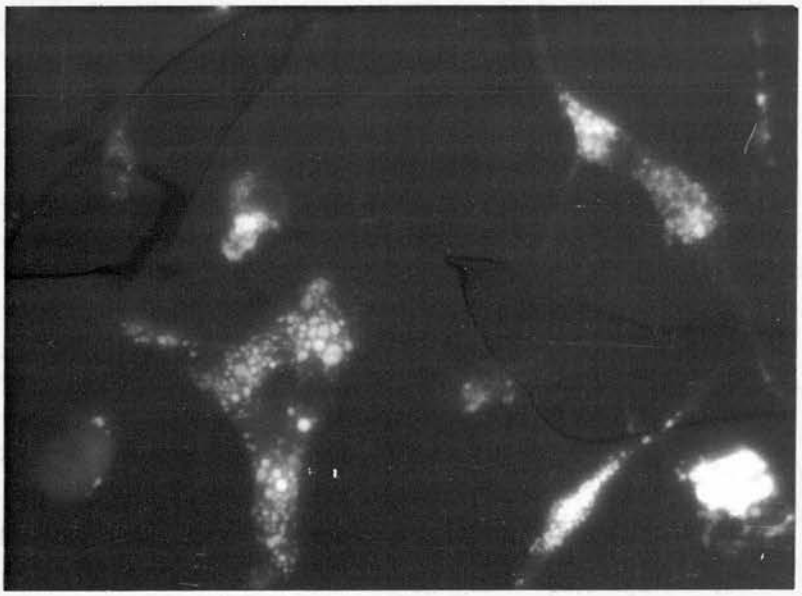

Fig.38a. Ultra-violet light has been used to show up the fluorescent material in both macrophages and reticular cells. The fluorescent material in the vacuoles of the reticular cell is probably due to the pinocytosis of dissociated labelled goat serum proteins. The soluble complexes were added 4 hours previously.

U.V. - Phase x 400

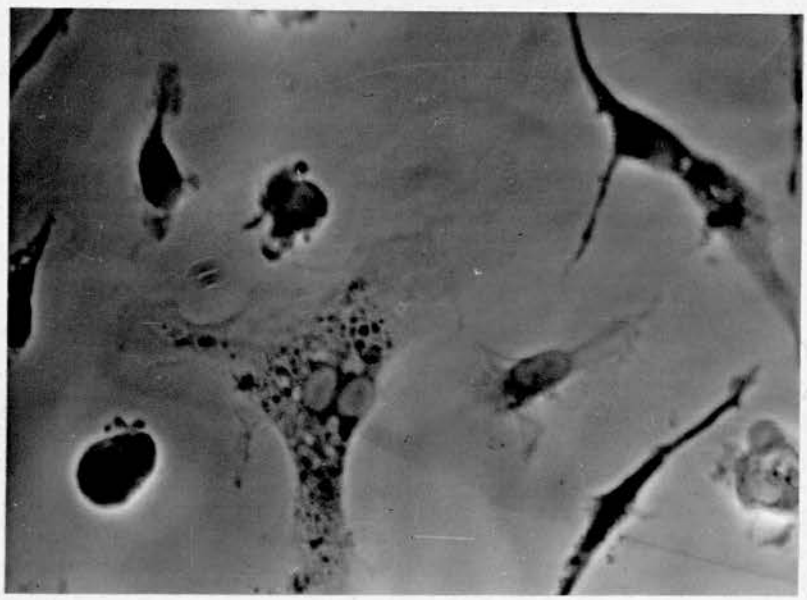

Fig.38b. Phase contrast appearance of the cells in Fig.36a.

Phase x 400

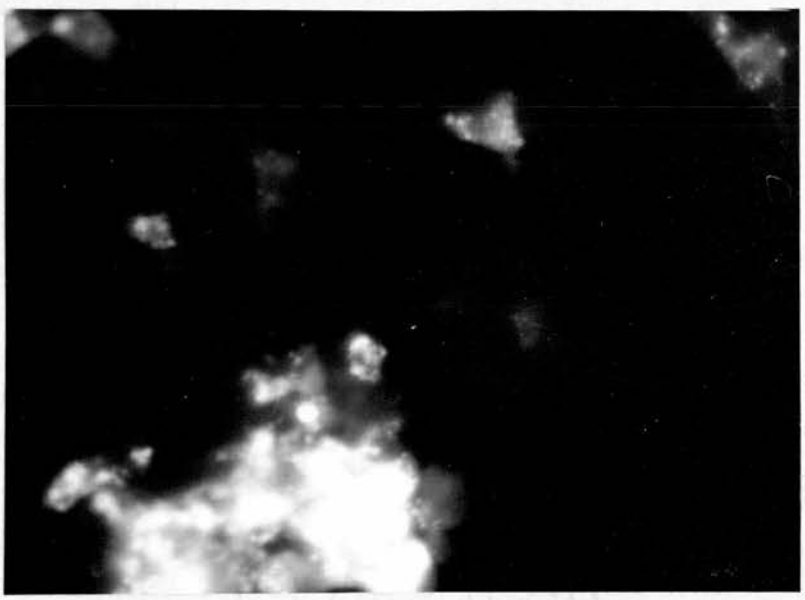

Fig.39a. Note the absence of fluorescent vacuoles in the reticular cell 4 hours after the cultures had been washed to remove uningested material. The large masses of brightly fluorescent material are aggregates of macrophages (soluble antigen-antibody complexes added 8 hours previously to a 3 day old culture of human peritoneal cells).

U.V. - Phase x 400

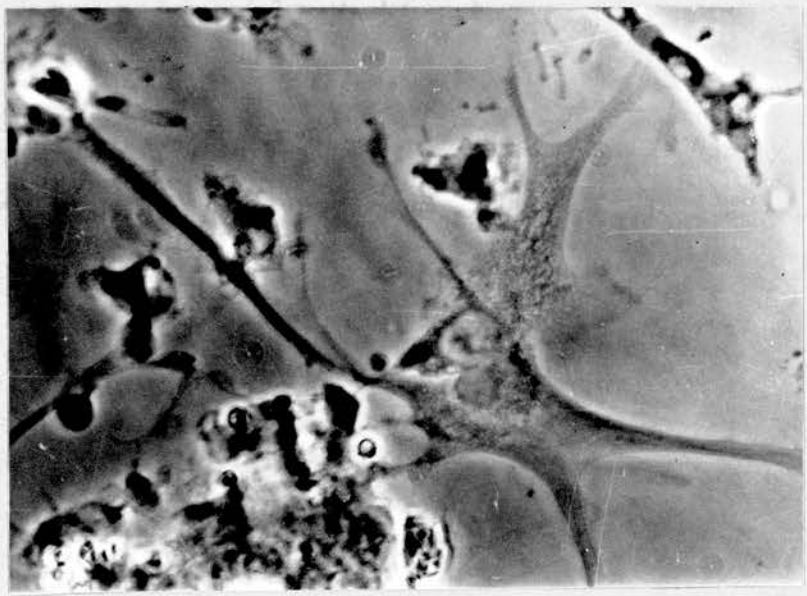

Fig.39b. Phase contrast appearance of the cells in Fig.39a.

Phase x 400

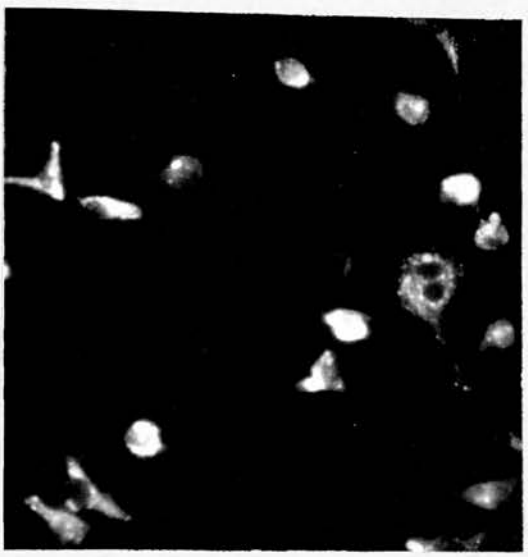

Fig.40a. The soluble antigen-antibody complexes were added 36 hours previously. Note the points of fluorescence in the cytoplasm of the binucleate cell, including a few in the processes.

U.V. - Phase x 250

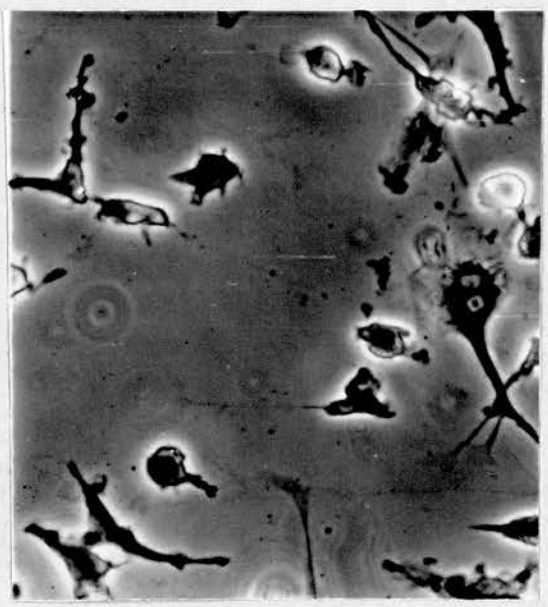

Fig.40b. Phase appearance of the cells in Fig.40a.

Phase x 250

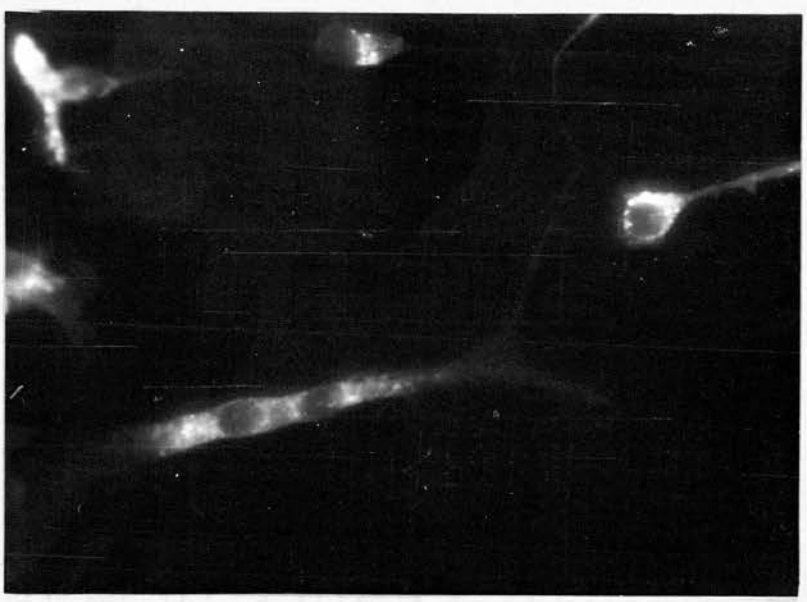

Fig.41a. The soluble antigen-antibody complexes were added 48 hours previously. Although there are no macrophages to be seen, the reticular cells still contain brightly fluorescent, granular material especially in the perinuclear area.

U.V. - Phase x 250

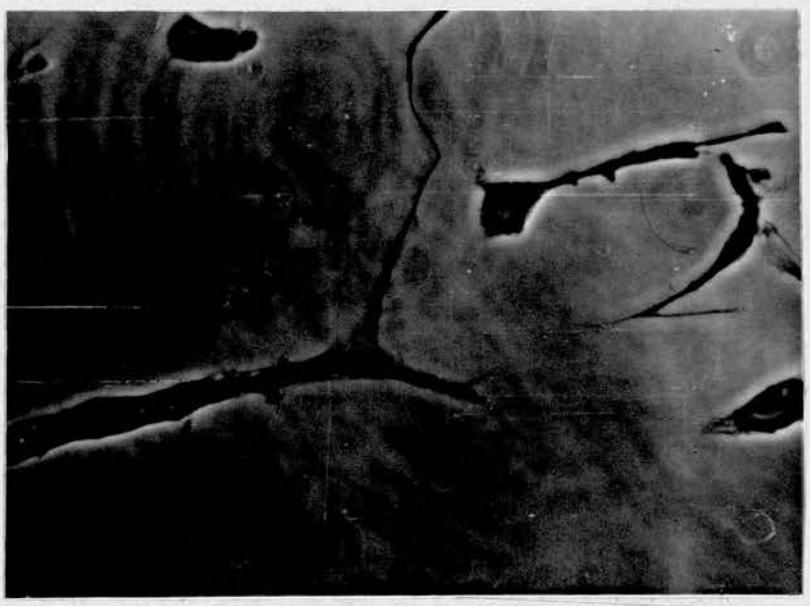

Fig.41b. Phase appearance of the cells in Fig.41a.

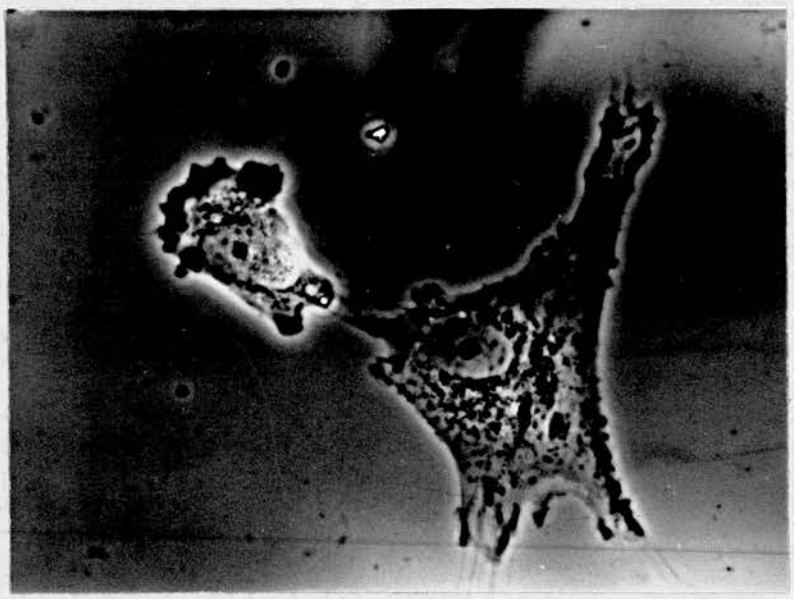

Fig.42. This illustrates the cytoplasmic connections which occur in culture between cells derived from human peritoneal fluid.

Phase x 200

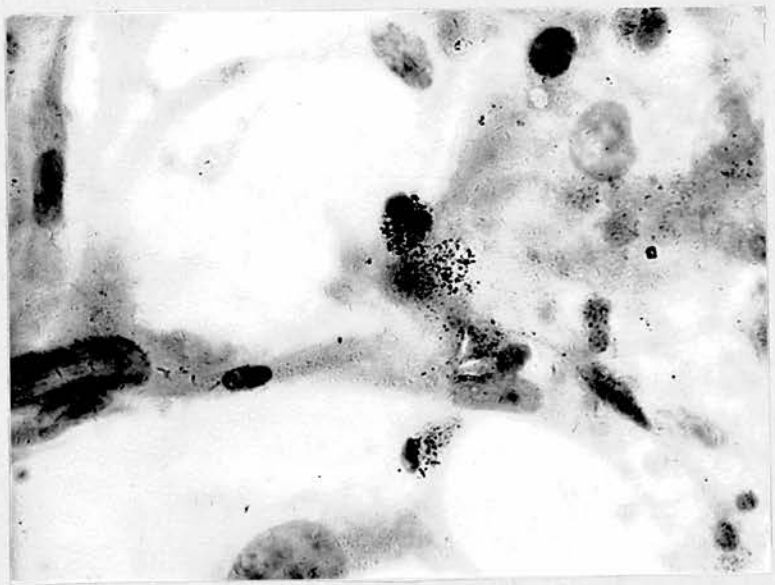

Fig.43. This shows macrophages in close association with reticular cells. This clustering was a prominent feature in cultures which had received the radio-labelled antigen-antibody complex.

Haematoxylin x 525

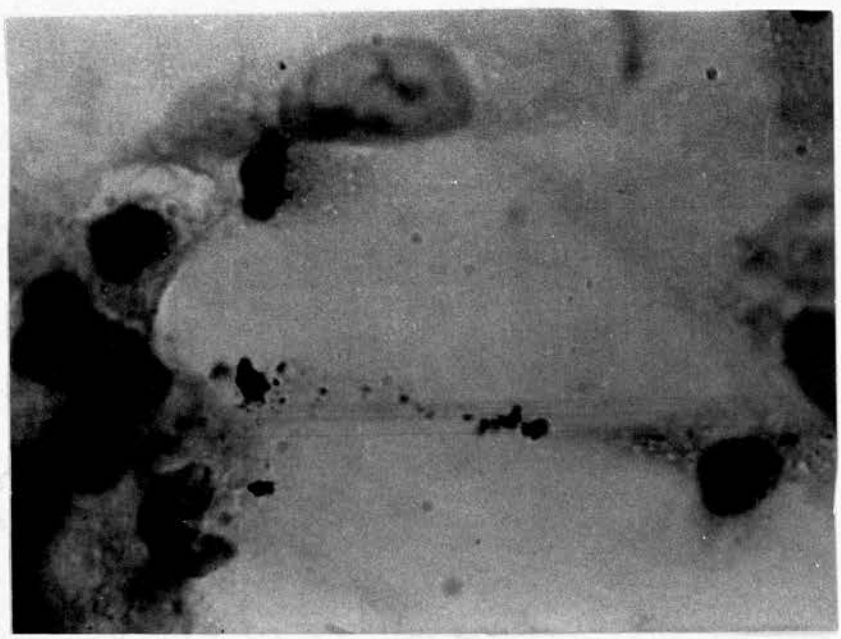

Fig. 44. This shows the labelled complex in a process linking a cluster of cells to a reticular cell. In the cluster there are heavily labelled macrophages.

Haematoxylin x 1000

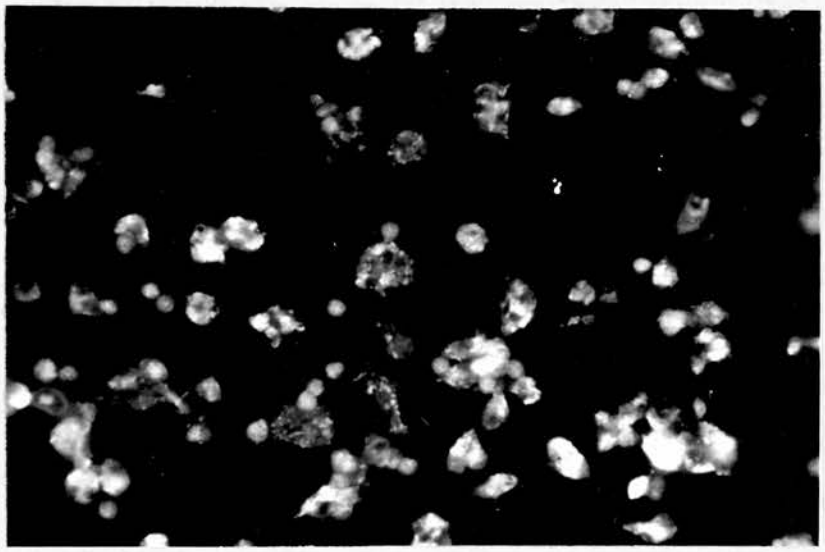

Fig.45a. Macrophages containing Cl. septicum after 4 hours' incubation. The bacteria are still bacillus-shaped.

U.V. - Phase x 250

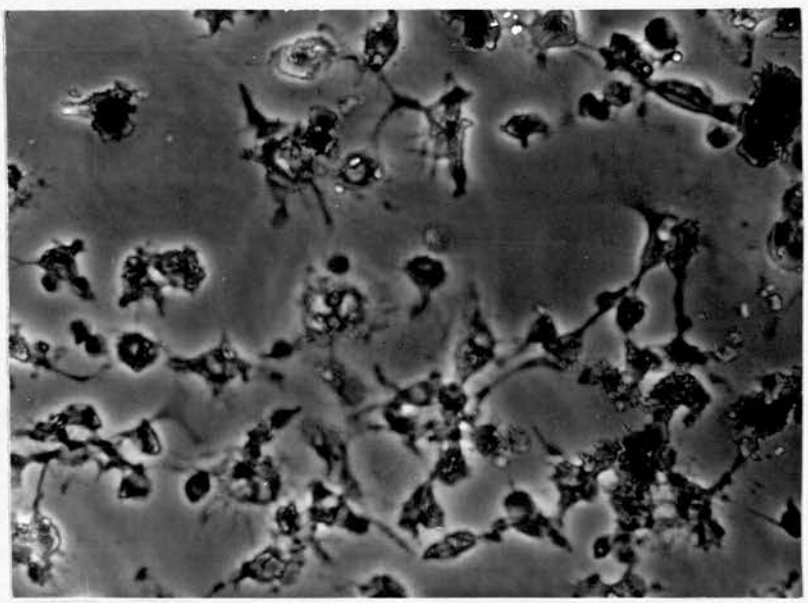

Fig.45b. Phase contrast appearance of the cells in Fig.45a.

Phase x 250

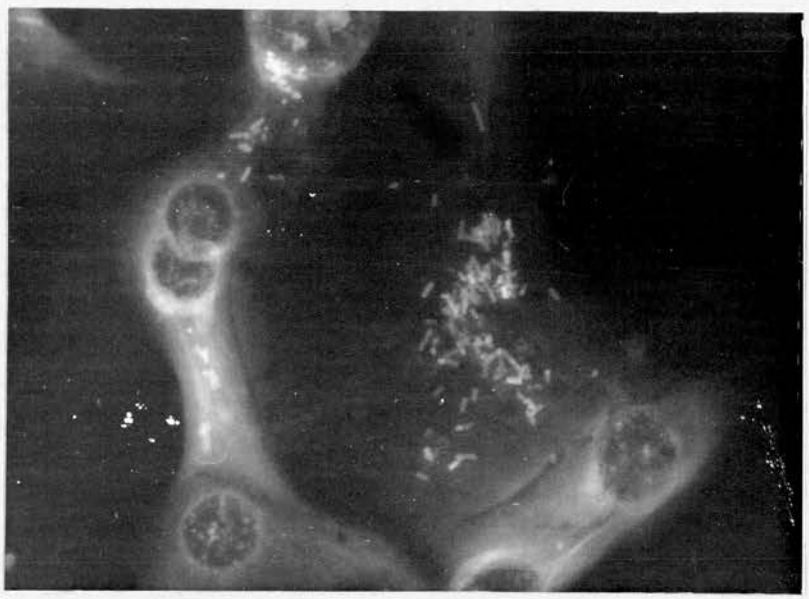

Fig.46a. There are recognisable bacilli in the reticular cells after 7 days. The tumour cell is showing a little autofluorescence but none specific for clostridial antigens.

U.V. - Phase x 400

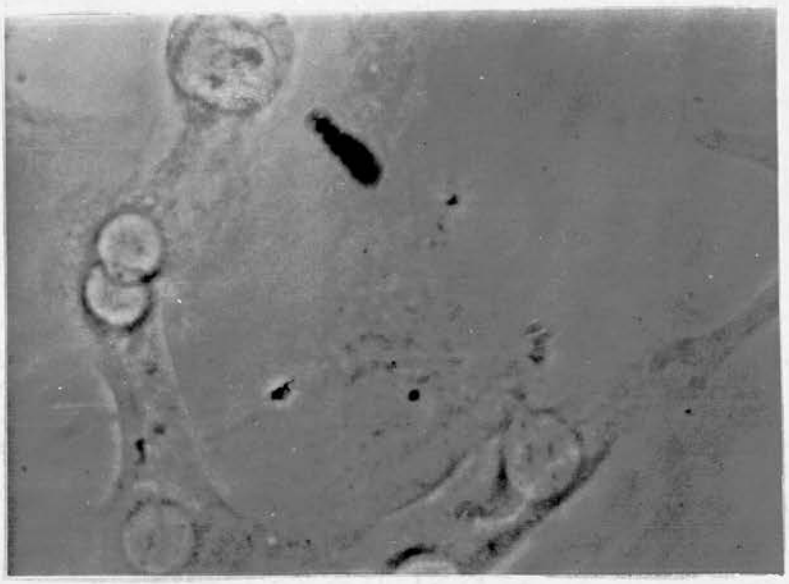

Fig.46b. Phase contrast appearance of cells in Fig.46a.

Phase x 400

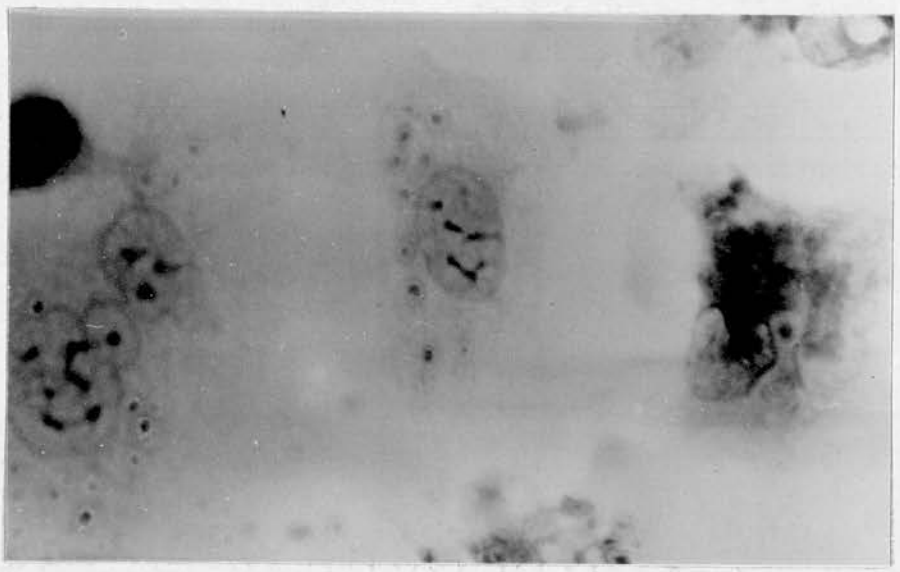

Fig.47. On the extreme top left of the illustration there is a macrophage containing partially digested yeast while the reticular cells contain recognisable yeasts.

P.A.S. Haematoxylin x 600

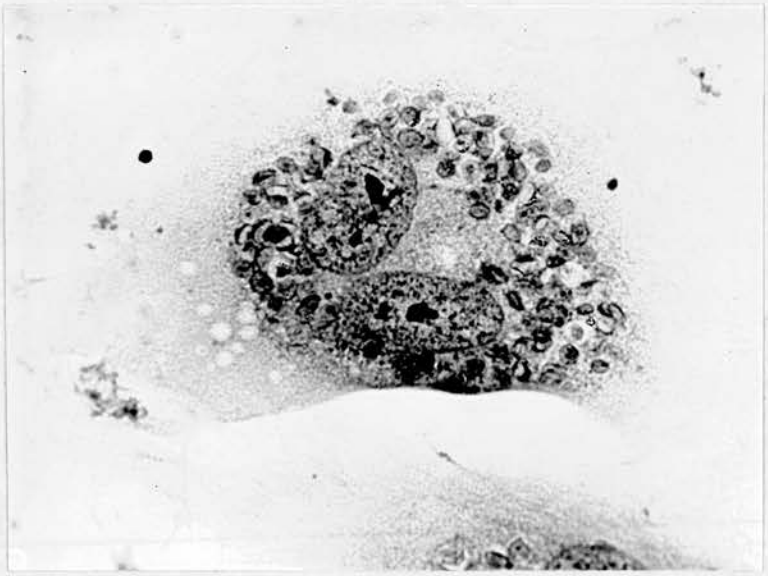

Fig.48. A binucleate reticular cell containing yeasts 20 days after the particles were added to the culture. The yeasts have lost their intense P.A.S. +ve reaction and only the cell wall is clearly visible.

P.A.S. Haematoxylin x 700

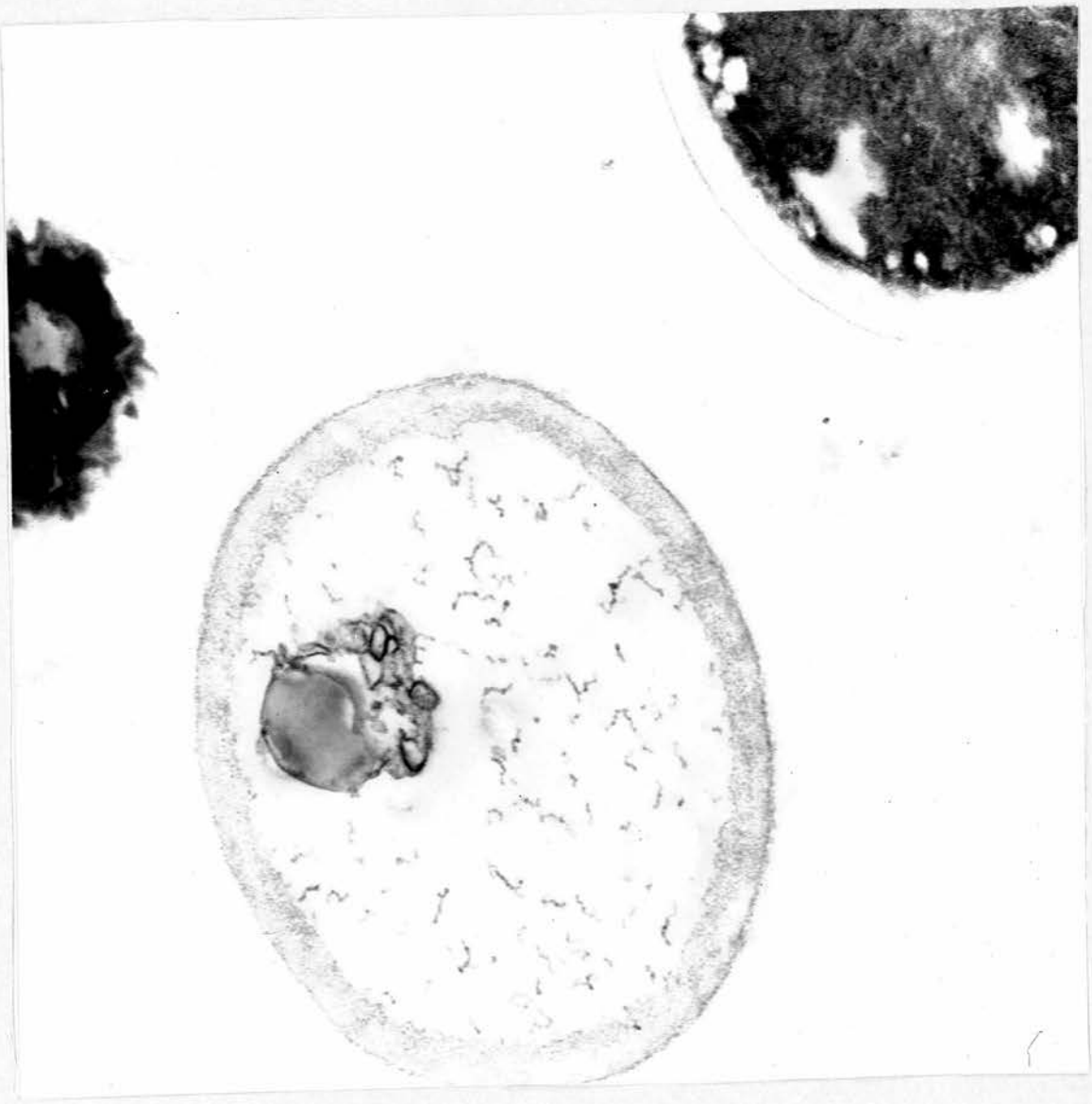

Fig.49. Yeasts from the suspension added to the culture of reticular cells.

E.M. x 40,000

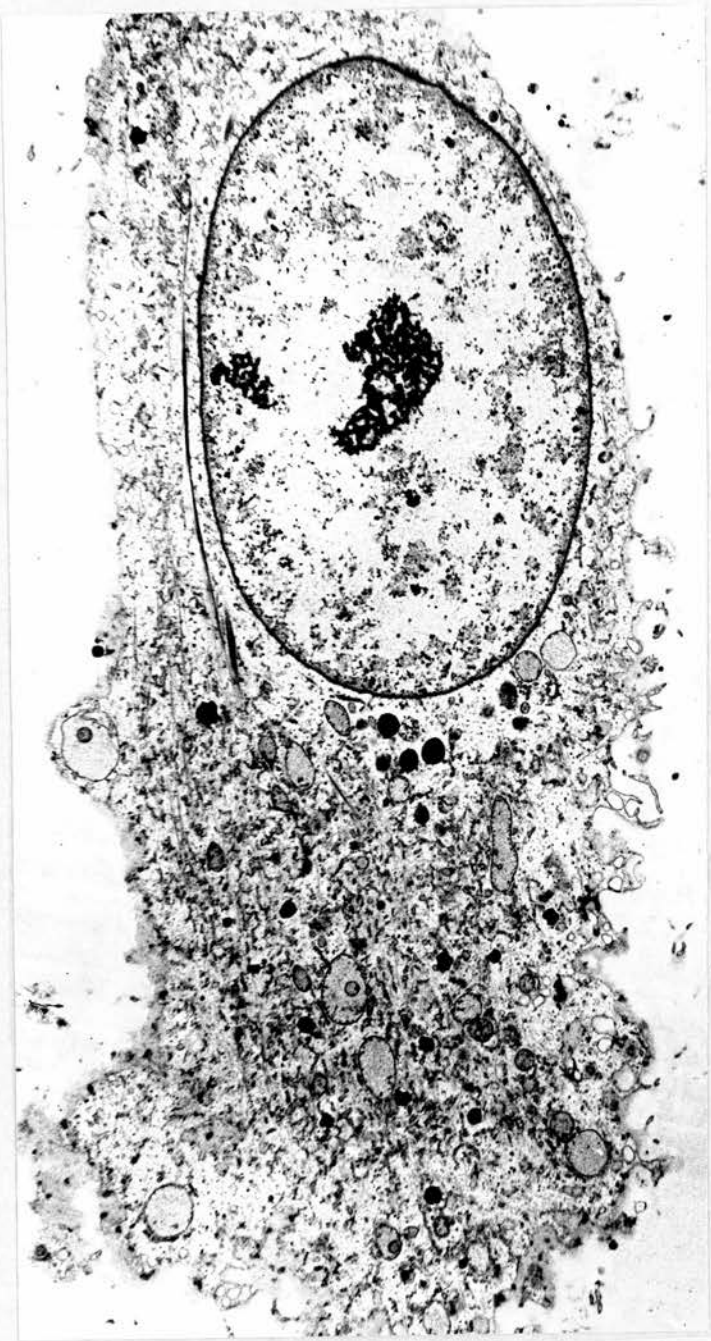

Fig.50. This is from a 14 day old culture of human peritoneal cells to which yeast had been added and incubated for 3 hours, then uningested material removed. Note the tube-like fibrous structure in the perinuclear area and the yeasts scattered throughout the cytoplasm.

E.M. x 3,000

Fig. 51. A high power of a yeast in the cytoplasm of a reticular cell. Note the absence of a distinct vacuole around the ingested particle.

E.M. x 32,000

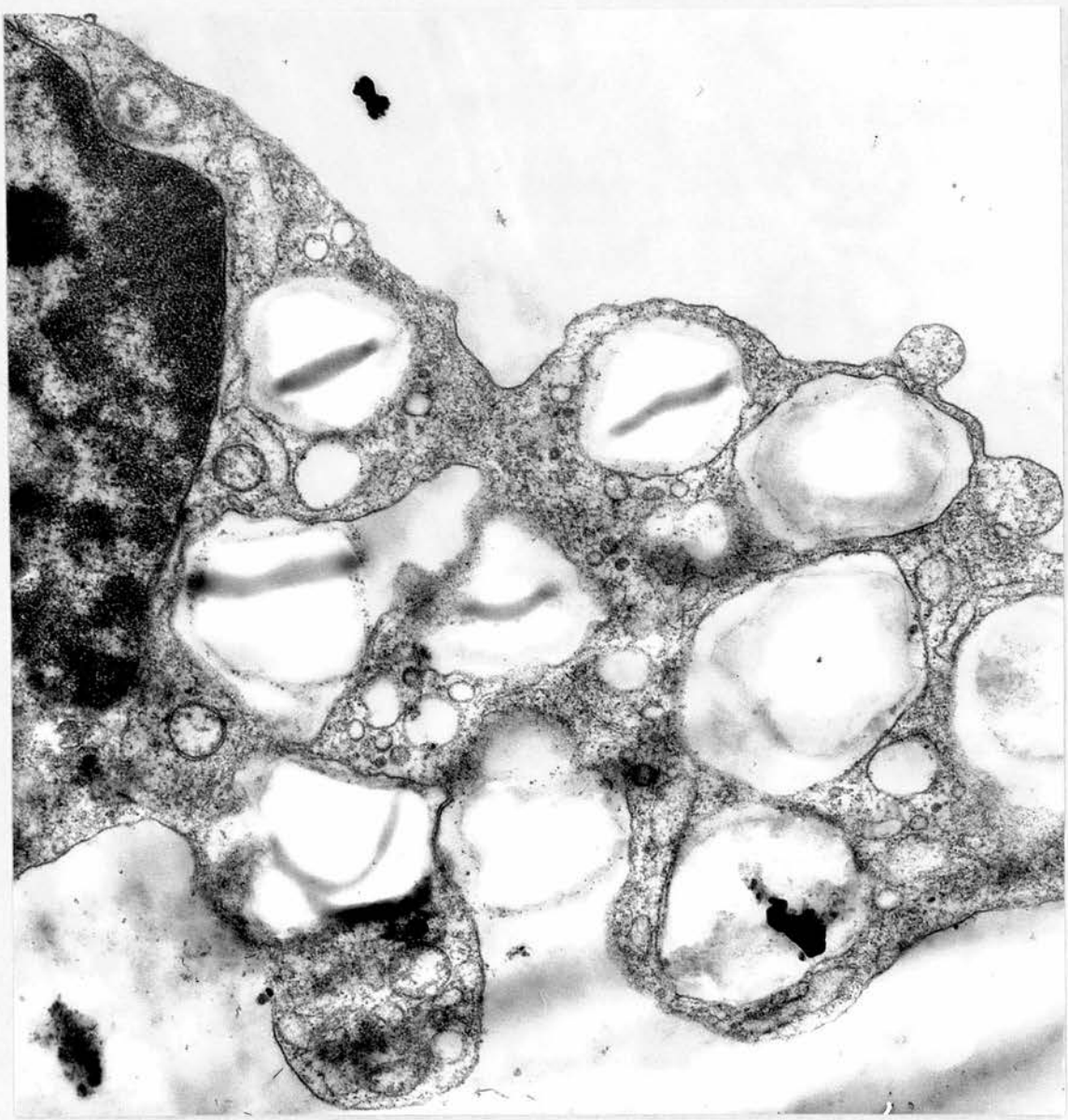

Fig.52. Part of the cytoplasm of a macrophage which had ingested numerous starch granules. Each granule is in a vacuole, unlike the yeast in Fig.49.

E.M. x 10,000

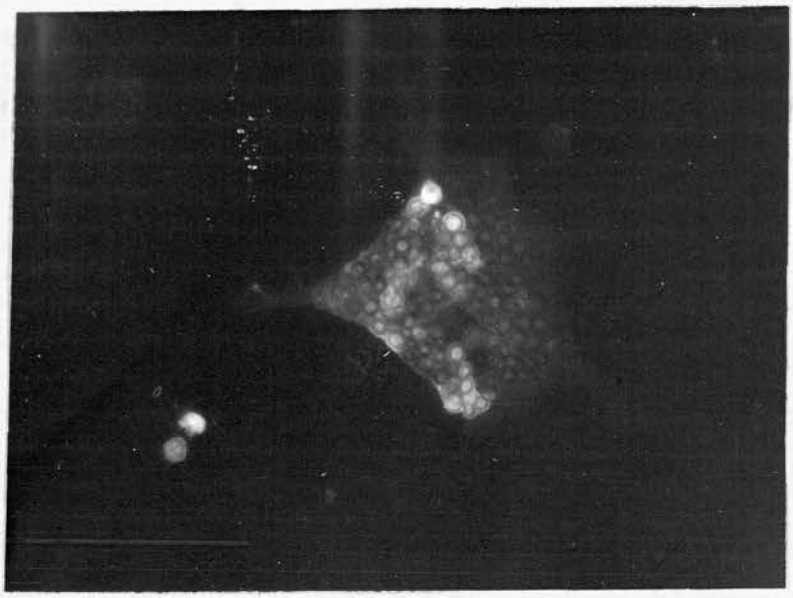

Fig.53. Preparation of reticular cells which had ingested yeast 17 days previously. The culture has been fixed and stained by the fluorescent sandwich technique using rabbit anti-yeast serum followed by fluorescent goat anti-rabbit serum.

U.V. - Phase x 630

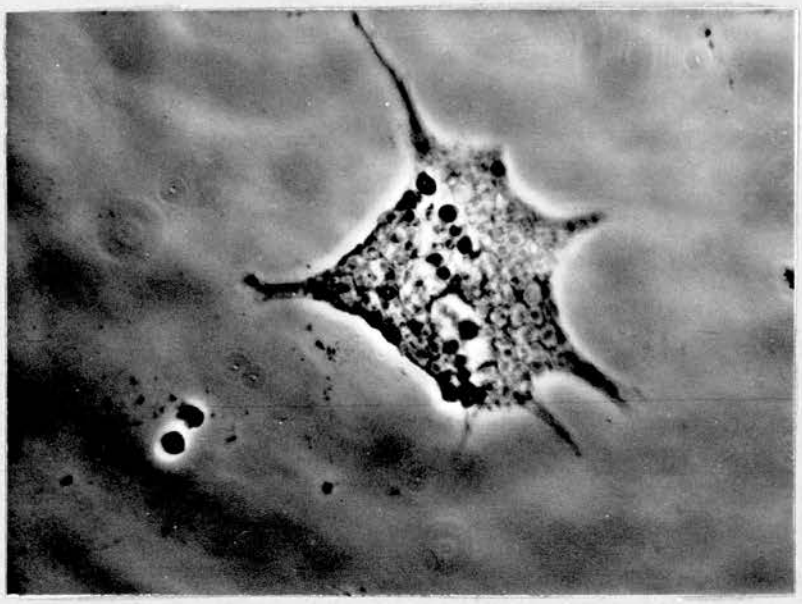

Fig.54. Phase contrast appearance of the same field as Fig.53. The yeasts can be seen as small phase dark dots.

Phase x 630

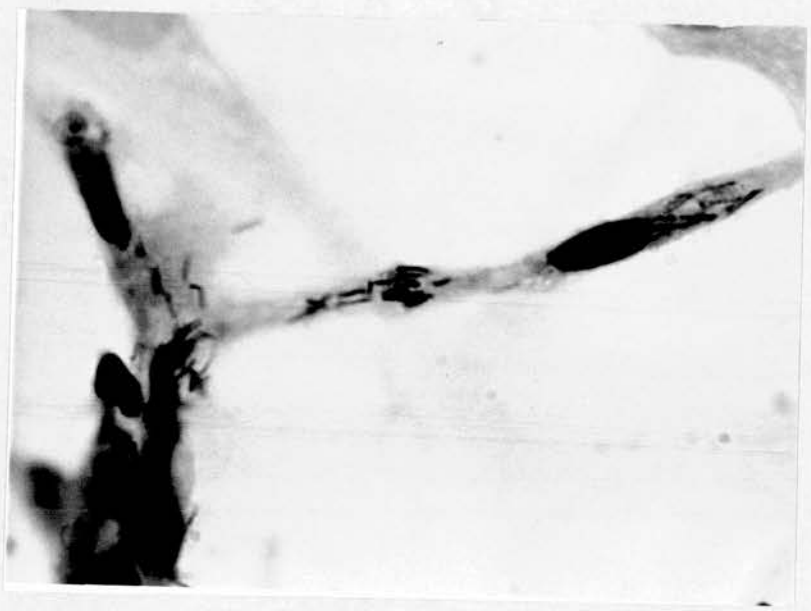

Fig.55. This illustrates the presence of B.C.G. in intercellular processes.

Fite-Faraco x 1000

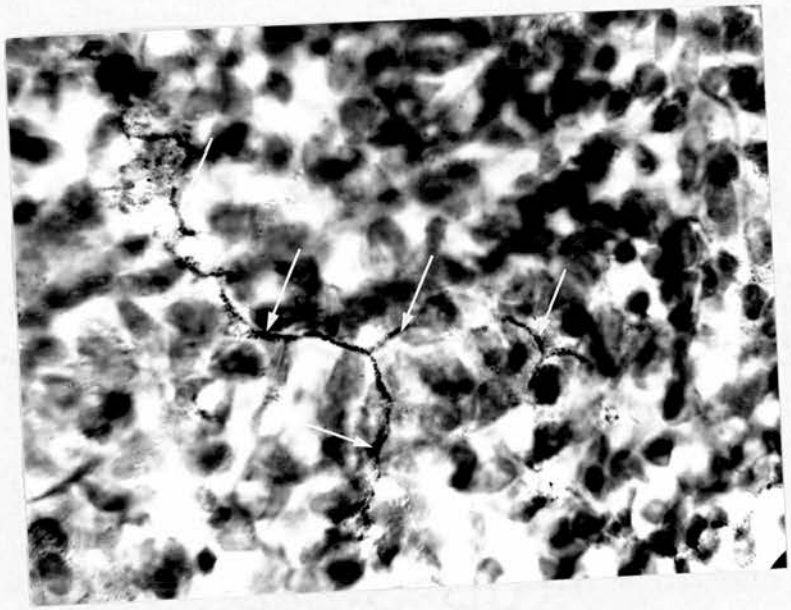

Fig.56. Typical lace-like pattern of radio-labelled H.S.A. in Malpighian body of guinea-pig 7 days after injection. (Arrows)

Autoradiograph, Haematoxylin x 700

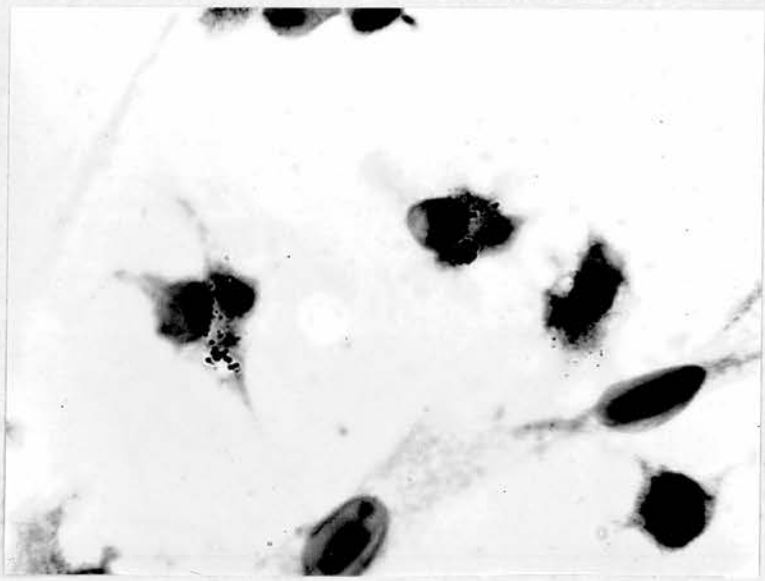

Fig.57. Radio-labelled macrophages in 7 day culture of guinea-pig spleen.

Autoradiograph, Haematoxylin x 700

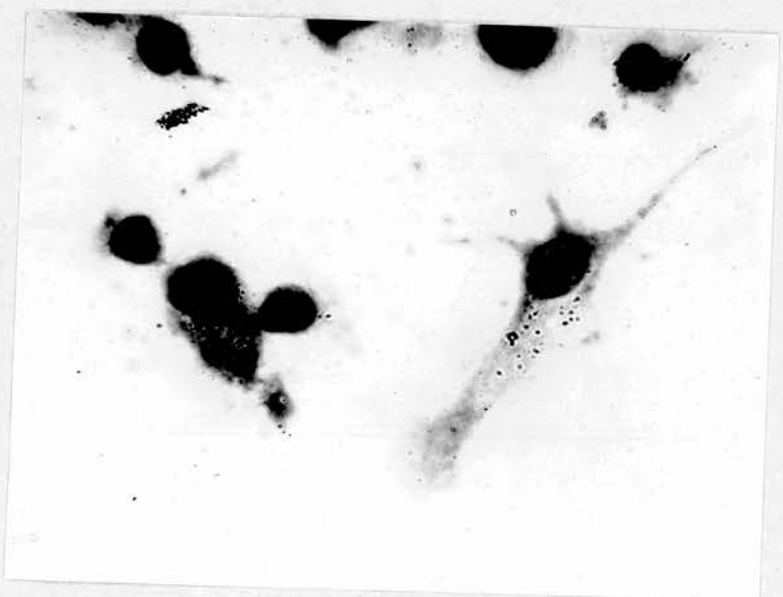

Fig.58. Reticular cell containing radio-label. Note the large size of this cell, the prominent nucleolus and extensive cytoplasm with branching processes.

Autoradiograph of guinea-pig spleen culture.

Haematoxylin x 200

CHAPTER V

EFFECT OF TRANSFORMING AGENTS ON CELLS FROM THE HUMAN PERITONEAL CAVITY

Reticular cells have been regarded as being pluripotential (Maximow and Bloom, 1938), giving rise to fibroblasts, haemopoietic and even lymphoid cells. They are regarded as a stem cell in leukaemic processes, and in Hodgkin's disease the proliferating reticular cells are thought to differentiate into myelocytes, fibroblasts and plasma cells. It therefore seemed worthwhile to investigate the response of reticular cells obtained from the human peritoneum to agents known to cause blast cell formation and mitotic division in lymphocytes. The transforming agents used were phytohaemagglutinin (PHA) and a cell-free filtrate from a culture of Staphylococcus aureus. These agents would alter the micro-environment and might result in differentiation to one or other of the cell types or give some other indication of potential lines of development. PHA was found to have blastogenic and mitogenic properties by Nowell (1960). Nowell said "Physiologic factors such as oxygen tension, carbon dioxide tension and plasma concentration were varied individually but proved to have only minor quantitative effects on mitotic activity in the cultures. Instead, an apparently non-physiologic mitogenic agent was discovered, the plant extract phytohaemagglutinin (PHA). This substance was originally employed for its erythrocyte agglutinating ability in obtaining leukocytes from whole blood. The present studies, however, indicate that it also has the ability to initiate mitosis among these leukocytes. . ."

Ling and Husband (1964) showed that an exocellular protein produced by Staphylococci is also capable of stimulating peripheral lymphocytes/

lymphocytes, the degree of stimulation being limited only by the amount of activity material added.

Although there has been much work on the effects of transforming agents on lymphocytes there are few reports of their effects on other cell types. The major work in this respect is that of Ioachim (1966), using PHA. When he added PHA to cells in suspension, agglutination occurred in all types of cultures. These clumps did not attach to glass until after 48 hours had elapsed.

When PHA was added to established monolayers ". . . the growth pattern as well as the cellular morphology was altered. The cells . . . appeared to be re-arranged in separate or coalescent islands with multilayered areas. . . . Mitoses in different stages were frequent and the mitotic indices were higher in the PHA-treated cultures than in controls. PAS-positive material was significantly increased in PHA-treated HEp No.2 cultures." (Ioachim, 1966).

MATERIALS AND METHODS

Phytohaemagglutinin (PHA) was supplied by Burroughs Wellcome & Co., 5 ml. dried PHA (MR10) was reconstituted with distilled water. The effects of this substance were tested at two dose levels and at two time intervals. The doses were 0.1 ml. of the stock solution and 0.1 ml. of this diluted 1/10. Each dose was added to cultures when they were set up at time zero and to cultures already established for 48 hours.

Cells treated with PHA were examined for morphological changes and/

and tested for alterations in phagocytic ability and staining reactions. Staphylococcal filtrates were made by growing Staphylococcus aureus in antibiotic free medium 199 (Burroughs Wellcome) for 5 days, then passing the fluid through a millipore filter for sterilisation and removal of bacterial debris. Doses of 0.1 ml. of the solution of the toxins as they came through the filter were added to cultures at time zero and at time 48 hours. Higher doses of the filtrate were tested and found to be toxic. The cultures were examined in the same way as those treated with PHA.

Details of the technique of autoradiography using H^3 labelled thymidine are given in the appendix. Thymidine was used to assess the degree of mitotic activity induced by the transforming agents.

RESULTS

High doses of PHA (0.1 ml. of stock solution) when added to the cultures at the time of cell settling were toxic and the cells failed to grow. Low doses (0.1 ml. 1/10) were also toxic to the majority of cells when added at the time of settling. However, a few cells survived, and when these attached to glass they showed little change in morphology.

When the high dose was added to established monolayers the results were dramatic. The cells aggregated within 24 hours. After another two or three days the cells had separated into two distinct groups - one which formed fibre-like strands, the other giant cells (Fig.59). Similar results were obtained using staphylococcal filtrates./

filtrates.

The giant cells had large numbers of pale-staining nuclei with nucleoli, and an extensive, very thin cytoplasm which stained poorly (Fig.60). Staining with periodic acid Schiff reagents did not show a marked increase in PAS-positive material in these cells, nor were there microscopic signs of an increase in mitotic activity. The clusters and the giant cells remained unchanged for up to one week in culture, suggesting that the clusters do not progress to become giant cells. The appearances of strands of cells and giant cells were quite abnormal, never having been observed in control cultures from these batches of cells. However, occasional batches of cells did show giant cells but they were of a different type. They had peripheral nuclei and a limited cytoplasm (Fig.61).

Neither of the transforming agents had an effect on the phagocytic activity of the cells. This was tested by the addition of antibody-coated erythrocytes and starch particles as described in Chapter III. The macrophages in the fibre-like aggregates were phagocytic whereas the giant cells showed little or no phagocytosis. This suggests that the giant cells may be derived from the reticular cells since these cells are known to be poorly phagocytic.

Although no microscopic evidence of mitotic activity was seen, some experiments using H^3 labelled thymidine were done. The thymidine was incubated with the culture for 6 hours and then autoradiographs prepared. When the thymidine was added at or before 4 days in culture, no specific labelling occurred. After this time a/

a few cells showed sparse label, in both control and test cultures. No densely labelled cells were ever seen. It appears that both PHA and staphylococcal toxin cause alterations in the morphology of human peritoneal cells. These substances do not increase mucopolysaccharide production (as shown by PAS staining) and do not increase mitotic activity.

DISCUSSION

Ioachim (1966) reported that the effects of PHA on cells in culture were varied, affecting not only lymphocytes but also many different cell types. "With the demonstration of PHA effects on a variety of cells, most of them unrelated to immunologic phenomena, a mode of action based on a presumed antigenic nature becomes more unlikely. The broad range of cell types affected, as well as the intensity of this effect, indicate the non-specific nature of this stimulation. A capacity to act on the cell surface as previously suggested could possibly explain some of the effects of PHA such as cell agglutination and induction of mitoses. This would be in accord with the finding that the leuco-agglutinogenic and mitogenic properties of PHA could not be separated by adsorption with leucocytes."

Maclaurin (1969) studied in detail the effects of PHA and tuberculin on primary cultures of macrophages. He used rat peritoneal and human peripheral blood macrophages at 24-48 hours after initial isolation, and found that the toxic effects of various batches/

batches of PHA differed considerably. He found that the staphylococcal filtrates caused the macrophages to round up in contrast to the aggregation and cytoplasmic linkage or bridging which was induced by PHA.

In my studies I noticed not only that there were aggregates of cells, but also that many giant cells were formed. This was not found either by Ioachim or Maclaurin. This would suggest that the giant cells were formed from the reticular cells as would the absence of phagocytic activity.

M.R. Lewis (1925) described, in her hanging drop cultures of peripheral blood, the development of giant cells. These were of the Langhans' type, that is the nuclei were situated around the periphery of the cell, usually in the form of a horseshoe or a ring, and were not phagocytic. Sabin and her co-workers (1924) also described the formation of giant cells and thought that it occurred by fusion of or amitotic division in, the cells she called clasmatocytes.

In the 1920's the Lewis's (Lewis, M.R. and Lewis, W.H., 1925, 1926) studied the development of giant cells in peripheral blood cultures from many animals. "The amount of transformation (of mononuclears to macrophages to epithelioid cells) which occurred, varied considerably with the species. The changes occurring in the blood of the sculpen, the frog, the toad and the Californian lizard were the most marked of any in the entire series. The mononuclears of these forms appeared to increase in number and mitosis was occasionally noticed. The size and frequency of formation of giant cells was very striking and large plasmodial-like masses were often encountered/

encountered on the coverglass. . . The giant cells which develop in the cultures are of the Langhans' type. . ." They studied the mode of formation of the giant cells using "the cultures of a modified white blood cell tumor of the rat." The formation of the giant cells by fusion took about 15 minutes for one cell to fuse completely. In some cases there was an indication that the number of nuclei in a giant cell could increase by division of nucleus without the cytoplasm dividing.

In vivo giant cells are formed in tissues in response to the presence of a foreign body or as a result of some fungal, bacterial or viral infections. In the latter case it is thought to be the result of alteration to the surface of the cell. Perhaps the commonest example is the measles giant cell found in hyperplastic lymphoid follicles. Viruses can also mimic this in vivo effect when added to cell cultures. My cell culture work suggests that the reticular cells are not pluri-potential. On the contrary they are a stable cell type of limited reactivity.

The morphology and functional activity of the giant cells formed in response to phytohaemagglutinin and staphylococcal toxin indicate that they are derived from reticular cells and not from macrophages. What light does this shed on the origin of giant cells found in vivo, in the pathology of foreign body reactions? Whilst not denying the existence of giant cells derived from macrophages, it seems likely that in some cases they are formed from reticular cells.

My thesis has attempted to show that these cells are matrix producers, fibre formers and antigen storers. These properties are in accord with a biological function in defence reactions.

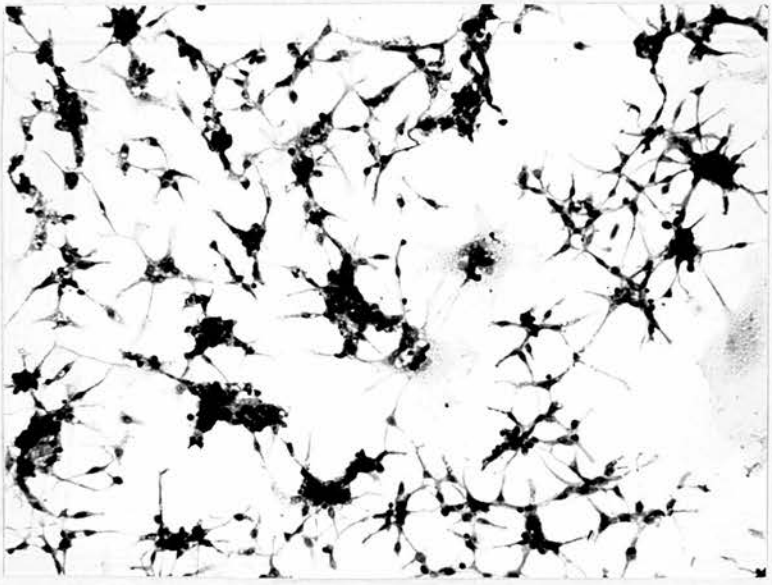

Fig.59. This shows the aggregation caused by staphylococcal toxin after its addition to an established (24 hour old) culture. Note the giant cells and the clusters of darkly staining cells.

Giemsa x 110

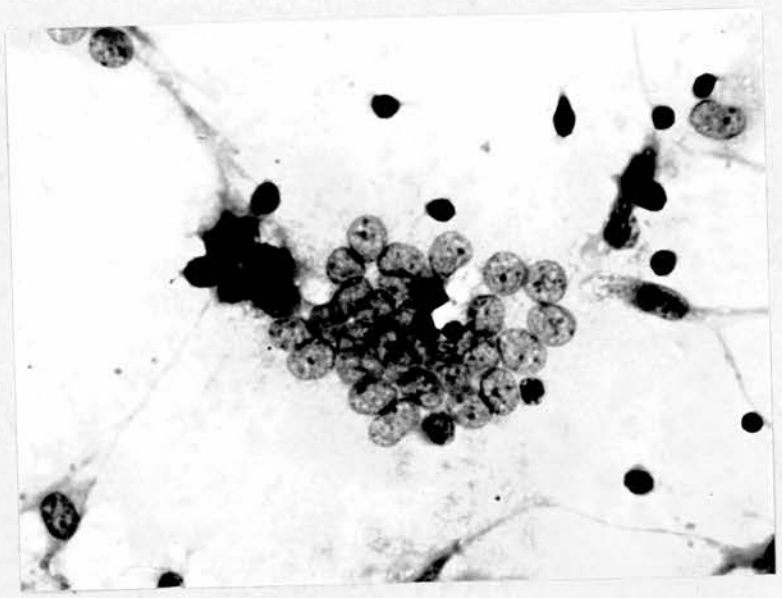

Fig.60. This is a giant cell which was induced by the addition of phytohaemagglutinin (PHA) to the culture. The nuclei are open with prominent nucleoli and are always placed in the centre of the extensive cytoplasm.

Giemsa x 525

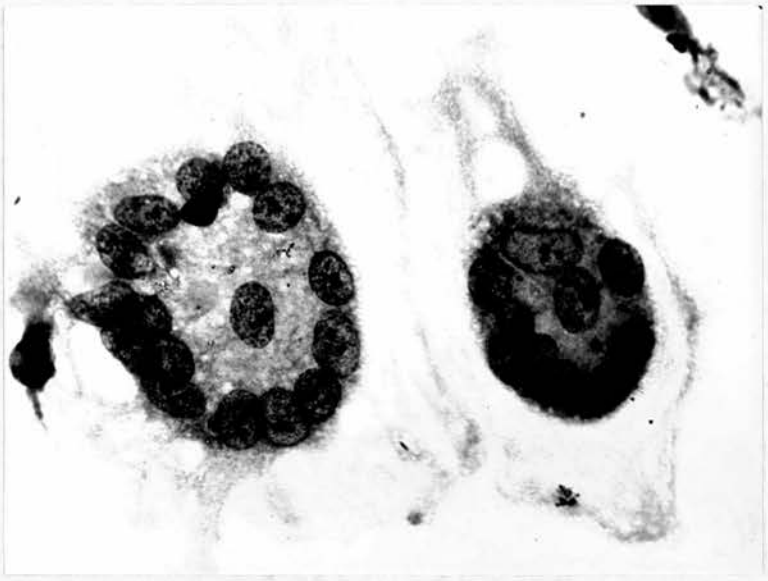

Fig.61. Multinucleate giant cell from an untreated culture of human peritoneal cells 5 days after isolation. Compare the distribution of the nuclei with those in the cell in Fig. 60.

Leishman x 700

CHAPTER VI

DISCUSSION

DISCUSSION

Ever since the description of phagocytosis by Metchnikoff (1884) there has been controversy over the significance of this function in immunological responses. Early workers (Cannon et al., 1929; Gay and Clark, 1924) attempted to prove that the macrophage is a necessary link in the chain of events leading to antibody production. In general they tried to block the functions of the reticulo-endothelial system using inert colloids such as carbon. This work was ably reviewed by Jaffé (1931). The results of these early experiments were inconclusive since at that time there were no reliable methods which would reveal the degree of blockade. More recent work by Stuart and Davidson (1964) showed that blockade of the reticulo-endothelial system with lipid emulsions such as cholesterol oleate or ethyl palmitate depressed the primary response in mice to sheep erythrocytes. Di Luzio and Wooles (1964) found that methyl palmitate had similar effects. Since then these findings have been confirmed and extended by Sabet and his co-workers (1968, 1969a, 1969b). They showed that both the dose of blocking agent and the time of its administration were critical if suppression of antibody response was to result. They also showed that the injection of carbon prior to a primary immunisation interfered with the development of immunological memory.

Other evidence for the importance of the macrophage system in immunological responses has come from the work on the induction of tolerance to protein antigens. Frei and his colleagues (1965) showed/

showed that antigen which had been "filtered" through an animal (thus removing the phagocytosable material) was tolerogenic when given to another animal. A similar finding was reported by Dresser (1962) who showed that supernatant from human serum albumin which had been ultracentrifuged was capable of inducing tolerance but injection of the deposit resulted in antibody production. Both of these reports deal with low zone tolerance. It is thought under these circumstances that the macrophage does not ingest the antigen which interacts directly with lymphocytes. High zone tolerance might be the result of overloading the macrophages, thus leaving the antigen free in the intercellular spaces. However this idea implies that antigen must be processed by macrophages. It has been shown that the macrophage is not essential in the immune response to flagellin while it is necessary in the response to erythrocytes (Shortman et al., 1970; Diener et al., 1970). This would suggest that high zone tolerance processing could be a result of a shift in the differentiating mechanism away from the production of memory cells to antibody producers, thus exhausting the supply.

Studies on B.S.A. labelled with I^{125} by Ada and his colleagues (1964) (given in tolerogenic doses) showed that the label is heavy in blood, lymphatic vessels and sinuses but that all areas of all lymphoid tissues are diffusely labelled. Other types of antigen, e.g., synthetic polypeptides and pneumococcal polysaccharides, are localized in phagolysosomes of reticulo-endothelial cells where they remain for long periods of time. Presumably these "packets" of antigen/

antigen act as a reservoir which releases antigen at a slow but constant rate, thus maintaining a state of tolerance among the lymphoid cells.

Other experiments have shown that the macrophages of the new-born, which can readily be made tolerant to protein antigens, were not so efficient at handling these antigens (Martin, 1966; Williams and Nossal, 1966). Williams and Nossal (1966) also showed that neonatal rats did not have an antigen trapping mechanism for flagellar antigens. Since Silverstein and his colleagues (1962 and 1963) have shown that foetal responses to antigen depend on the antigen and the age of the foetus, one must state the antigen and the species being used. As a general rule, neonatal animals do not respond to antigens in the same way as adults. Robbins (1963) assayed the total organ content of azo-compounds in neonatal and adult rabbits and found that the degradation rates were greater in adults. This difference was not apparent when rabbit serum albumin, presumably non-antigenic, was used. These findings led him to postulate that the macrophages of the new-born had an enzymatic defect.

The induction of tolerance could be prevented if the neonates were injected with adult macrophages at the same time as the antigen. Martin (1966) showed that in rabbits the induction of tolerance to protein antigens in the new-born was due to a defect in the macrophage system, since the injection of the same dose of B.S.A. without or with adult rabbit macrophages induced tolerance or immunity/

immunity respectively. There is, however, no conclusive evidence about the form this defect takes. It could be any one of the following.

1. Lack of macrophages.
2. An enzymatic defect (as suggested by Robbins, 1963).
3. A defect in the phagocytic capacity of individual cells.

McFadden (1968) using the property of metalophilia and the presence of acid phosphatase and non-specific esterase to identify the reticulo-endothelial cells, showed that the splenic white pulp of rats at birth and for the next 21 days is deficient in cells which contain both enzymes and are metalophilic, i.e., which are "fully-fledged" macrophages as described by Pettersen (1964), thus giving support to the first of the possibilities.

Antibody production by lymphoid tissue in vitro was first studied by Carrel and Ingebritsten (1912). They used guinea-pig lymph node grown in goat blood and showed that after a few days a substance was produced which lysed goat erythrocytes.

Some evidence for primary antibody production in vitro was given by Bloom (1927) when he showed that lymph node explants from normal rabbits cultured with pigeon red cells did not ingest the erythrocytes until a few days after the cultures were set up. However, if the antibody was present in the medium, ingestion of the erythrocytes was immediate. Both of these experiments used explant cultures which meant that the spatial arrangements and therefore cell contacts were kept intact. This appeared to be a necessary/

necessary condition for the induction of antibody synthesis. However, Fishman (1961) showed that it was possible to induce primary response to antigen in vitro more consistently if the antigen had been "processed" by macrophages. He did this by allowing macrophages to ingest bacteriophage and then prepared a cell-free extract, also free from detectable virus. This extract, when added to explant cultures of lymph nodes from unimmunised animals, induced antibody production. Shands (1962) in his review of the literature on macrophages and antibody production drew attention to the adaptive changes shown by macrophages in response to antigen and to the fact that macrophages produce RNAase sensitive material which can be shown to be immunogenic.

Gallily and Feldman (1967) showed that macrophages although not affected morphologically by irradiation, were damaged with respect to their ability to process bacteria (Shigella). They also showed that macrophages were essential for the production of antibody in reconstituted, irradiated animals. This work used both in vitro and in vivo systems. In the wholly in vitro system Mosier (1967) showed that macrophages were essential for antibody production to erythrocytes. However, Diener and his group (1970) showed that macrophages were not necessary for flagellar antigens. Using sheep R.B.C. this group confirmed that a mixed cell population, i.e., adherent (to glass) and non-adherent, was necessary in order to obtain antibody production in vitro and that it was difficult, if not impossible, to induce tolerance to R.B.C. in vitro although this was possible using flagellar/

flagellar antigens. This may simply be due to the size of the R.B.C. and if smaller fragments were prepared which were antigenic then antibody production might be macrophage independent (Palmer, 1970).

These experiments provide strong circumstantial evidence that the macrophage is necessary for the induction of a primary response to at least some antigens *in vitro*. Subsequent work has confirmed these findings and has also shown that macrophages retain immunogenic material longer than do polymorphonuclear leucocytes (Cohn, 1964). Unanue and Askonas (1968) have shown that immunogenic material can be retained by the macrophage in association with membranes for at least two weeks while Gill and Cole (1965) showed that macrophages were capable of concentrating antigen-antibody complex and therefore presumably also antigen, into vacuoles just at the surface of the cell.

The available evidence strongly supports the view that macrophages are an integral part of the mechanism for the induction of a primary response to some but not all antigens. However, they do not appear to be necessary for a secondary response since it has been shown (Harris, 1965) that cultures of lymphoid tissue from primed animals will respond to a subsequent challenge with antigen *in vitro*. The finding that lymphocytes will transform in response to antigens such as P.P.D. or pokeweed mitogen (Schrek, 1963; Chessin *et al.*, 1966; Douglas *et al.*, 1967) also supports the view that secondary responses are not mediated through macrophages.

The secondary immune response and the development of immunological/

immunological memory, however, appear to be unaffected by macrophages. Morphological studies (Conway, 1937; Ringertz and Adamson, 1950; Fagreus, 1948) have correlated the appearance of 7s antibody and immunological memory with the development of germinal centres, which develop in the lymphoid follicles which contain reticular cells or antigen-retaining cells.

Using silver impregnation methods, Marshall and White (1950) showed that in germinal centres there were multi-branched cells which they included in the reticulo-endothelial system even although they did not stain readily with vital dyes. These cells increased in number after antigenic stimulation. Ortega and Mellors (1957) showed that in normal animals, gamma-globulin is localised in germinal centres in a "lacy" pattern. Mellors and Brzosko (1962) showed that antigen-antibody complex was found in a similar pattern in the germinal centres of the white pulp of the spleen only when immune clearance of the injected antigen could be demonstrated and was therefore probably associated with antibody production.

Similar associations between reticular cells and antigen were described by Nossal's group (1964) and White (1963). Reticular cells were also found in primary follicles where Nossal (1967-68) described them thus "These aggregates of small lymphocytes and branching reticular cells are, in fact, most readily identified by their remarkable capacity to trap antigen-antigen complexes. Electron microscopic study reveals an extensive intertwining of delicate cell processes in these areas (Milanesi, 1965). Many of the processes arise/

arise from the dendritic reticular cells, others from lymphocytes." Furthermore, these cells can be demonstrated in the omentum (Fischer, 1970) where, 6 hours after an intraperitoneal injection of carbon, 85 per cent of the carbon-containing cells are dendritic cells. Similarly, French et al. (1960) demonstrated that there were phagocytic cells in the diaphragm (mesothelial cells), the ultrastructure of which resembled reticular cells. Fischer also found that the localization of carbon in the dendritic cells was depressed by irradiation. This is in accord with the findings reported by Nettesheim (1969). He showed that the trapping of antigen in the interstices of the web (on the surface of the reticular cells and their processes) is radio sensitive, and is also affected by immunosuppressive agents such as actinomycin D or cyclophosphamide. The administration of these chemicals to animals which already have antigen localized in the follicles reduces the concentration of antigen retained. Since the effects of these agents is limited to the spleen it appears that inhibition of phagocytosis is not a major factor. The electron microscope studies reported by this group showed that the reticular cells were affected morphologically by irradiation. This was seen as a lack of infoldings of the plasma membrane and also by some suggestion of cytoplasmic damage indicated by vacuolation and pyknosis. They also found that antigen localization in the follicles was dependent on the presence of 19s antibody. This dependence on antibody was expressed by Balfour (1967) thus ". . . it is now clear that, provided there is circulating antibody/

antibody present, available antigen will always, sooner or later, localize in germinal centres. Even a minute amount of antibody can alter the distribution of antigen in a lymph node." Nossal (1967) reviewed the work on antigen trapping in follicles and said "The predominant impression is of a complex, convoluted mass of internal and external cell membranes and interdigitating processes. Usually it is not possible to determine from which cell the processes are derived, but many may originate from reticular cells. Not uncommonly desmosome bridges can be seen between processes. . . . Most often of all, there are so many convoluted membranes in these areas that it is not possible to determine how much antigen is *in*, and how much *on*, cell processes. However, one thing is quite clear: in complete contrast to the appearances in the medulla, the antigen is not in lysosomes or similar inclusions. . . . I believe there is a strong possibility that induction of antibody formation is largely a matter of surface interaction between antibody combining sites on reactive lymphoid cells and cell-membrane-associated surface depots of antigens."

This doubt about the position of antigen with respect to the cell membrane was echoed by Schwartz *et al.* (1970) thus:- "The lymph node cortex contains a characteristic, fimbriated reticular cell, whose numerous cytoplasmic processes interdigitate and entwine with similar extensions thrust out by lymphocytes. The result is an extensive dendritic network or web. Antigen is retained on or in these dendritic processes. It has been claimed that the dendritic meshwork/

meshwork retains antigen extra-cellularly - on the plasma membranes of the cytoplasmic villi - but the method used, electron microscopic autoradiography, does not in our opinion, permit such a conclusion. The silver grains seen on the two-dimensional autoradiographs could have emanated from radio-labelled antigen within the cytoplasmic extension of the cell. Another interpretative difficulty arises from the fact that the entangled dendrites of reticular cells and lymphocytes are morphologically similar, making it difficult or impossible to identify the cellular origin of a given regiment of web."

Nossal's idea of depots of antigen reaction with antigen reactive cells is similar to that expressed by White (1967) who considered that "The suggestion is strong that this formation (of germinal centres) occurs by virtue of a mixed agglutination reaction between dendritic cells bearing antigen at their surface and lymphoid cells bearing complementary antibody at their surface. After this initial agglutination reaction the centre begins to enlarge by growth of individual cells and multiplication of the lymphoid cellular elements."

The storage of antigen in follicles has been associated with antibody by purely circumstantial evidence. This was well expressed by Abdou *et al.* (1970) who said "If macrophages perform an essential preliminary rôle by processing the antigen, then the movement of lymphocytes past the relatively sessile macrophages in the follicles would enable contact between these two cell types to take place and facilitate the transfer of information from the macrophage to the immunocompetent/

immunocompetent lymphocyte, thus initiating the sequence of events leading up to antibody formation."

Schwartz (1970) considered that antigen trapped in the follicle prevented the development of tolerance in the case of overloading by antigen. He also suggested that "In still other circumstances, the development of low zone tolerance might be abetted. It is conceivable, for example, that immunologic tolerance of certain auto-antigens, such as thyroglobulin, which are normally present in the circulation in small amounts, is maintained because they are sequestered and catabolised in macrophages."

Balfour and Humphrey (1967) showed that in truly tolerant animals no follicular localization occurs, which would support the idea expressed by Schwartz.

In all this work the reticular cells have been studied in vivo. Little work has been done on the origin of these cells. Everett and Tyler (1967) using H^3 labelled thymidine showed that it was unlikely that the reticular cell was the stem cell of the lymphoid tissue, since animals depleted of lymphoid tissue by radiation did not show labelled blast cells in the regenerating tissue even when the majority of the reticular cells was labelled. My work using agents known to cause blast cell formation in lymphocytes supports this finding.

It is possible that the reticular cells of the follicle are a specialised type of macrophage. Pearsall and Weiser (1970) equate the two cell types in lymphoid tissue and say "Macrophage-cytophilic antibodies/

antibodies are retained for long periods of time on dendritic reticular cells in lymph nodes, where they may promote the immune response by trapping and retaining antigen", and later in their book, - "In the cortex of lymph nodes, trapped antigen is adsorbed to fine dendritic processes of reticular cells, which appear to be non-phagocytic macrophages." The work of Gill and Cole (1965) and Unanue et al., (1968) has shown that ten per cent of some antigens is retained within the cell. Thus it is possible that antigen-retaining macrophages migrate to the follicle and there become reticular cells. This form of "transformation" or "modulation" of one cell into another has been said to occur in inflammation where fibroblasts modulated to macrophages. Rhodin (1967) expressed this idea thus: "It has been claimed that fibroblasts can transform into macrophages in inflammation, but it is most likely that the fibroblast is normally the precursor of the macrophage. The reason for this assumption is based on the situation which exists in lymphoid organs like the spleen, the lymph nodes and the bone marrow. Here, special cells, reticular cells, have the ultrastructure and the function of macrophages. In addition they are the only cells available which can be assumed to produce the collagen fibrils as well as the reticular fibrils of these organs." If this transformation does occur then it should be possible to use the techniques of Huber et al. (1969) to detect an IgG marker on the cells. However, this uses only one parameter to define a cell type and it has long been known that several should be used. Willmer (1965) emphasised that great/

great caution should be exercised in assuming that one cell could transform into another. He said "Only when a single cell can be followed by the cine-camera throughout the whole transformation does the evidence become at all convincing and, even then, it must be supported by other evidence of identification. For example, in the fibroblast macrophage problem the ability of the fibroblast to liberate tropocollagen and to produce collagen fibres, or to liberate hexosamines and uronic acids and produce extracellular mucopolysaccharides, to effect contact inhibition with neighbouring cells, to respond favourably to treatment with embryo juice and unfavourably to doses of sodium arsenite which leave macrophages unscathed, should all be established before the identity of the cell can be assured. Similarly, the development of intracellular acid phosphatase, rapid pinocytosis, phagocytosis and the segregation of vital dyes, especially neutral red and trypan blue, would all tend to establish the identity of a cell as a macrophage, especially if the cell showed an avoiding reaction with respect to other cells of its kind."

The reticular cells studied in this work have been found to have functional and staining characteristics similar to those of the reticular cell <u>in vivo</u> (Lennert, 1961). However, it has not been possible to obtain unequivocal proof that they are identical.

In the discussion above, little has been said about the possible function of antigen retained on the dendritic cell in the development of immunological memory. As mentioned above, it has been shown that macrophages are not necessary for a secondary response (Harris, 1965). Lymphocytes/

Lymphocytes, or at least one of the cells of the small lymphocyte series, is the memory cell (Gowans and Uhr, 1966). This was confirmed by La Via (1969), who thought that the memory cell was formed from a plasma cell after it had released its antibody. He presented electron microscopic evidence that the "bare" plasma cell nucleus could resynthesise cytoplasm and a cytoplasmic membrane. To my mind this is in contradiction to the more probable finding of Cunningham (1969) that memory cells were not of the same line as antibody-producing cells. He also supported the findings by Sercarz and Byers (1967) that memory developed only at the same time as maximum plaque forming cell activity occurred. Maximum antibody-production followed a few days later. Celada (1967) suggested that memory was a result of two lines of differentiation from the one cell which had been stimulated by antigen. Mitchison (1968) suggested that direct access of antigen to lymphocytes resulted in tolerance while antigen which had first been processed by antigen resulted in antibody production.

In view of the more recent work by Shortman *et al.* (1970) that macrophages may not be necessary for all antigens, the follicular localization of such "macrophage independent" antigens assumes new importance since the association of antigen with the membranes of the multibranched reticular cell would provide maximum opportunity for antigen-reactive lymphocytes to come in contact with antigen. This is similar to the idea of "agglutination" as the start of germinal centre formation expressed by White (1967).

The/

The electron microscopic and conventional light microscopic studies on the localization of antigen give at best morphological definition of the type of cell but do not clearly define its functional characteristics. The main characteristics defined by these studies are -

1. The reticular cell is a primitive cell, i.e., it does not contain well-developed cytoplasmic structures (Fresen, 1960; Kajikawa, 1964; Roberts and Latta, 1964).
2. The nucleus usually has at least one nucleolus.
3. The cell has branches with cytoplasmic connections with other cells of the same type.
4. The cell stains lightly by silver impregnation methods but does not take up vital dyes under normal conditions in vivo (Marshall and White, 1950).
5. It is found in intimate contact with small lymphocytes (White, 1967).
6. Experimental work has shown that it retains antigen on its membranes for long periods of time (White, 1963; Nossal, 1964; Szakal and Hanna, 1968).

The reticular cell which has been studied in the experiments reported in this thesis has many of these characteristics. It is a branched cell which showed movements typical of the amoebocyte rather than the mechanocyte class of cell. The nucleus had one or more well-developed nucleoli, the cytoplasm contained mitochondria but few other well-developed organelles. It stained faintly with silver/

silver and took up vital dyes only slowly. It has been shown that lymphocytes will adhere to the processes. The reticular cell has the full complement of degradative enzymes.

This evidence classified the reticular cell as a cell of the reticulo-endothelial system with properties very similar if not identical with those of the antigen-retaining cell *in vivo*.

In addition to characterising this cell *in vitro*, I have reported the following findings:

1. This cell, although it has the full complement of enzymes, did not degrade yeast in the same manner as did the macrophage. Electron microscopic studies showed that yeasts were not ingested into a vacuole but that nuclear or cell debris was. It has previously been shown that virus can exist free in the cytoplasm of HeLa cells (Epstein *et al.*, 1964) and that ferritin need not be segregated into vacuoles (Patterson *et al.*, 1965).
2. The reticular cells were found to be insensitive to gamma-globulin-coated particles in that they were not ingested by the cells. Macrophages avidly ingested gamma-globulin-coated particles.
3. Macrophages with ingested material, e.g., antibody-coated erythrocytes or B.C.G., migrated rapidly towards the reticular cells in culture. After this had happened, the reticular cells were found to contain the material. Whether the reticular cell obtained this material by ingesting the whole macrophage or by the macrophage transferring material across intracellular bridges is not yet known. Light microscopic and cinemicrophotographic studies suggest that transfer/

transfer does occur. Antigenic material once inside the reticular cell could be transferred from cell to cell since cinematographic evidence has shown that cytoplasmic connections between reticular cells are common.

The experimental evidence I have obtained is not conclusive proof that this cell is the dendritic cell described by White and Nossal but suggests very strongly that it is.

APPENDIX

GIEMSA

Fixative - Absolute Methanol

Method

1. Coverslips were washed twice in 0.85% saline and fixed in absolute methanol.
2. Methanol was removed from the tubes and replaced with 1 ml. of Giemsa stain diluted in phosphate buffer pH 7.2. 1/15 dilution for 1 hour or 1/60 dilution overnight.
3. After staining, the coverslips were washed in distilled water.
4. Coverslips were differentiated in 0.25% colophonium in methylated spirit dehydrated through graded alcohols and blotted between sheets of filter paper.
5. Coverslips were cleared in xylol and mounted in D.P.X.

FAT STAIN - SUDAN IV

Fixative - 10% formal saline

Method

1. The coverslips were taken from 10% formal saline, rinsed in distilled water, then in 70% alcohol and placed in staining solution for 20 mins.
2. The coverslips were rinsed twice in 70% alcohol and counter-stained in Mayer's haemalum for 2-3 mins., then 'blued' in lithium carbonate.
3. The coverslips were then washed and mounted in glycerine jelly.

Stock solution

Saturated solution of Sudan IV in absolute alcohol.

Staining solution

Stock solution - 70 ml.

Distilled water - 30 ml.

Add 1 pellet of sodium hydroxide.

The staining solution was shaken well and filtered through a double filter paper immediately before use. A clear red solution was used. A solution which contained precipitate was discarded.

PERIODIC ACID SCHIFF

Fixative - 10% formal saline

Method

1. After fixation, coverslips were rinsed in water and oxidised in 1% aqueous periodic acid - 10 mins.
2. Then they were washed well in water and treated with Schiff reagent for 10-30 mins.
3. The coverslips were transferred directly to three sulphite rinses of 2 mins. each and then washed for 10 mins. in running tap water.
4. The preparations were counterstained with Lillie's haematoxylin, 'blued' in lithium carbonate, dehydrated, cleared and mounted in D.P.X.

Staining solution

de Thomasi Schiff reagent

1. 1 g. of basic fuschin was dissolved in 200 ml. of boiling distilled water and then shaken for 5 minutes.
2. The solution was cooled to 50°C and 20 ml. N/1 hydrochloric acid added.
3. After further cooling to 25°C, 1 g. sodium metabisulphate was added.
4. The solution was then stored for 18-24 hours in the dark, after which time 2 g. of activated charcoal were added.
5. The reagent was filtered to remove the charcoal and stored in the dark at 0-4°C.

KURNICK'S MODIFICATION OF METHYL GREEN-PYRONIN STAIN (KURNICK 1955)

Fixative - 10% formal saline

Method

1. Coverslips were immersed in dye mixture for 45 minutes.
2. Coverslips were blotted dry between filter paper and immersed in two changes of n-butyl alcohol. 5 minutes each.
3. Cleared in xylol 5 minutes, and mounted in D.P.X.

   Result D.N.A. - green.

Staining solution

25% pyronin Y (chloroform extracted)	-	12.5 ml.
2% methyl green (chloroform extracted)	-	7.5 ml.
Distilled water	-	30 ml.

LENDRUM'S DI-ARGENTINE CARBONATE FOR RETICULIN FIBRES

Fixative - 10% formal saline

Method

1. The coverslips were rinsed in water and treated with 30% aqueous phosphotungstic acid for 5 minutes.
2. The coverslips were washed in water and rinsed in 95% alcohol.
3. The coverslips were treated with tannic acid sensitiser for 2 minutes and rinsed in distilled water.
4. The coverslips were placed in di-argentine carbonate for 5 minutes and then washed in distilled water.
5. The coverslips were treated with formalin reducer for 30 seconds and then with 5% hypo for 30 seconds.
6. The coverslips were washed in water and toned in gold chloride.
7. The coverslips were washed in water and treated with 5% hypo for 60 seconds.
8. The nuclei were stained with Scarba red, dehydrated, cleared and mounted.

Result

Reticulin fibres - black

Nuclei - red

STAINING SOLUTIONS

Tannic Acid Sensitiser

Tannic acid - 1 g.
Acetic acid - 2 ml.
Absolute alcohol - 100 ml.

Formalin Reducer

Formalin - 10 ml.
Distilled water - 90 ml.
Di-argentine carbonate - 1 ml.

Gold chloride toner

Gold chloride - 2 g.
Distilled water - 100 ml.

Di-argentine carbonate

5 ml. 10% silver nitrate and 5 ml. saturated aqueous lithium carbonate were pipetted into a 200 ml. conical flask and mixed thoroughly. The precipitate was washed by adding 50 ml. distilled water. The precipitate was allowed to settle and the supernatant was decanted. The washing was repeated twice. 10 ml. distilled water was added to the precipitate and dissolved by adding ammonia drop by drop. The flask was left uncovered for several hours to allow the ammonia to evaporate. 10 ml. 10% silver nitrate was pipetted into the flask, mixed, washed, suspended and dissolved as before. An excess of ammonia was added by allowing a little precipitate to remain undissolved. The final volume was made up to 50 ml. with distilled water.

WEIL - DAVENPORT

Fixative - 10% formal saline

Method

1. After fixation, the coverslips were rinsed in distilled water then placed in silver solution for 3-4 mins.
2. The coverslips were then transferred directly to 15% formalin until they were slightly brown in colour.
3. The preparation was then toned in 0.2% gold chloride by dipping it into the solution approximately 5-6 times.
4. The coverslips were then dehydrated, cleared and mounted in D.P.X.

Silver solution

To 2-3 ml. of strong ammonia in a flask, add 10% silver nitrate drop by drop until 16-18 ml. have been added. The final solution should be slightly opalescent. Dilute solution to 40 ml. with distilled water.

HEIDENHAIN'S IRON HAEMATOXYLIN

Fixative - Palade

(1% osmium tetroxide in veronal-acetate buffer pH 7.3)

Method

1. Coverslips were washed twice with saline and transferred to bijoux bottles containing Palade fixative at 4°C for 2 hours.
2. Coverslips were washed well in distilled water and placed in 5% iron alum for 1-2 hours.
3. Coverslips were rinsed rapidly in water and transferred to the haematoxylin solution for 1-2 hours.
4. Coverslips were rinsed in water before differentiating in 2% iron alum.
5. Coverslips were washed in running water for 5 minutes to remove iron alum and then dehydrated, cleared and mounted.

Staining solutions required

Iron alum solution

Ferric ammonium sulphate - 5 g.

Distilled water - 100 ml.

Haematoxylin solution

Haematoxylin - 0.5 g.

Absolute alcohol - 10 ml.

Distilled water - 90 ml.

Solution must be kept for 4-5 weeks to ripen.

THE CALCIUM-COBALT METHOD FOR ALKALINE PHOSPHATASE

(Gomori, 1952)

Method

1. The coverslips were rinsed twice in 0.85% saline warmed to 37°C.
2. The coverslips were incubated for 2 hours at 37°C in the following medium:

3% sodium β-glycerophosphate	-	10 ml.
2% sodium diethyl barbiturate	-	10 ml.
distilled water	-	5 ml.
2% calcium chloride	-	20 ml.
5% magnesium sulphate	-	1 ml.

3. The coverslips were rinsed in water and then treated with 2% cobalt nitrate for 3-5 minutes.
4. The coverslips were rinsed in distilled water and then treated with 1% aqueous ammonium sulphide for 1-2 minutes.
5. The coverslips were washed in water, dehydrated in alcohol and cleared in xylol, and then mounted in D.P.X.

Result

Alkaline phosphatase activity - brownish black or black.

STANDARD COUPLING AZO DYE TECHNIQUE FOR ACID PHOSPHATASE

(Pearse 2nd edition)

Method

1. Coverslips were rinsed twice in 0.85% saline.
2. The monolayers were incubated at 37°C for 30 minutes in the following medium: 10 mg. of sodium α-naphthyl phosphate was dissolved in 10 ml. of 0.1M acetate buffer (Walpole). 10 ml. of Fast Garnet G.B.C. salt was added. The mixture was shaken well and filtered.
3. The coverslips were washed in water for 2 minutes.
4. The coverslips were counterstained in Mayer's haemalum, washed in water and mounted in glycerine jelly.

Result

Sites of acid phosphatase activity appeared - reddish brown.

Nuclei - blue.

A MODIFIED COUPLING AZO DYE METHOD FOR ALKALINE PHOSPHATASE

(Pearse 2nd edition, 1960)

Method

1. The coverslips were rinsed twice in 0.85% saline warmed to 37°C.
2. The coverslips were incubated at 17-22°C for 1 hour in the following medium: 20 mg. sodium α-naphthyl was dissolved in 20 ml. 0.1M stock tris buffer pH 10.4. 20 mg. Fast Red TR was added and the mixture was filtered before use.
3. The coverslips were washed in water and the nuclei were counterstained in Mayer's haemalum for 1-2 mins.
4. The coverslips were washed well in water and mounted in glycerine jelly.

Result

Sites of activity - brown

Nuclei - blue

TWEEN METHOD FOR LIPASE

(Modification of Richterich 1952)

Method

1. The coverslips were rinsed twice in 0.85% saline warmed to 37°C.
2. The monolayers were incubated for 6 hours at 37°C.

   The incubation medium was prepared as follows: to 68 ml. boiled, distilled water, was added -

10% calcium chloride	-	7 ml.
0.5 M tris buffer pH 7.4	-	10 ml.
5% aqueous solution of Tween 60	-	15 ml.

   The pH of the incubating medium was adjusted to between pH 7 and 8.
3. The coverslips were rinsed in distilled water.
4. The coverslips were treated with 2% lead nitrate for 10 minutes and then were washed in three rinses of distilled water.
5. The coverslips were placed in 1% ammonium sulphide for 3-5 minutes and then washed in water.
6. The coverslips were mounted in glycerine jelly.

Result

Sites of activity appeared brownish-black.

ESTERASE - THE NAPHTHOL A S ACETATE METHOD

(Gomori, 1952)

Method

1. The coverslips were rinsed twice in saline warmed to 37°C.
2. The coverslips were incubated at 17-20°C for 30 minutes in the following medium:

   1% naphthol AS acetate in acetone - 0.1 ml.

   M/20 phosphate buffer pH 7.0 - 10 ml.

   5-chloro-toluidine - 10 mg.
3. The monolayers were rinsed in distilled water and the nuclei stained in Mayer's haemalum.
4. The coverslips were rinsed and mounted in glycerine jelly.

Result

Sites of activity - red

Nuclei - blue

β-GLUCURONIDASE

(Pearse 2nd edition, 1960)

Method

1. The coverslips were rinsed twice in 0.85% saline warmed to 37°C.
2. The coverslips were incubated for 5 hours at 37°C in the following medium:

   30 mg. 6-bromo-2-naphthyl-β-D-glucopyraronoside (glucuronide) was dissolved in 5 ml. absolute methanol. To this was added 20 ml. phosphate-citrate buffer pH 4.95 and 75 ml. distilled water.
3. The coverslips were washed in water for 1 minute and placed in freshly prepared solution of Fast Blue B (1 mg/ml in 0.02 M phosphate buffer pH 7.5-7.8) for 3-5 minutes.
4. The coverslips were rinsed in distilled water and mounted in glycerine jelly.

Result

Sites of activity indicated by reddish precipitate.

METHOD FOR LEUCINE-AMINOPEPTIDASE

(Nachlas, Crawford and Seligman, 1957)

Method

1. The coverslips were rinsed twice in 0.85% saline warmed to 37°C.
2. The coverslips were incubated at 37°C for 1¾ hours in the following medium:

8 mg/ml L-leucyl-β-naphthylamide	- 0.5 ml.
0.1 M acetate buffer (Walpole) pH 6.5	- 5.0 ml.
0.85% sodium chloride	- 4.0 ml.
0.13% potassium cyanide	- 0.5 ml.
Fast Blue B	- 5.0 mg.

   The mixture was filtered before use.
3. The coverslips were rinsed in 0.85% saline and treated with 0.1 M copper sulphate for 2 minutes.
4. The coverslips were rinsed in two changes of 0.85% saline and mounted in glycerine jelly.

Result

Sites of activity indicated by a purplish colour.

METHODS FOR LACTATE AND MALATE DEHYDROGENASES

(Hess, Scarpelli and Pearse, 1958)

Method

1. The coverslips were rinsed twice in 0.85% saline warmed to 37°C.
2. The coverslips were incubated for 1½ hours at 37°C in the following medium:

1.0 M sodium DL-lactate		
or		
1.0 M sodium L-malate	-	0.1 ml.
0.1 M diphosphopyridine nucleotide (D.P.N.)	-	0.1 ml.
0.1 M sodium cyanide	-	0.1 ml.
0.05 M magnesium chloride	-	0.1 ml.
0.06 M phosphate buffer pH 7.5	-	0.25 ml.
1 mg/ml nitro-BT	-	0.25 ml.
distilled water	-	0.1 ml.
polyvinyl pyrrolidine (M.W. 11,000)	-	75 mg.

   The final pH was adjusted to 7.4 with 0.2 M tris buffer pH 10.4
3. The coverslips were rinsed in 0.85% saline and fixed in 10% formal saline - 10 minutes.
4. The coverslips were rinsed in distilled water and mounted in glycerine jelly containing 0.5 M cobaltous acid.

Result

Sites of activity indicated by blue formazan.

DIRECT FLUORESCENT ANTIBODY TECHNIQUE

(to detect Cl. septicum)

Fixative - 90% ethanol

Method

1. The lyophilised fluorescent antibody was made up in 1 ml. distilled water. The antiserum was diluted to 1 in 20 with phosphate buffered saline (PBS) pH 7.1, and was adsorbed for 2 hours at room temperature with rat liver powder. (This is made up as described by Nairn, 1969). The mixture was centrifuged to remove the liver powder and with it the non-specific fluorescent material.
2. The coverslips were rinsed in saline, fixed for 90 seconds and air dried.
3. The coverslips were flooded with the adsorbed antibody and then incubated for 30 minutes at 37°C in a humid atmosphere. The coverslips were washed in PBS for 15 minutes at 37°C again in a humid atmosphere. The washing was repeated twice more. The coverslips were mounted in buffered glycerine (9 pt. glycerol, 1 pt. PBS) and ringed with clear nail varnish before they were examined.

Nairn, R.C., 1969. Fluorescent protein tracing 3rd ed. Pub. E. and S. Livingstone, p.304. Adsorption with tissue preparations.

INDIRECT FLUORESCENT OR SANDWICH TECHNIQUE

(to detect yeast antigens within cells)

Fixative - 90% ethanol

Method

A potent anti-yeast serum was prepared by repeated injection of an autoclaved 1% suspension of yeast into rabbits. The rabbits were bled, the serum separated and then the immunoglobulins were salted out using ammonium sulphate.

The fluorescent anti-rabbit γ-globulin was prepared in goats (Hoechst Pharmaceuticals Ltd.), and supplied as a lyophilised powder. The antiserum was made up and prepared in the same way as described in the method for direct tracing.

The coverslips were rinsed in saline, fixed for 90 seconds and air dried.

The coverslips were covered in the rabbit anti-yeast serum, incubated for 30 minutes at 37°C in a humid atmosphere then washed in PBS for 15 minutes at 37°C in a humid atmosphere. The washing was repeated twice more. The coverslips were flooded with the fluorescent anti-rabbit serum, incubated and washed as before.

After the last wash they were mounted in buffered glycerol (9 pt. glycerol, 1 pt. PBS), ringed with clear nail varnish and examined.

To control the specificity of this reaction, one set of coverslips was incubated in fluorescent antiserum without the anti-yeast serum.

Nairn, R.C. Fluorescent Protein Tracing, 3rd ed., 1969.
Pub. E. and S. Livingstone, p.303. Obtaining γ globulin fraction from serum.

AUTORADIOGRAPHY

The cultures of human peritoneal cells and guinea-pig spleen cells treated with radio-labelled complex, were fixed in 10% formal saline. Blocks were processed routinely, sections were cut, the wax removed, and then they were taken back to water.

Method

The coverslips and spleen dabs were rinsed in 0.85% NaCl. to remove the fixative. The sections were taken straight from water. Ilford L4 emulsion was prepared by melting the emulsion and adding an equal volume of distilled water. The mixture was left at 40°C in a water bath. The coverslips were dipped in the emulsion at least four times, put on a slide, cell side up, and placed in a rack. The rack was put inside a light-proof box and a stream of warm air blown in (from a hair dryer) to dry the slides. The dried slides were wrapped in black polythene and put into a container which was sealed. The coverslips were exposed for 3-4 weeks at 4°C, developed in 1D11 or 1D36 diluted 1 in 4 for 8-10 minutes, washed in water, fixed in Ilfofix (1 in 5) for 10-15 minutes, washed again, then stained with Mayer's haemalum, dehydrated, cleared and mounted in D.P.X.

FIXATION, DEHYDRATION AND EMBEDDING SCHEDULE FOR MATERIAL EXAMINED WITH THE ELECTRON MICROSCOPE

Fixative - 3% gluteraldehyde in phosphate buffer pH 7.2

Method

The material (cultures, suspensions and tissue blocks) was fixed for between 4 and 24 hours then washed three times (30 mins. per wash) in phosphate buffer. Post fixation was done in 1% osmium tetroxide in phosphate buffer for 2 hours. The specimens were then dehydrated according to the following schedule:

3 x 15 mins. in 10% alcohol

1 x 20 mins. in 50% alcohol

3 x 30 mins. in absolute alcohol

2 x 20 mins. in propylene oxide (1:2 epoxypropane)

After this, the specimens were impregnated overnight with embedding araldite, and then embedded in fresh embedding araldite which was polymerised at 56°C for 48-72 hours.

Phosphate buffer

Solution A - 1/15 M KH_2PO_4

Solution B - 1/15 M Na_2HPO_4, $12H_2O$

Buffer solution pH 7.2 mix 28.5 ml. Solution A and 71.5 ml. Solution B.

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TABLE I.

HISTOCHEMISTRY

ENZYME	2 days	5 days	7 days
Acid phosphatase	+	++	++
Esterase	+	+	+
Leucine amino-peptidase	+	±	+
Succinic dehydro-genase	+	+	+ (weak)
Lactic dehydrogenase	+	+	++
Malic dehydrogenase	+	+	+
Beta glucoronidase	-	+ (reticular cells only)	-
Lipase	+	+	+

All controls were negative.

Alkaline phosphatase was negative in all cultures by all methods.

FITE FARACO MODIFICATION OF ZIEHL-NEILSON STAIN

Fixative - 10% formal saline

Method

1. The coverslips were left in a mixture of equal parts of xylol and corn oil for 10 minutes, blotted dry and then washed.
2. The coverslips were then stained in carbol fuchsin at room temperature for 25 minutes then washed in water.
3. They were then decolourised in 10% sulphuric acid, the nuclei stained with haematoxylin and 'blued' in lithium carbonate.
4. The coverslips were then blotted dry, cleared in xylol and mounted in DePex.

Results

Bacilli - bright red

Nuclei - blue