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The fate of phytohaemagglutinin-activated human lymphocytes following their peak proliferative activity

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INTRODUCTION

Following exposure to phytohaemagglutinin, lymphocytes in culture undergo a burst of proliferative activity lasting from the second to about the sixth day, after which mitosis ceases. The morphological changes accompanying lymphocyte activation and proliferation are well documented (Inman & Cooper, 1963; Douglas, Hoffman, Borjeson & Chessin, 1967; Chapman, Gough & Elves, 1967; Tokuyasu, Madden & Zeldis, 1968) but very little information is available about the subsequent fate of the cells and it is usually stated that after proliferation ceases the cells show indications of degeneration and eventually die (Bessis, 1973). However, the authors have observed that different cell types become evident in the later stages of cultures and several apparently healthy cells persist even after 17 days.

The purpose of this study is to investigate the fine structure of the different cells which appear in the later stages of phytohaemagglutinin-stimulated lymphocyte cultures. This could shed light on the fate of activated lymphocytes after the phase of proliferation has ceased.

MATERIALS AND METHODS

Human lymphocytes were separated on Ficoll-hypaque density gradient from the peripheral blood of five normal, healthy donors and cultured in RPMI 1640 medium †(Gibco) supplemented with fetal calf serum (15%), phytohaemagglutinin M (Gibco: 0·15ml per 10 ml medium), penicillin (100 u/ml) and streptomycin (100 μg/ml). The initial cell concentrations were adjusted to about $1{\cdot}75 \times 10^6$ cells/ml. The medium was changed every three or four days by centrifugation, removal of the supernatant with a volumetric pipette and replacement with an equal volume of fresh medium. For each specimen of blood, the following procedures were performed on the cultures before incubation and at intervals between 1 and 17 days.

(*a*) Cell counts were performed using a Neubauer counting chamber.

(*b*) For light microscopy, cytocentrifuge smears were stained with Giemsa stain.

(*c*) For acid phosphatase (light microscopy) cytocentrifuge smears were incubated, either unfixed or after fixation in buffered formalin, in a medium containing naphthol AS–B1 phosphate and hexazotised pararosanilin in acetate buffer at pH 5·0

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† Trademark.

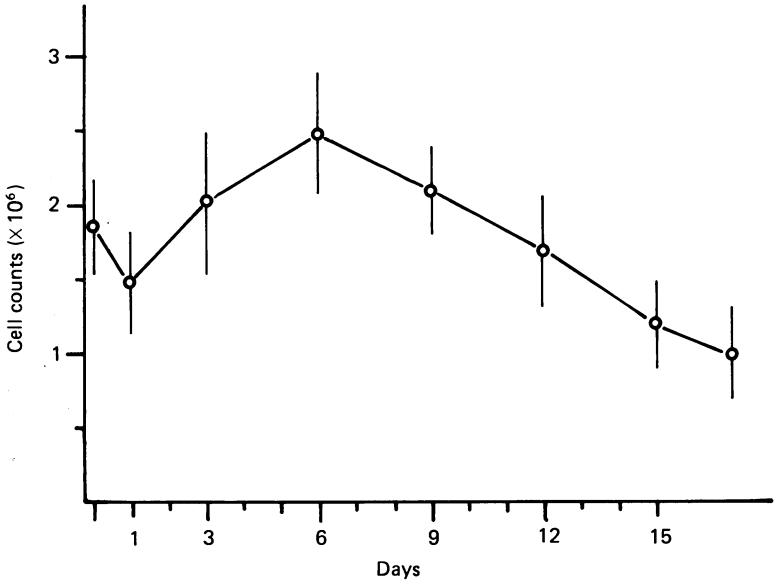


Fig. 1. Cell counts of phytohaemagglutinin-stimulated lymphocyte cultures at various intervals. The values represent the averages of five experiments. In each case the initial count was adjusted to about $1{\cdot}75 \times 10^6$ cells/ml.

(Goldberg & Barka, 1962) for 1 hour at 37 °C. Controls were incubated in medium containing 10^{-4} M sodium fluoride or 0·045 M tartaric acid as inhibitors.

(*d*) For electron microscopy, the cells were fixed in 1·5 % glutaraldehyde in 0·06 M phosphate buffer, pH 7·2, at room temperature for 1 hour, postfixed in 1 % osmium tetroxide, dehydrated in graded ethanol solutions and embedded in Araldite. Ultrathin sections were cut on an LKB ultratome III, stained in uranyl acetate and lead citrate and viewed in a Jeol 100CX electron microscope.

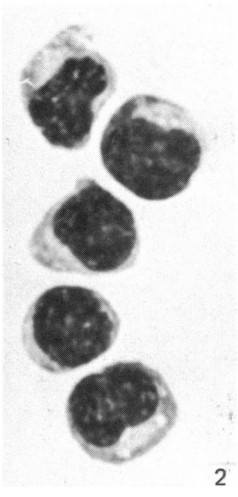
(*e*) For acid phosphatase electron histochemistry, cells were fixed for 30 minutes in 2 % paraformaldehyde and 0·5 % glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, at room temperature. The cells were incubated for 30 minutes at 37 °C in a β-glycerophosphate and lead nitrate medium (Barka & Anderson, 1963), postfixed in osmium tetroxide and processed for electron microscopy as stated above. The sections were viewed without counterstain.

Figs. 2–4. Giemsa-stained cytocentrifuge smears. × 1300. Fig. 2. Unstimulated lymphocytes. Fig. 3. Activated lymphocyte from 3 day culture. Fig. 4. Phagocytic cell (centre) and two activated lymphocytes from a 3 day culture.

Figs. 5–7. Acid phosphatase preparations. × 1300. Fig. 5. Unstimulated lymphocytes. Fig. 6. Activated lymphocytes from 3 day cultures; the cell on the left shows much greater activity than the two cells on the right. Fig. 7. Phagocytic cell from a 3 day culture; note the activity between the vacuoles.

Fig. 8. Cluster of cells from a 6 day culture showing cellular heterogeneity. Note one cell (open arrow) with small cytoplasmic vesicles and another cell (closed arrow) with abundant non-basophilic cytoplasm. × 1200.

Fig. 9. Giant cell from a 12 day phytohaemagglutinin culture showing multiple nuclei or nuclear lobes joined by thin strands of chromatin material and several small azurophilic granules in the cyroplasm. This cell is surrounded by small lymphocytes. × 1300.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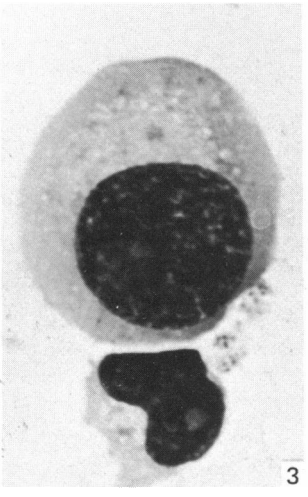
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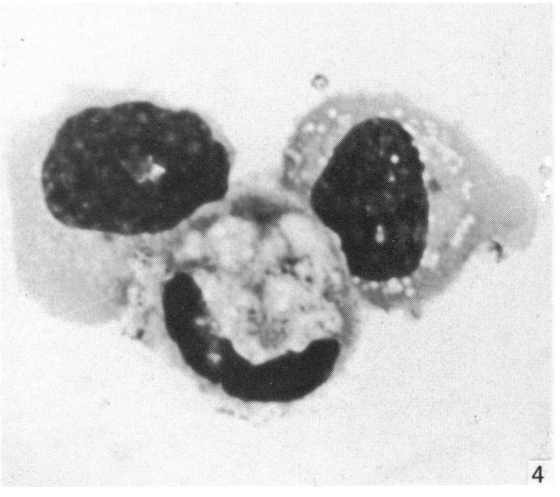
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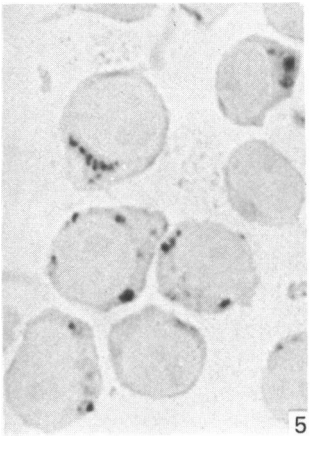
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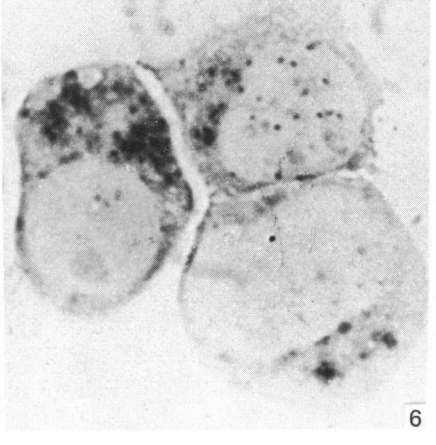
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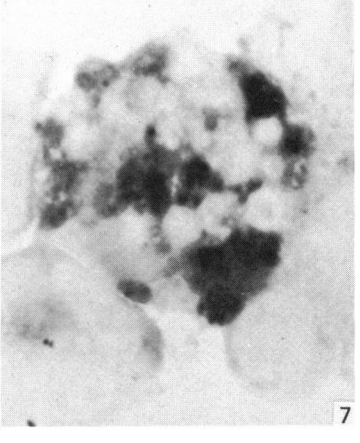
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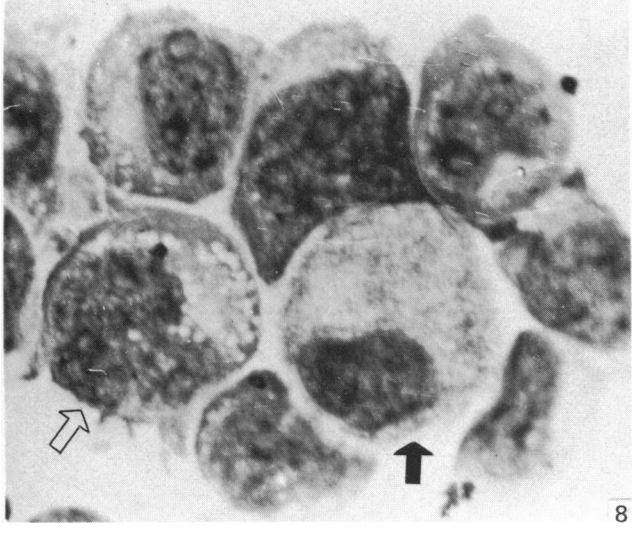
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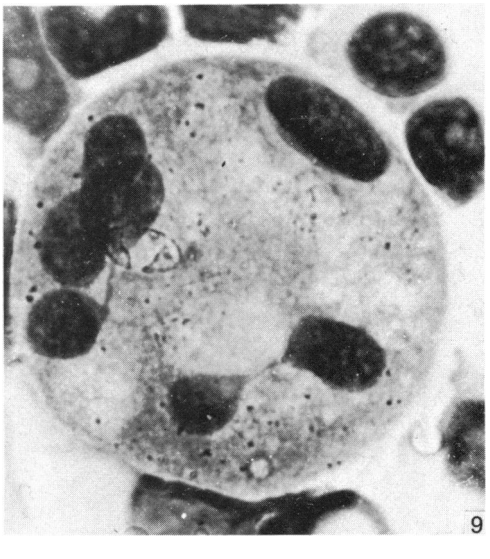
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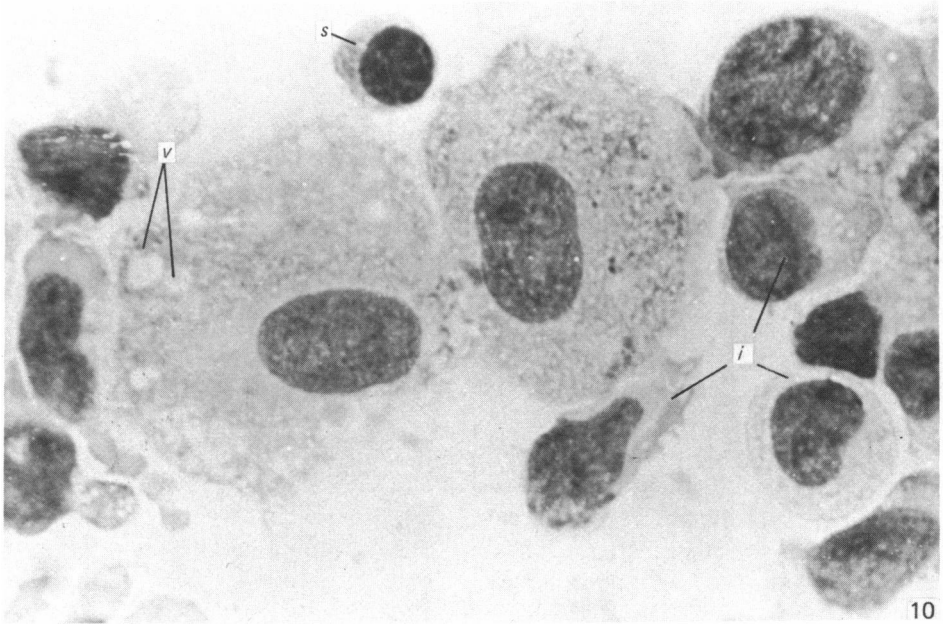


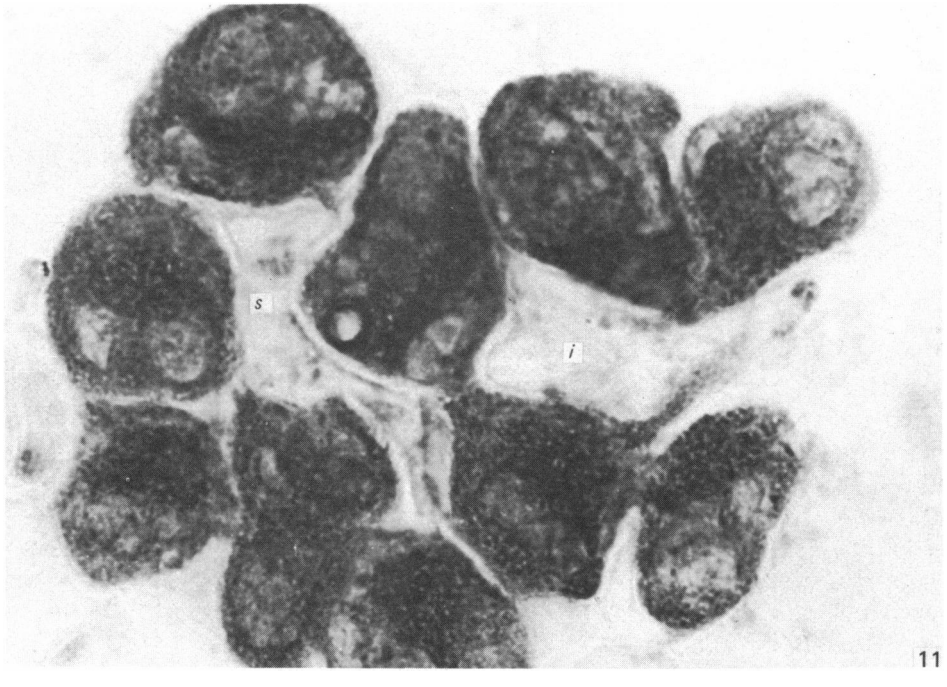


Fig. 10. Nine day culture showing two large cells containing eccentric nuclei, abundant finely granular cytoplasm and a few vacuoles (*v*) in one of the cells. A small lymphocyte (*s*) and some intermediate cells (*i*) are also shown. × 1300.

Fig. 11. Nine day culture showing intense acid phosphatase activity in the large cells and very weak activity in the intermediate (*i*) and small (*s*) lymphocytes. × 1300.

RESULTS

Light microscopy

The averages of the cell counts at different stages of culture are given in Figure 1.

The initial cell population consisted of 85–90 % lymphocytes (Fig. 2) and 10–15 % monocytes. At 24 hours, many of the lymphocytes showed morphological changes and at 3 days most of them were activated (Fig. 3). They measured 20 to 30 μm in diameter and had a large open faced nucleus with two to six nucleoli and a basophilic cytoplasm containing a perinuclear area of pale staining, foamy cytoplasm and a variable number of clear vesicles. Mitotic figures were frequent. At 3 days, 5–10 % of the cells were small, apparently unstimulated lymphocytes; 8–14 % of the cells were phagocytic cells containing large vacuoles of engulfed material (Fig. 4) and less than 1 % were typical monocytes. In 6 day cultures (Fig. 8), the cells were generally similar to activated lymphocytes but contained numerous cytoplasmic vesicles or abundant, non-basophilic, finely granular cytoplasm and an eccentric nucleus.

In 9 to 15 day cultures, the cells showed great variations in size and structure. Large cells (Fig. 10) with a diameter of 25–40 μm had abundant, non-basophilic, finely granular cytoplasm containing scattered azurophilic granules, occasional phagocytic vacuoles and an indented, eccentric nucleus with one or two nucleoli. A few giant cells contained multiple nuclei or nuclear lobes connected by thin strands (Fig. 9). The small cells were structurally similar to unstimulated lymphocytes, while many cells were intermediate in size and structure between activated and small lymphocytes (Fig. 10). In addition, phagocytic cells similar to those observed earlier were still present. The wide range of variation and overlap in the size and structure of the various cells precluded the performance of meaningful differential counts in Giemsa-stained preparations.

At 15 and 17 days, large cells were less numerous than in earlier stages and many of them showed evidence of disruption. The proportion of small cells was relatively greater, and at 17 days most of the cells were structurally similar to normal unstimulated lymphocytes, with only an occasional large cell and abundant cellular debris.

Acid phosphatase using light microscopy

Uncultured lymphocytes and monocytes showed weak granular activity (Fig. 5). At 1 and 3 days, the activity was slightly greater in most of the activated lymphocytes and markedly greater in a few of them (Fig. 6). Strong activity was also present in the phagocytic cells (Fig. 7).

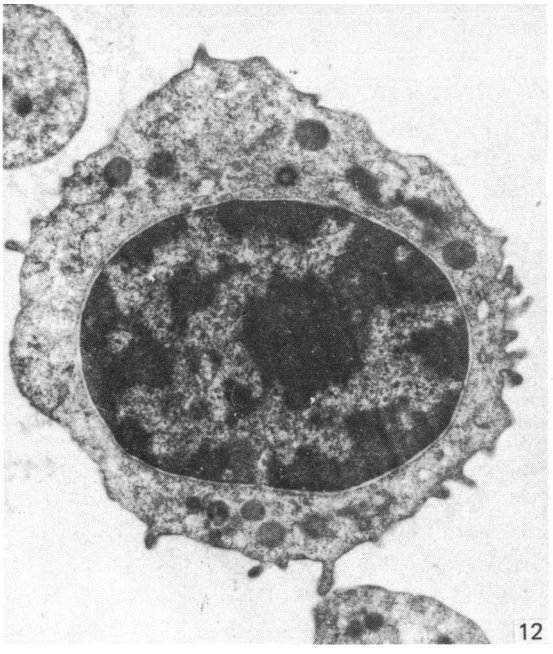
At 6 to 15 days, the large cells showed intense granular activity occupying the whole of the cytoplasm (Fig. 11). Intense activity was also present in the phagocytic cells, but it was often difficult to distinguish between the two cell types because of the intensity of the reaction. In contrast, small and medium sized lymphocytes showed weak activity comparable to that present in unstimulated lymphocytes.

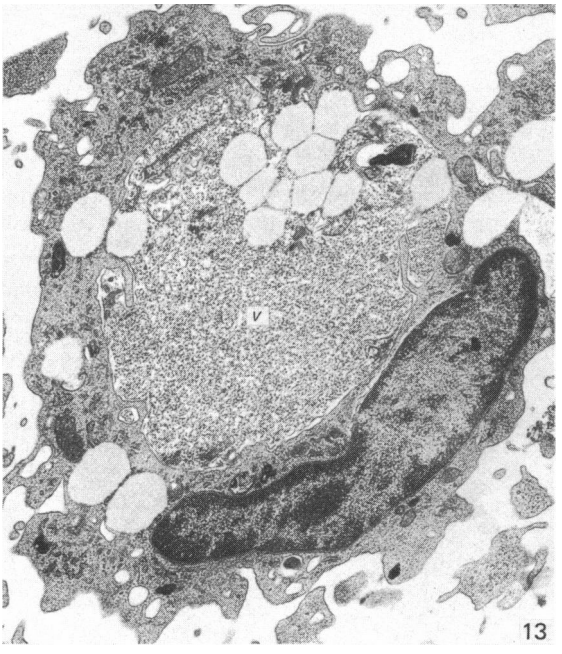
The cells with intense activity, including the phagocytic cells, formed 25–30 % of the total cells at 6 days, 20–30 % of the cells at 9 days and 10–20 % of the cells at 12 days. In later cultures, such cells were infrequent while small lymphocytes with weak activity predominated.

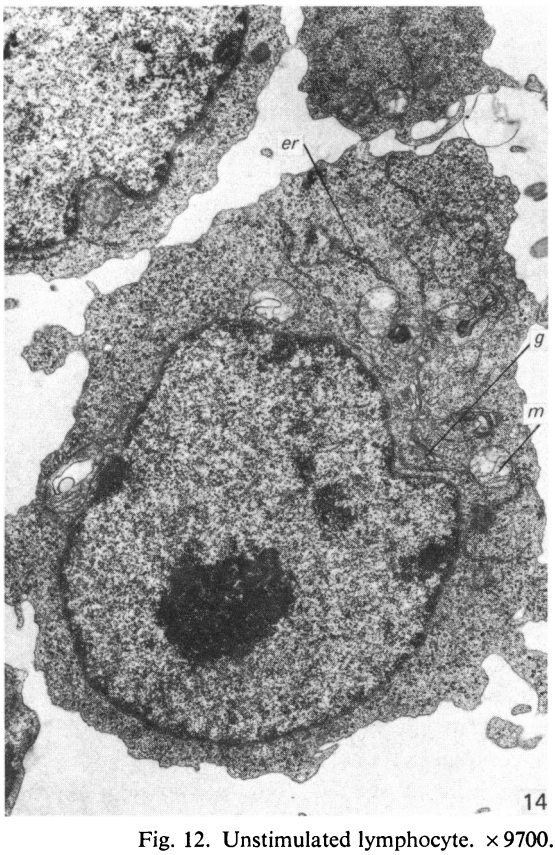
At all stages the acid phosphatase reaction was completely inhibited by sodium fluoride or sodium tartrate.

Electron microscopy

A typical uncultured lymphocyte from the initial cell population is shown in Figure 12.







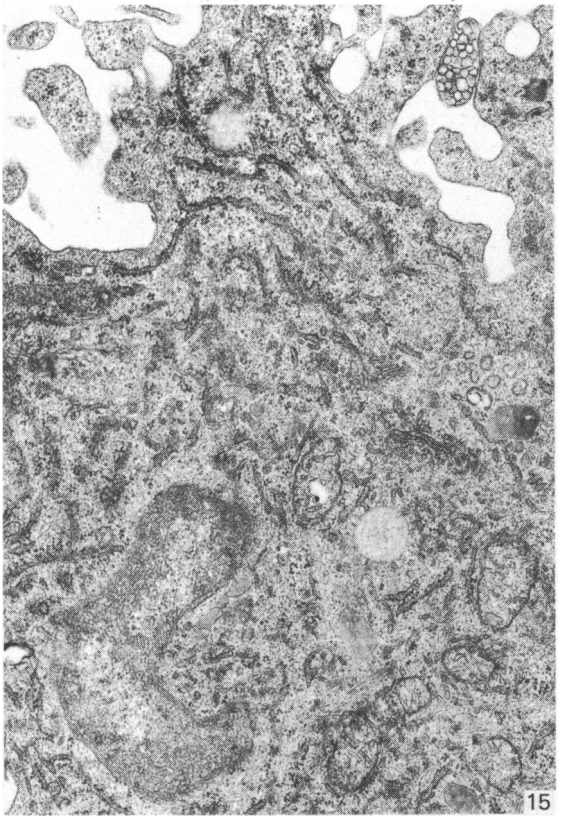


Fig. 12. Unstimulated lymphocyte. ×9700.

Fig. 13. Phagocytic cell from a 1 day culture. Note the irregular cell outline, flattened nucleus with condensed chromatin and a large phagocytic vacuole (*v*). ×8000.

Fig. 14. Activated lymphocyte from a 3 day culture showing a large euchromatic nucleus with nucleolus, a few profiles of endoplasmic reticulum (*er*), Golgi apparatus (*g*) and mitochondria (*m*). ×8300.

Fig. 15. Activated lymphocyte from a 3 day culture, containing abundant endoplasmic reticulum. ×16500.

Activated lymphocytes in 3 day cultures (Fig. 14), had a smooth outline with few short projecting microvilli. The nucleus was euchromatic and showed prominent nucleoli. The cytoplasm contained a well developed Golgi apparatus, moderate amounts of endoplasmic reticulum, mitochondria, free ribosomes and pinocytotic vesicles. Lysosomes were sparse in most cells but were quite numerous in a few cells. Occasional cells contained extensive endoplasmic reticulum (Fig. 15).

Phagocytic cells, present in all stages of culture, had several broad surface projections and contained large phagocytic vacuoles, occupying most of the cytoplasm (Fig. 13). The irregular nucleus had peripheral clumps of heterochromatin. The cytoplasm, around and between the phagocytic vacuoles, contained numerous lysosomes, residual bodies with heterogeneous contents and a few lipid bodies.

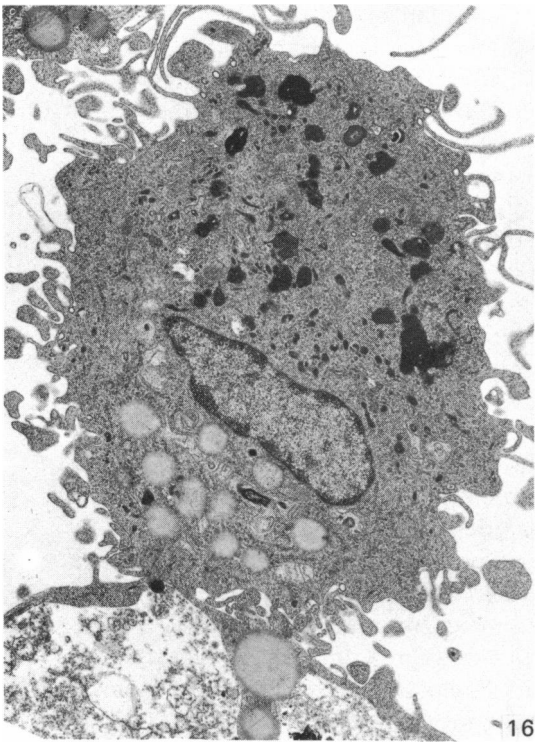
The large cells in 6 to 15 day cultures (Figs. 16, 20) had a very irregular outline with numerous broad cytoplasmic projections and long slender microvilli. The nucleus was deeply indented, mostly euchromatic with small clumps of peripheral heterochromatin and one or more nucleoli. The abundant cytoplasm to one side of the eccentric nucleus contained numerous, heterogeneous, electron-dense bodies. They varied from small oval or elongated structures with finely granular contents to large, irregular bodies composed of discrete, uniform, closely packed granules arranged in regular geometric arrays of parallel lamellae (Fig. 21). The large and small dense bodies were frequently in continuity with one another. A few lysosomes with membranous contents or myelin figures were also present. The Golgi apparatus was well developed and usually centrally placed among the dense bodies. At the pole of the cell close to the nucleus there were clusters of spherical bodies with homogeneous pale centres and slightly denser rims (Fig. 17). Microfibrils, numerous free ribosomes and pinocytotic vesicles were regularly present, but mitochondria and endoplasmic reticulum were sparse. Small phagocytic vacuoles were rarely observed (Fig. 17).

Intermediate sized cells in 6 to 12 day cultures generally had a smooth outline with occasional microvilli, a round euchromatic nucleus with nucleoli and a paucity of organelles. Apart from free ribosomes, the cytoplasm was quite featureless (Fig. 19) and contained a few mitochondria, sparse endoplasmic reticulum and occasional lipid bodies (Fig. 22). A remarkable feature of these cells was the presence of cell contacts (Fig. 23) in which the apposed plasma membranes were thickened and dense. Very occasionally, cells with fairly abundant endoplasmic reticulum and a well developed Golgi apparatus were observed.

Small cells with condensed nuclear chromatin and scanty cytoplasm (Fig. 19) were present with increasing frequency in older cultures. They were generally similar to normal lymphocytes but some contained numerous mitochondria (Fig. 18).

Ultrastructural localisation of acid phosphatase

The reaction product was localised within the lysosomes and sometimes in the Golgi region. Acid phosphatase-containing lysosomes were few in activated lymphocytes at 3 days (0–3 per cell profile). They were numerous in phagocytic cells where the lysosomes were clustered together in the cytoplasm between the phagocytic vacuoles (Fig. 24) and in the large epithelioid cells at 6 to 12 days, where they were scattered throughout the cytoplasm (10 to 25 per cell profile) (Fig. 25).



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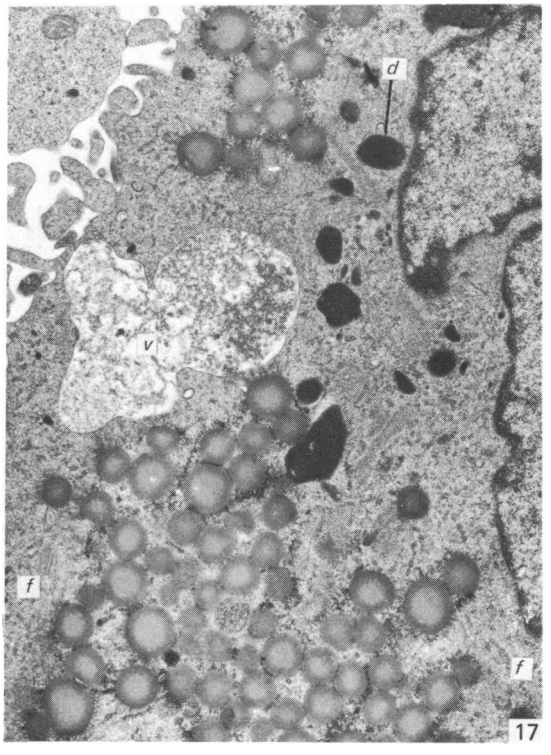

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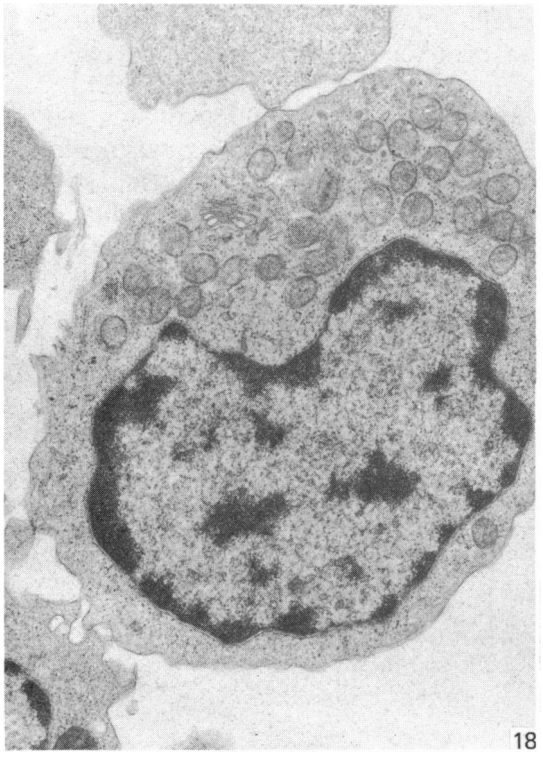

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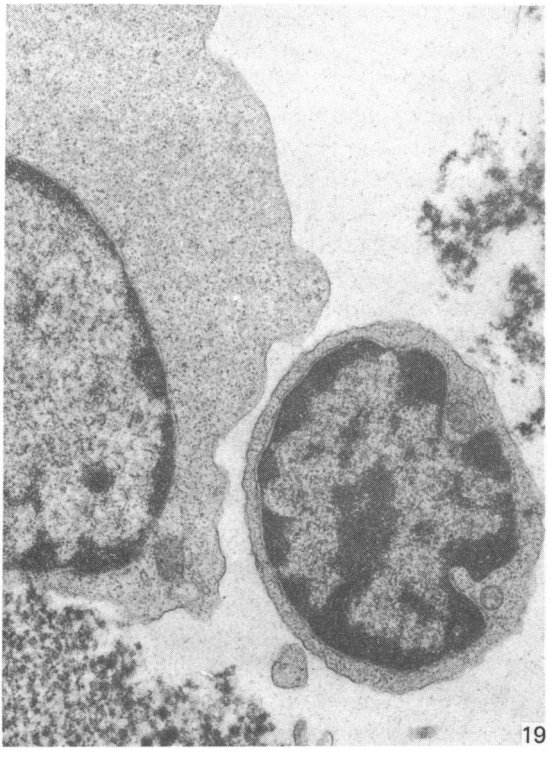

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DISCUSSION

During the period of proliferation, phytohaemagglutinin-activated lymphocytes show rather small morphological variations, so that classification of cell types is indistinct and arbitrary. It is also difficult to interpret to what extent the morphological heterogeneity reflects varying degrees of activation of the cells, their position in the cell cycle or the functional capabilities or commitments of the lymphocytes from which they arise. The present results indicate that in the later stages of culture, after proliferation has ceased, distinct cell types are recognisable.

The large cells which develop in the later stages of culture have morphological characteristics highly suggestive of epithelioid cells in which phagocytosis is very infrequent. This is in contrast to the actively phagocytic cells which are present throughout the culture period and are presumably derived from monocytes. Epithelioid cells may be derived from macrophages which are no longer phagocytic and have become secretory (Papadimitriou & Spector, 1971). However, the monocytes present in the initial cell inoculum could not numerically account for all the large cells with intense acid phosphatase activity unless it is postulated that they have proliferated. The monocytes form 10–15% of the initial cell inoculum, whereas the cells with intense acid phosphatase activity form 25–30% of the cells at 6 days. Taking into account the increase in total cell number between 0 and 6 days, the absolute number of these cells at 6 days is 2·7–3·3 times the absolute number of monocytes initially present.

Monocytes may sometimes be difficult to distinguish from lymphocytes, so that the number of monocytes in the initial cultures may have been underestimated. It is unlikely, however, that errors in counting would account for such a large discrepancy. The results suggest, therefore, that some of the epithelioid cells could have been derived from activated lymphocytes. In 6 day cultures, early epithelioid cells show a remarkable similarity to activated lymphocytes.

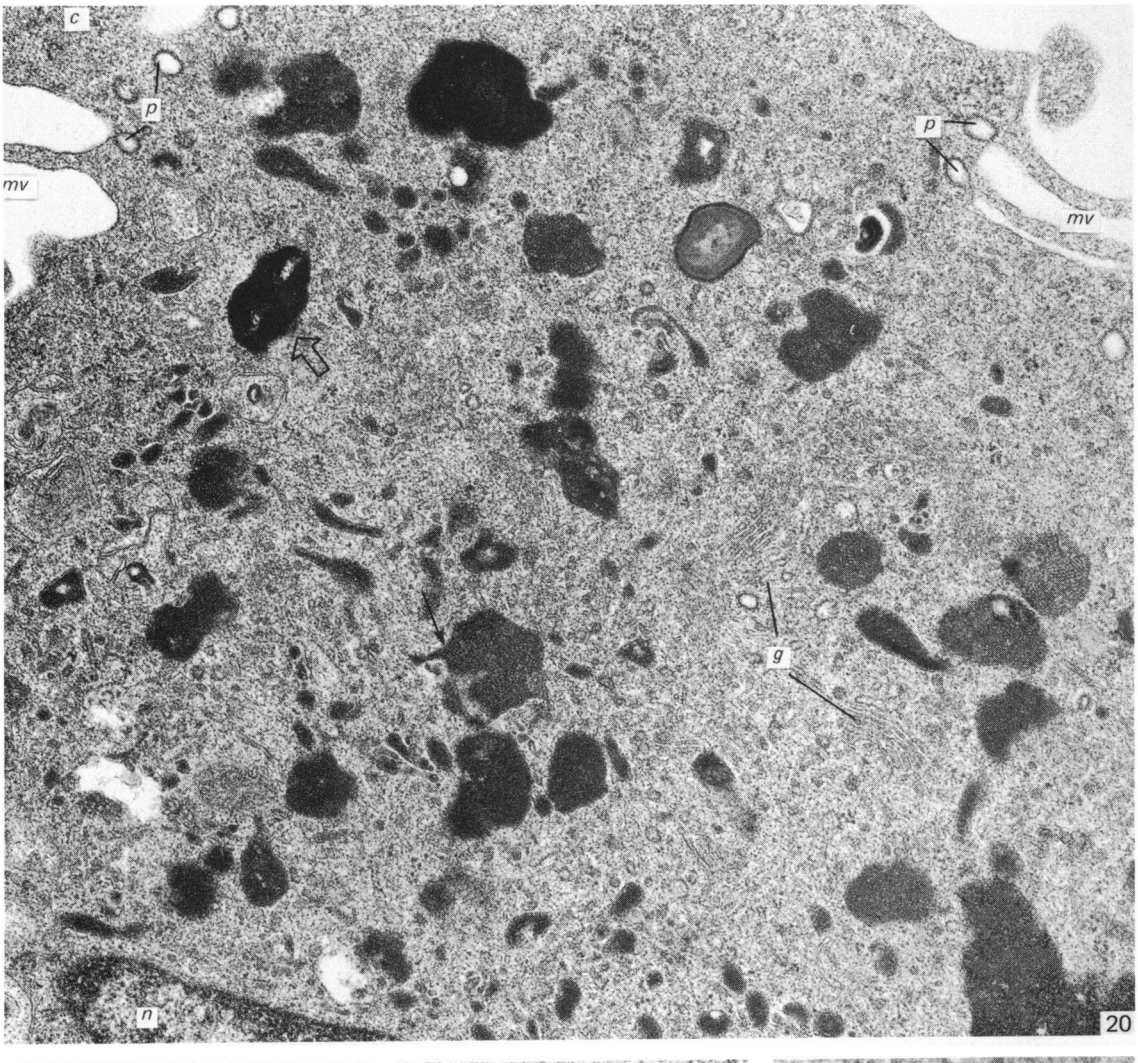
The development of large epithelioid cells in mitogen-stimulated lymphocyte cultures has not been previously described because the later stages of culture have not been studied. Nevertheless, the existence of heterogeneity among mitogen-activated lymphocytes has been noted (Ling & Kay, 1975). On the basis of light microscopy, Yoffey, Winter, Osmond & Meck (1965) distinguished three types of 'blast' cells, one of which is larger than the other types, has a lower nuclear–cytoplasmic ratio and pale non-basophilic cytoplasm. This type of cell fits the description of early epithelioid cells observed in this study. The ultrastructural study of Douglas *et al.* (1967) illustrated, among others, some cells from 4 days old phytohaemagglutinin and pokeweed mitogen cultures with abundant cytoplasm and numerous dense bodies surrounding the Golgi apparatus, but did not classify them as a distinct cell type.

Fig. 16. Large cell from a 9 day culture showing numerous microvilli and broad surface projections, pinocytotic vesicles, numerous electron-dense bodies on one side of the nucleus and spherical bodies with pale homogeneous contents on the other side of the nucleus. × 6000.

Fig. 17. Part of a large cell from a 9 day culture showing numerous spherical bodies with a pale central area and slightly denser rim, cytoplasmic filaments (*f*), a few electron-dense bodies (*d*) and a small phagocytic vesicle (*v*). × 8200.

Fig. 18. Small cell from a 12 day culture. The nucleus has peripherally condensed chromatin. Numerous mitochondria and a prominent Golgi apparatus are present at one pole of the cell. × 15000.

Fig. 19. Twelve day culture showing a small lymphocyte with scanty cytoplasm and peripherally condensed nuclear chromatin and an intermediate cell with a euchromatic nucleus and finely granular cytoplasm almost completely devoid of organelles. × 17600.



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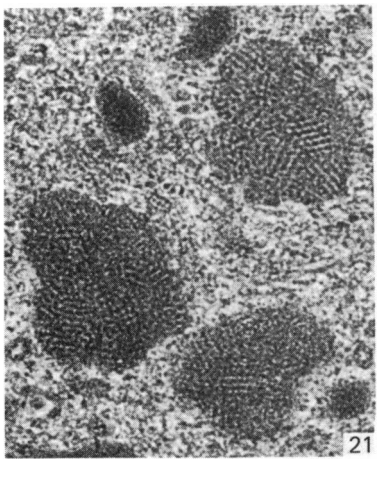

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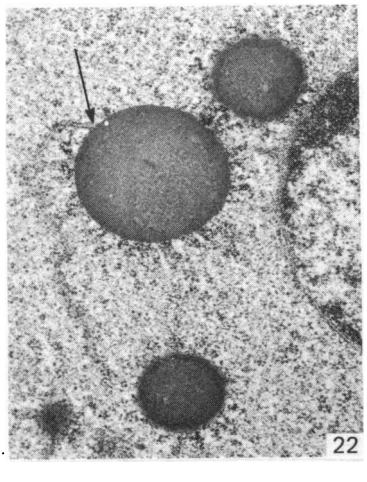

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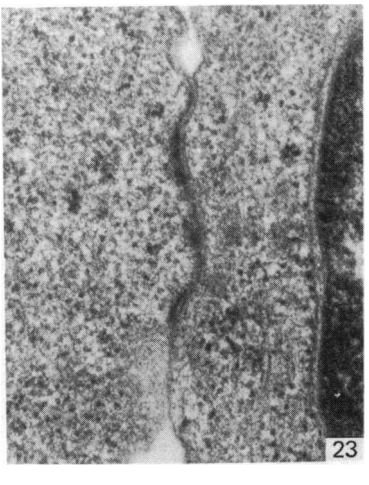

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Although lymphocytes are not normally phagocytic, it has been observed that, in 3 day phytohaemagglutinin cultures, ferritin is endocytosed by activated cells and accumulates within multivesicular and dense bodies (Biberfield, 1971). Possibly such cells and those with a high content of lysosomes and acid phosphatase could be the precursors of the epithelioid cells in later cultures.

The development of macrophages from lymphocytes has been shown to occur in unstimulated lymphocyte cultures *in vitro* or in subcutaneously implanted diffusion chambers *in vivo* (Bartfield & Juliar, 1964; Gough, Elves & Israels, 1965; Czerski, 1967; Elves, 1972). Some of these macrophages have been shown to possess few phagocytic vacuoles but are laden with dense bodies containing apparently 'crystalline' materials with a regular periodicity (Chapman *et al.* 1967). Such dense bodies are very similar to those described in this study in the epithelioid cells. Similar bodies have also been observed in lymphocytes of disseminated lupus erythematosus (Prunieras & Grapper, 1970; Haas & Yunis, 1970), in various syndromes associated with antibody deficiencies (Huhn & Tymper, 1970), in lymphocytic leukaemia and, occasionally, in lymphocytes of normal individuals (Bessis, 1973).

Activated lymphocytes with an unusually well developed endoplasmic reticulum were occasionally observed in this study. It has been suggested that such cells could be immunoglobulin secreting cells derived from B-lymphocytes (Douglas *et al.* 1967). Although phytohaemagglutinin stimulates preferentially the T-cells there is evidence that it can also induce a small degree of B-cell stimulation (Greaves, Owen & Raff, 1974).

The results of the present study also suggest that many of the activated cells subsequently reverted to small and medium sized lymphocytes. In previous studies such a reversion has been observed after removal of the mitogen and is more marked and complete if the time of exposure to the mitogen is limited to a few hours (Yamamoto, 1966; Polgar, Kibrick & Foster, 1968; Hardy & Ling, 1973). Some lymphocytes respond to mitogens more slowly than others (Gerhart, Mills, Monticone & Paetkan, 1976) but once committed they proceed to mitosis even if the mitogen is removed (Younkin, 1972). The present results suggest that in cultures continuously exposed to mitogen, reversion to small lymphocytes occurs after proliferation ceases.

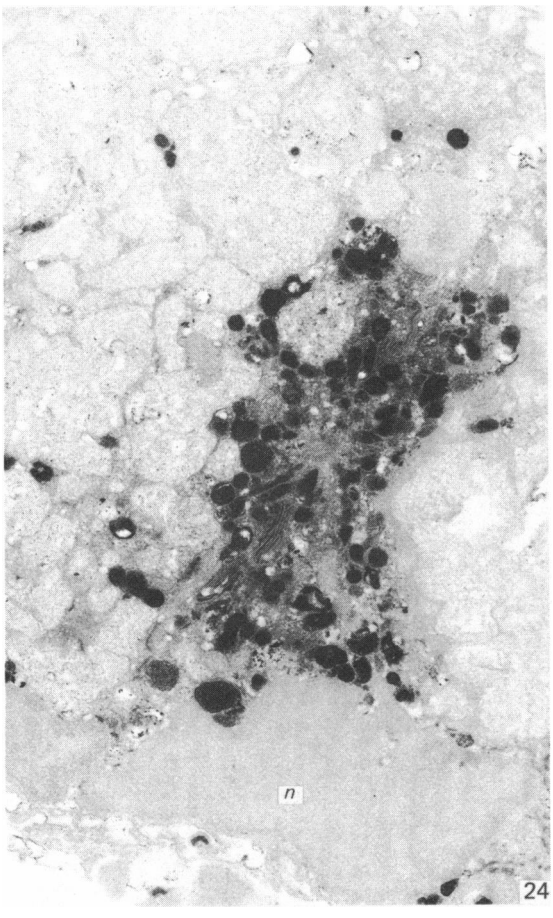
A model for the mechanism of T-cell activation and proliferation (Smith, Gillis & Baker, 1980) implicates the initial interaction of lectins with T-cells which stimulate the monocytes or macrophages to release lymphocyte-activating factor. This in turn stimulates a special subset of lymphocytes to produce T-cell growth factor (TCGF), which is solely responsible for proliferation of activated lymphocytes (TCGF-responsive cells). T-cell growth factor is actually utilised by the cells and so becomes rapidly depleted from the medium, and further proliferation ceases.

Fig. 20. Part of a large cell from a 9 day culture showing numerous dense bodies of variable size and shape. Note continuity between a small elongated and a large irregular dense body (arrow). A dense body has membranous contents (open arrow) while the majority of dense bodies have a granular structure (see Fig. 21). The Golgi apparatus (*g*) lies in the centre of the cell. Note pinocytotic vesicles (*p*) near the plasma membrane, microvilli (*mv*) and a thick surface projection of cytoplasm (*c*). Part of the nucleus (*n*) is shown at the bottom of the Figure. × 28000.

Fig. 21. Dense bodies from a large cell in a 9 day culture showing a granular structure with the granules arranged in parallel rows. × 53500.

Fig. 22. Intermediate cell from a 12 day culture showing a few spherical bodies (possibly lipid) and a profile of endoplasmic reticulum closely associated with one of the bodies (arrow). × 17300.

Fig. 23. Cell contact between two intermediate cells showing thickening of the apposed plasma membranes. × 40000.



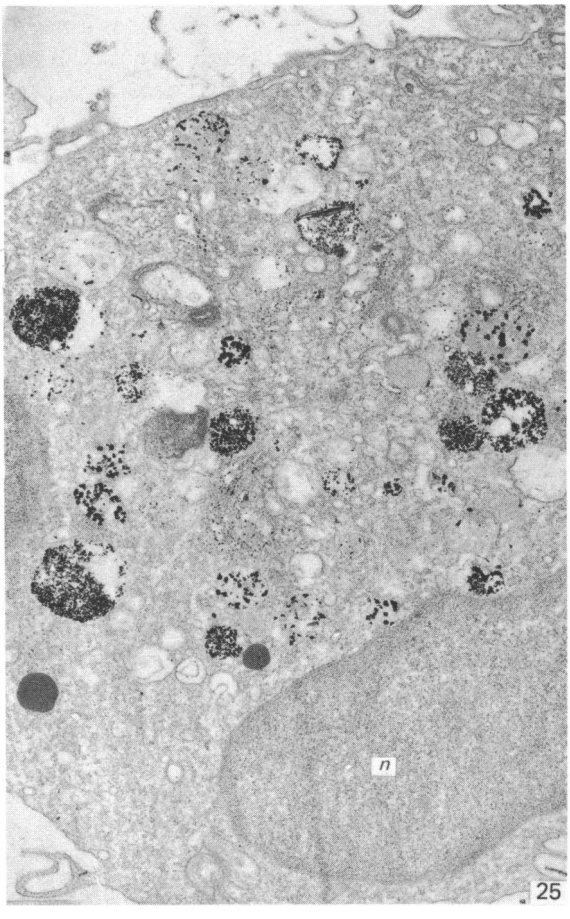


Fig. 24. Phagocytic cell from a 3 day culture showing acid phosphatase activity localised in the Golgi apparatus and in numerous small lysosomes in the cytoplasm between the phagocytic vacuoles. *n*, nucleus. × 10900.

Fig. 25. Large cell from a 9 day culture showing acid phosphatase activity in the form of coarse precipitate localised in numerous large bodies on one side of the nucleus (*n*). × 10800.

The epithelioid cells in later stages of phytohaemagglutinin cultures are not primarily phagocytic, but their contained organelles indicate considerable metabolic activity. Secretory macrophages may have diverse functions including the release of acid hydrolases, proteinases and lysozyme (Davies & Allison, 1976) and of cytostatic factors which act on rapidly proliferating lymphocytes and other cells (Keller, 1976; Nelson, 1976). It has been suggested that lymphoid cells provide for their own homeostasis (Nelson, 1976). Possibly, the epithelioid cells in later stages of phytohaemagglutinin cultures may exert such a cytostatic effect.

SUMMARY

Morphological and histochemical studies and cell counts were performed on phytohaemagglutinin-stimulated lymphocyte cultures at intervals from 1 to 17 days. Following a phase of proliferative activity lasting from the second to the sixth days, different cell types became evident in the later stages of culture. Some of the cells were large and characterised by an abundant cytoplasm containing numerous electron-

dense bodies with a characteristic internal structure, a well developed Golgi apparatus, a highly irregular plasma membrane, numerous pinocytoses and intense acid phosphatase activity localised within the dense bodies. These cells resembled epithelioid cells in which phagocytic vacuoles were very infrequent. In contrast, phagocytic cells distended with phagocytosed debris were present throughout the period of culture.

In later cultures many of the cells were smaller than activated lymphocytes. Intermediate sized cells were characterised by a paucity of organelles and low acid phosphatase activity, and small cells were similar to unstimulated lymphocytes but frequently contained numerous mitochondria.

A few cells with a highly developed endoplasmic reticulum were observed at 3 days and also in later stages.

The large cells with intense acid phosphatase activity constituted 20–30% of the total cells at 6 days but were more sparse in later stages when intermediate and small lymphocytes predominated. The total cell count on the seventeenth day was 50% of the initial cell count and the cells were almost entirely small, apparently healthy lymphocytes.

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