J. Anat. (1985), **140**, 1, pp. 93–104
With 21 *figures*
Printed in Great Britain

Surface morphology of mitogen-activated human lymphocytes and their derivatives *in vitro*

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(*Accepted* 17 *May* 1984)

INTRODUCTION

Scanning electron microscopy has shown considerable heterogeneity in the surface structure of human lymphocytes. Normal peripheral blood lymphocytes are usually spherical cells covered with microvilli which are somewhat variable in number, length and thickness, but lymphoid cells from thymus, tonsil and lymph nodes are mostly smooth-surfaced (Kelly & Nockolds, 1977; Newell, 1980).

Various studies have been performed on the surface structure of mitogen-stimulated lymphocytes *in vitro* (Biberfield, 1971; Clarke, Salsbury & Willoughby, 1971; Gormley & Ross, 1972; Holt, Pal, Catorsky & Lewis, 1972; Polliack *et al.* 1975; Criswell, Rich, Dardano & Kimzey, 1975; Hoffman, Moore, Shih & Blakley, 1977; Newell & Roath, 1978). However, these have given inconsistent results and many of them require cautious interpretation because of the use of air drying or aspiration filtration of living cells, procedures which have subsequently been shown to cause artefacts or loss of surface structure (Boyde, Weiss & Vesely, 1972; Alexander & Wetzel, 1975). Besides, all these studies have been limited to the first three to five days in culture when the activated lymphocytes are at their peak proliferative activity. However, it has been observed (Cuschieri, Mughal & Kharbat, 1985) that after proliferation ceases the cells undergo further morphological changes and give rise to different cell types.

In the present study the surface structure of the cells in mitogen-stimulated lymphocyte cultures has been investigated at various stages from one to seventeen days in order to determine the changes which accompany lymphocyte activation, proliferation and subsequent differentiation in culture.

MATERIALS AND METHODS

Lymphocytes were separated on 'lymphoprep' from heparinised human peripheral blood from six normal healthy donors. After washing three times in Hank's balanced salt solution the lymphocytes were cultured at 37 °C in RPMI 1640 medium† (Gibco) containing fetal calf serum (15 %), penicillin (100 u/ml), streptomycin (100 μg/ml) and either phytohaemagglutinin M (Gibco, 0·15 ml per 10 ml medium) or pokeweed mitogen (Gibco, 0·01 ml/10 ml medium). Control cultures of unstimulated lymphocytes were prepared in medium lacking mitogen. The cultures contained $1{\cdot}5 \times 10^6$ cells

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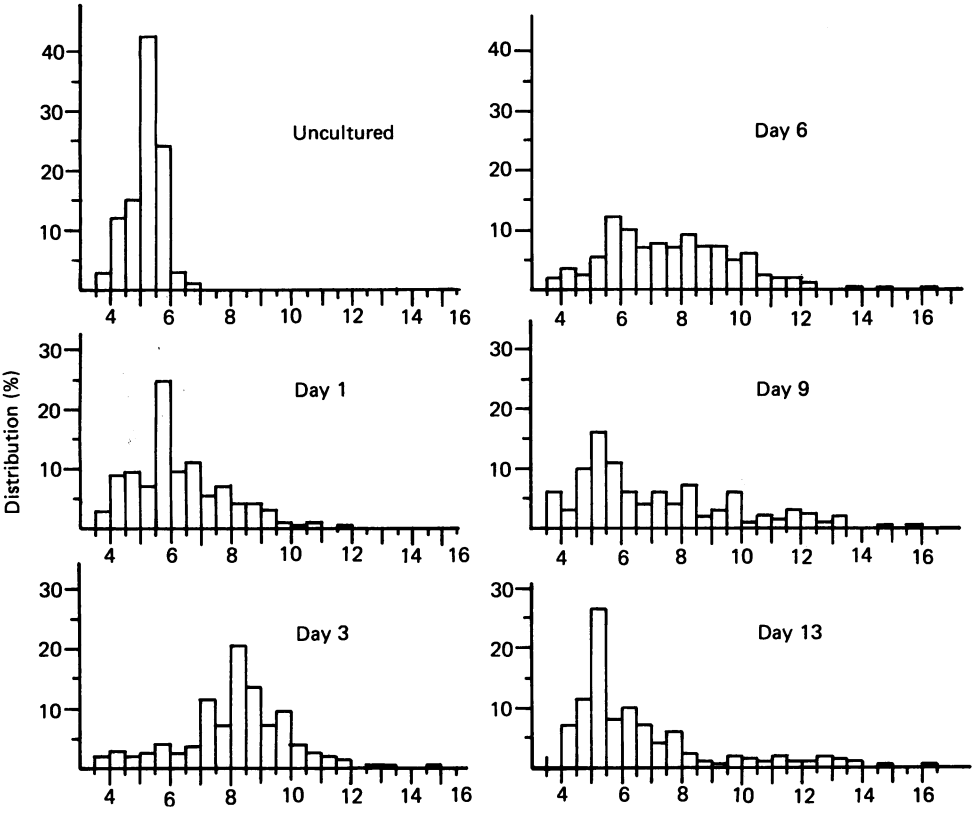
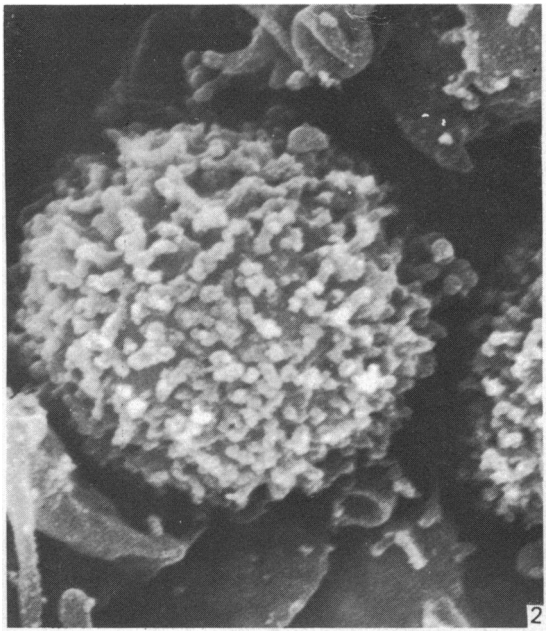
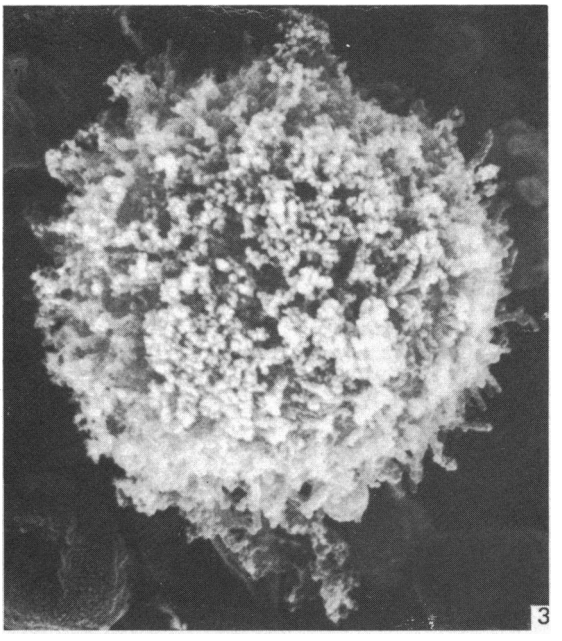


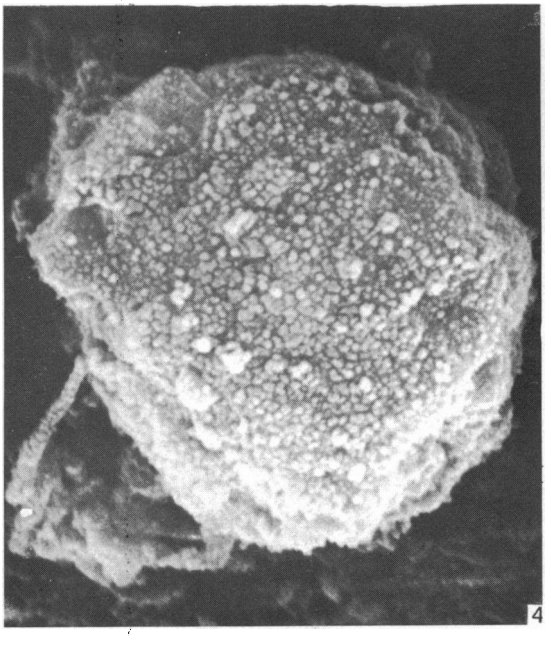
Fig. 1. Histograms showing distribution of cell diameters in normal uncultured lymphocytes and lymphocytes from 1, 3, 6, 9 and 13 days phytohaemagglutinin-containing cultures.

per ml and were set up in 8 ml aliquots in tightly closed plastic universal containers. The medium was changed every three or four days.

Uncultured lymphocytes and lymphocytes harvested from phytohaemagglutinin, pokeweed mitogen or unstimulated cultures at 1, 3, 6, 9, 13 and 17 days were centrifuged and resuspended in 3 ml of medium. An equal volume of 3 % glutaraldehyde in 0·12 M phosphate buffer, pH 7·2 at room temperature was added. After initial fixation for 15 minutes, 2 ml of the cell suspension was placed in a syringe barrel fitted to a Nucleopore filter holder containing a 13 mm silver membrane and very slowly aspirated over a period of 10–15 minutes using a water vacuum pump. When filtration was almost complete the membranes were transferred to fresh fixative for a further 60 minutes. They were then washed in phosphate buffer and postfixed in 0·25 % buffered osmium tetroxide for 30 minutes. Following further washes in buffer and distilled water, the membranes were treated successively with a saturated solution of thiocarbohydrazide for 30 minutes and 1 % aqueous osmium tetroxide for 30 minutes with washes in distilled water after each step. The specimens were dehydrated in a graded series of cold acetone solutions and critical-point dried from carbon dioxide in a Sorvall critical-point drying apparatus. The dried specimens were mounted on copper stubs using adhesive copper tape and coated first with carbon in a Denton evaporator and then with gold in a JEOL ion sputter coater.







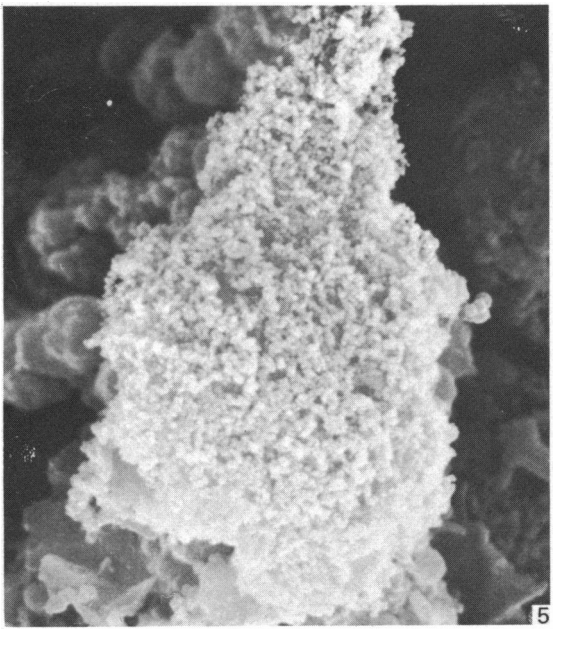
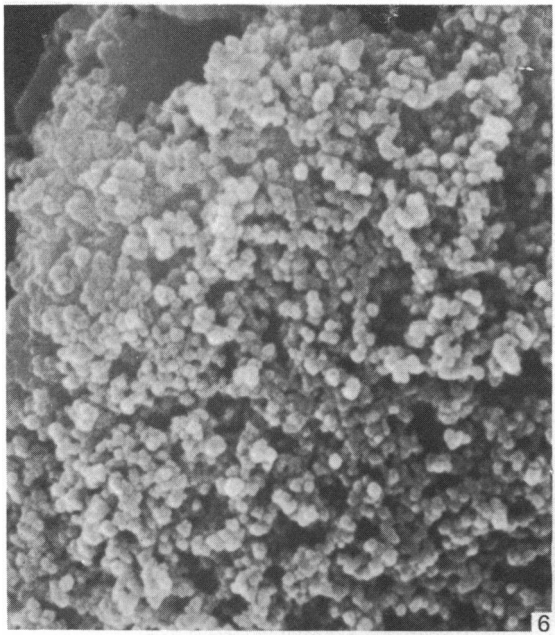


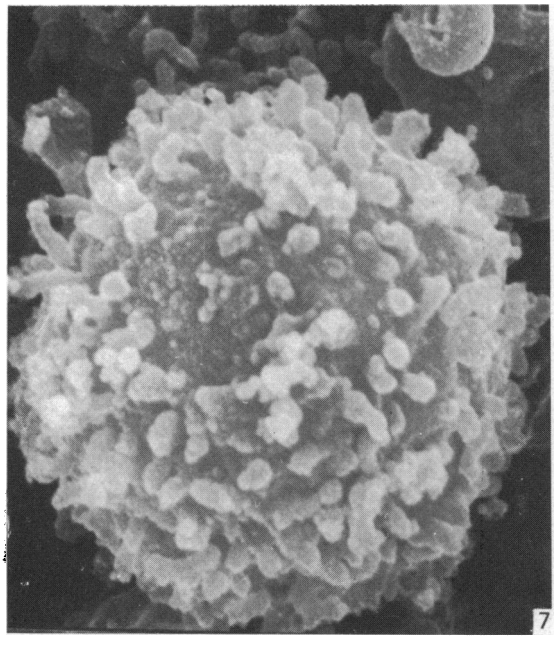
Fig. 2. Normal uncultured lymphocyte. × 9500.

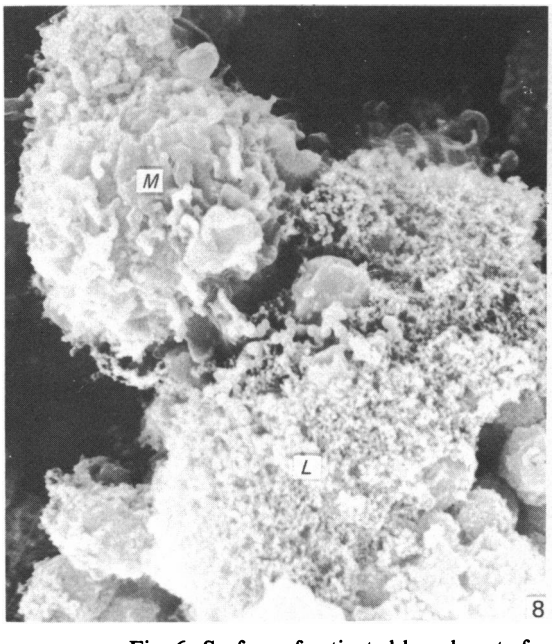
Fig. 3. One day phytohaemagglutinin culture: large spherical lymphocyte showing very fine, short microvilli and longer finger-like microvilli. × 5500.

Fig. 4. One day phytohaemagglutinin culture: lymphocyte showing short stub-like villi. × 11000.

Fig. 5. Three day phytohaemagglutinin culture: large irregular activated lymphocyte uniformly covered with very fine microvilli. × 5500.







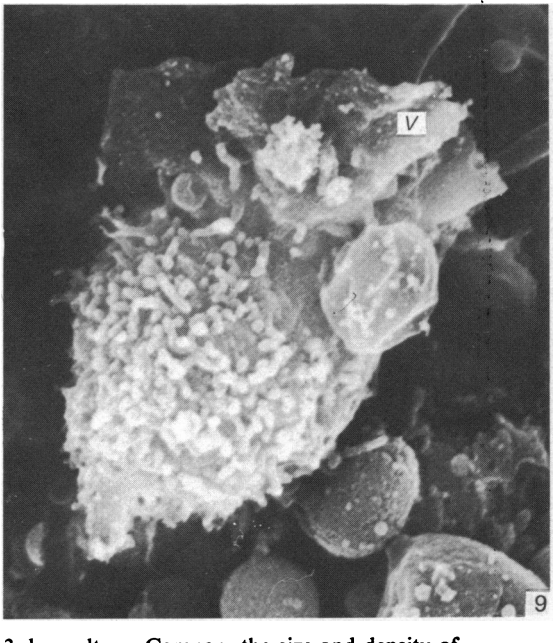
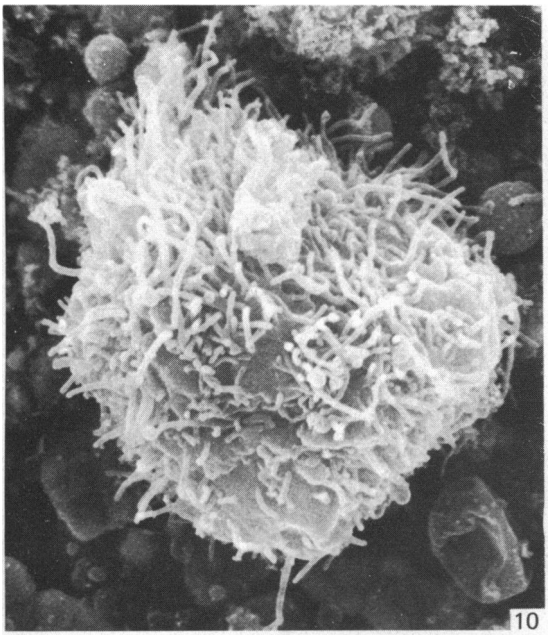


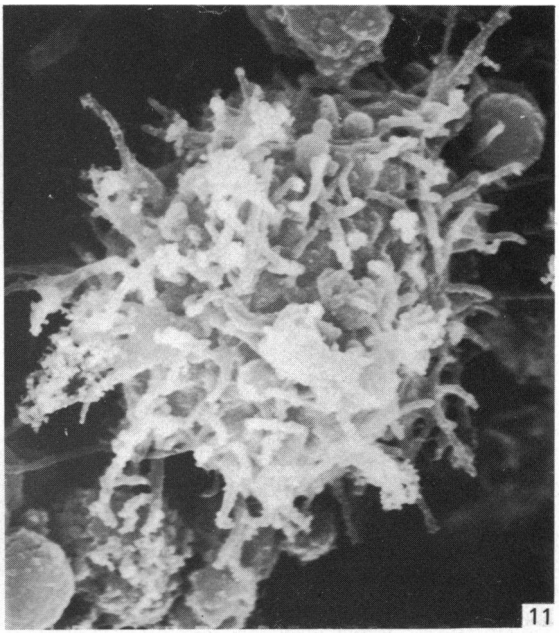
Fig. 6. Surface of activated lymphocyte from 3 day culture. Compare the size and density of these microvilli with those of a normal lymphocyte (Fig. 7) photographed at the same magnification. × 13000.

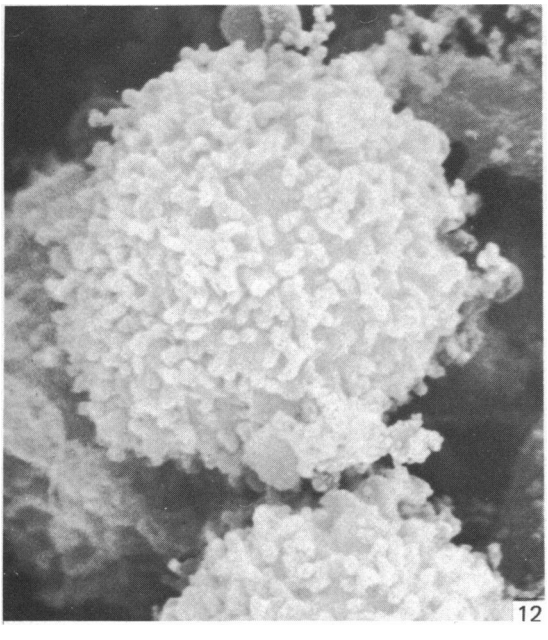
Fig. 7. Surface detail of normal unstimulated lymphocyte. × 13000.

Fig. 8. Three day phytohaemagglutinin culture: activated lymphocytes (*L*) covered with fine microvilli and monocyte (*M*) with surface ruffles. × 4200.

Fig. 9. Three day phytohaemagglutinin culture: motile cell displaying veils (*V*) at one pole of the cell and microvilli on the rest of the surface. × 6500.







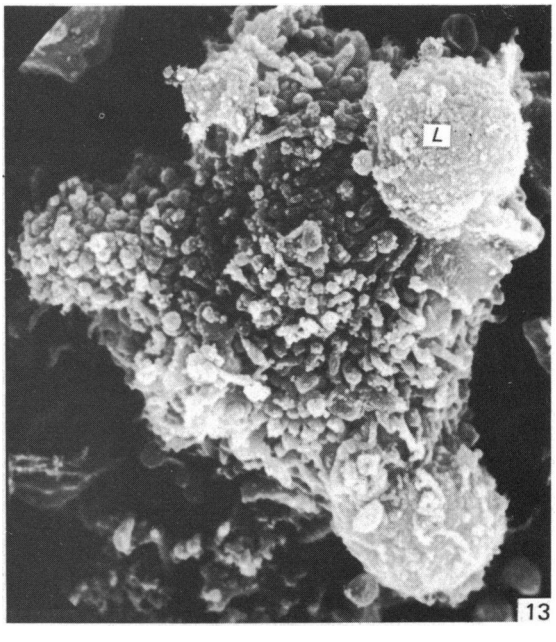
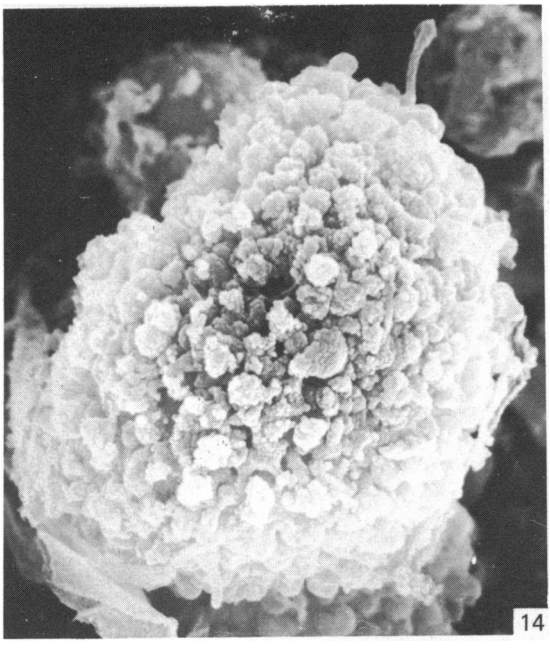


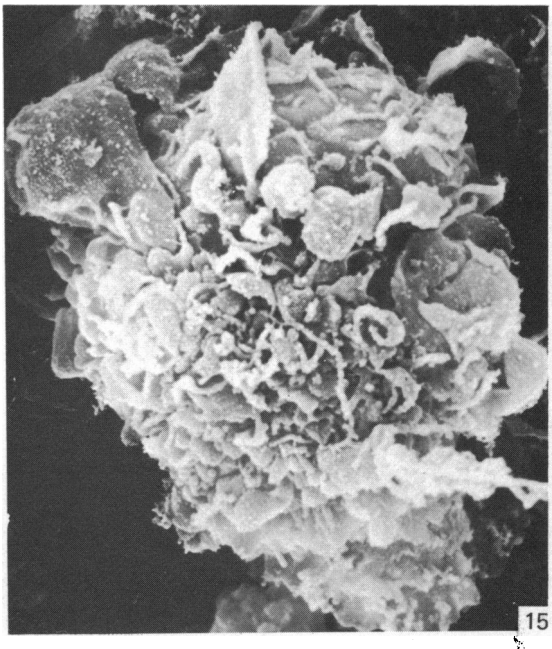
Fig. 10. Six day phytohaemagglutinin culture: large cell with long, slender microvilli. ×4000.

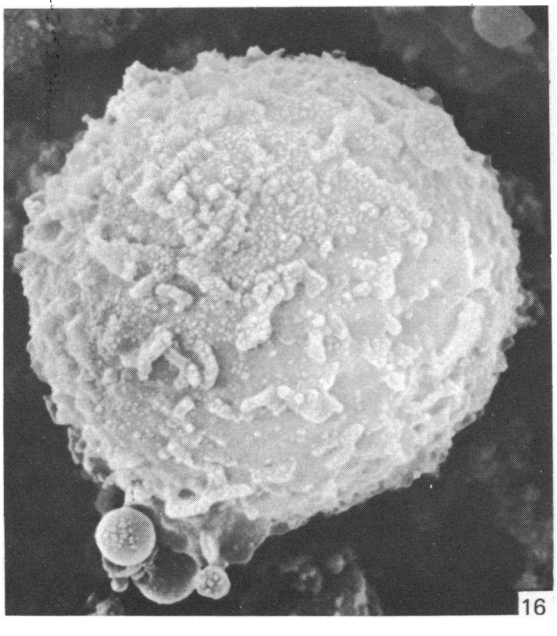
Fig. 11. Six day phytohaemagglutinin culture: cell with irregular microvilli. ×7000.

Fig. 12. Thirteen day phytohaemagglutinin culture: small cell with surface structure closely resembling that of normal lymphocyte. ×9500.

Fig. 13. Nine day phytohaemagglutinin culture: large cell covered mainly with microvilli and a few blebs. A small lymphocyte (*L*) lies on the top right corner of the cell. ×4000.







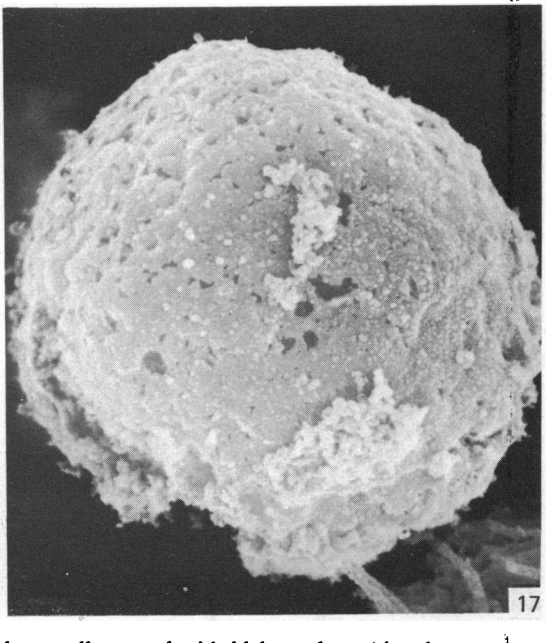
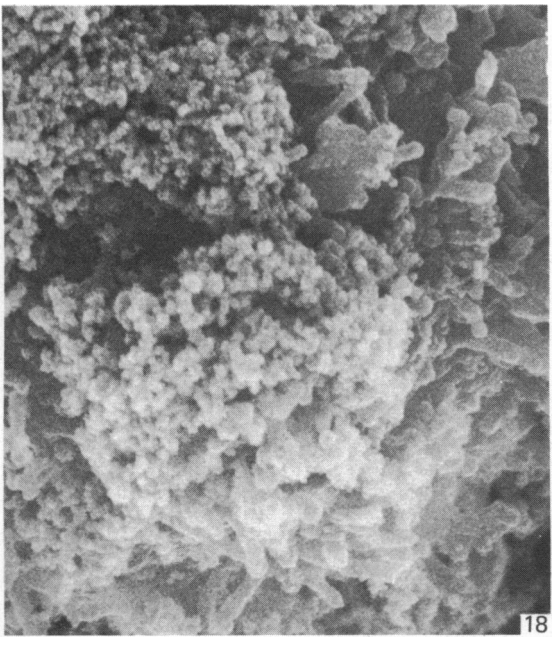


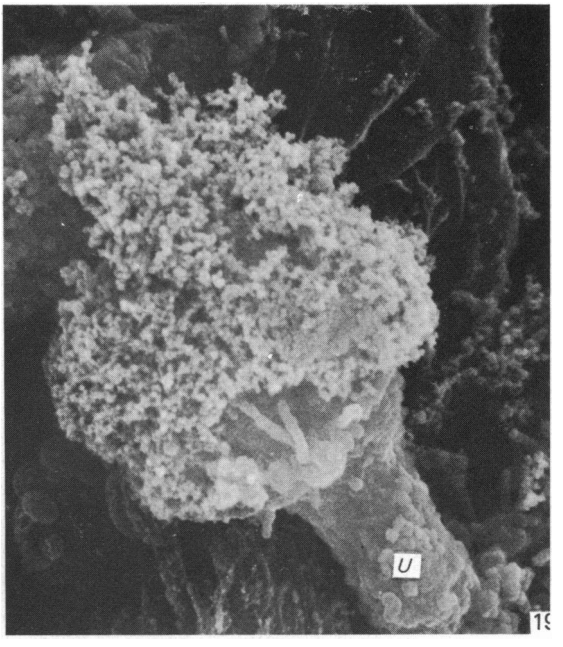
Fig. 14. Nine day phytohaemagglutinin culture: large cell covered with blebs and occasional microvilli. × 5100.

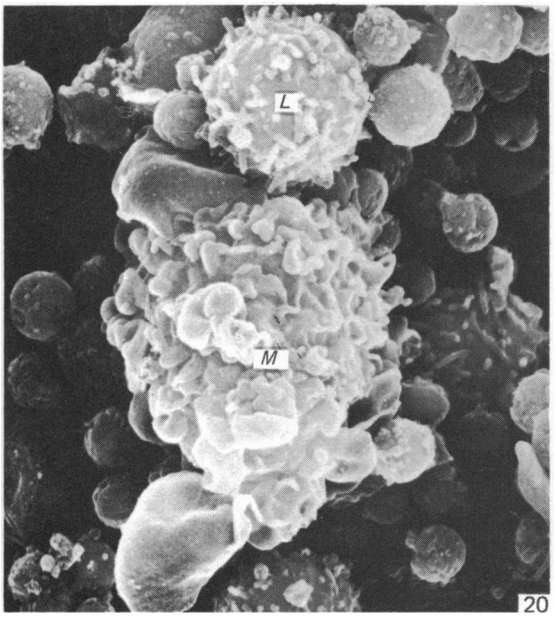
Fig. 15. Nine day phytohaemagglutinin culture: large cell with prominent ruffles, possibly a monocyte. × 4000.

Fig. 16. Thirteen day phytohaemagglutinin culture: spherical cell with ridges and few short microvilli. × 8000.

Fig. 17. Thirteen day phytohaemagglutinin culture: large smooth-surfaced cell showing surface clefts indicative of degeneration. × 6500.







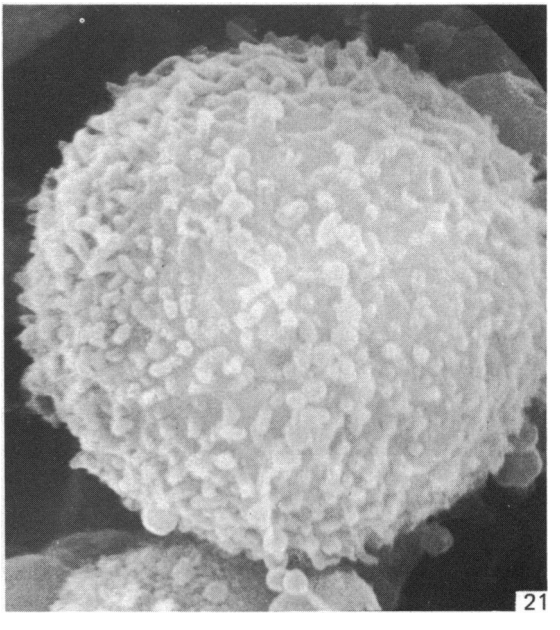


Fig. 18. Three day pokeweed mitogen culture: detail of activated cell showing a mixture of very fine and coarser microvilli. × 10500.

Fig. 19. Three day pokeweed mitogen culture: probably motile cell with a uropod (*U*) and fine microvilli. × 5700.

Fig. 20. Three day unstimulated culture showing a monocyte (*M*) and lymphocyte (*L*) with few microvilli. × 3600.

Fig. 21. Nine day unstimulated culture: large villous cell, occasionally seen in unstimulated cultures. × 7500.

The specimens were viewed in a JEOL JSM 35 scanning electron microscope and photographed on Polaroid P/N 55 film.

Measurements of cell diameters were made on the screen of the scanning electron microscope and calculated from the magnification scale bar on the microscope screen calibrated against a standard grid; 150–200 consecutive cells were measured from each of the specimens at 1, 3, 6, 9 and 13 days of phytohaemagglutinin culture.

RESULTS

The diameters of the cells in the cultures at different stages showed great variations, which are detailed in the histograms of Figure 1.

Normal uncultured lymphocytes were spherical cells, uniformly covered with microvilli (Fig. 2). In spite of some variations in the length, diameter and density of their microvilli, it was not possible to categorise different subpopulations of lymphocytes on the basis of their surface structure. Smooth-surfaced lymphocytes were very rare. Monocytes were readily distinguishable by the presence of typical surface ruffles. They measured 5·6–7·0 μm in diameter and constituted 8–10 % of the total number of cells.

In phytohaemagglutinin-stimulated cultures there was a considerable overlap in cell morphology at various stages. The predominant cell types of each stage are described below.

In 1 day cultures, most of the cells were spherical or slightly irregular and were covered with microvilli. The majority of these cells contained patches of very fine, densely packed, microvilli interspersed among areas of larger but less numerous microvilli (Fig. 3). Some cells had short, stub-like microvilli (Fig. 4) and a few resembled normal lymphocytes but had a diminished density of microvilli.

In 3 days old cultures, the majority of cells were large, irregular in outline and covered with numerous very fine and closely packed microvilli (Fig. 5), which were much thinner and shorter but more numerous than those on uncultured lymphocytes (compare Figs. 6 and 7). Monocytes with characteristic surface ruffles (Fig. 8) constituted 8–10 % of the cells in 1 and 3 day cultures. Some cells were identified as motile cells by the presence of a uropod or of thin veils (Fig. 9). On these cells, microvilli were frequently clustered at one pole but were sparse over the rest of the cell surface. In 3 day cultures, a few cells had long villi similar to those in the cells from 6 day cultures.

In 6 days old cultures (Figs. 10, 11), the majority of cells were large and irregular and were covered with a moderate number of long finger-like or conical microvilli, much bigger than those of uncultured lymphocytes. Cells similar to those which predominated in 3 and 9 day cultures were occasionally observed.

In 9 and 13 days old cultures, the cells could be classified into two broad categories. The first category consisted of small and intermediate sized cells (up to 8 μm in diameter) which were mainly spherical and had a uniformly villous surface closely resembling that of normal uncultured lymphocytes in the size and density of their microvilli (Fig. 12). A few of these cells, however, had few microvilli or were smooth-surfaced and showed degenerative changes. The second category consisted of large and irregular cells which had a rich and heterogeneous surface contour displaying villi, blebs, ridges, ruffles or various mixtures of these structures (Figs. 13–15). A few of the cells had typical monocytic features with prominent ruffles but few villi or blebs. Others were covered mainly with villi or blebs and lacked ruffles. However,

most of the cells showed so much heterogeneity and overlap in their surface structure that it was not possible to categorise them as monocytic or not. A few very large spherical cells were predominantly smooth-surfaced with only a few shallow ridges and an occasional villus (Fig. 16); they often showed irregular surface clefts indicative of degeneration (Fig. 17).

In 17 days old cultures the cells were similar to those at nine and thirteen days but the proportion of large and small cells showing degeneration changes was considerably greater.

Pokeweed mitogen cultures at 1 and 3 days were generally similar to phytohaemagglutinin cultures, but in most of the large activated cells the very fine microvilli occurred in irregular areas interspersed among areas having thicker, finger-like microvilli (Fig. 18). Some of these cells occasionally also showed a few surface blebs.

Motile cells (Fig. 19), cells with very long microvilli and large cells with heterogeneous surface features similar to those in phytohaemagglutinin cultures were also observed. However, it was evident that many cells had smooth surfaces and irregular clefts, indicating that there was extensive cellular degeneration and very few healthy cells could be recognised at 9 days.

Unstimulated cultures at 3 days contained mostly small lymphocytes with a diameter of 3·2–6·0 μm. Most of them had few microvilli and some were smooth-surfaced. Typical monocytes constituted 15–20 % of the cells. By the sixth day there was a great decrease in the total cell number and practically all the lymphocytes were either smooth-surfaced or showed obvious degeneration. The monocytes were, however, very prominent against the background of degenerating lymphocytes. They were very large cells, 10–17 μm in diameter, and had a strikingly constant and typical surface structure consisting of prominent ruffles with few or no villi (Fig 20). They lacked the heterogeneity of features observed in the large cells of phytohaemagglutinin cultures. Occasional large spherical cells uniformly covered with finger-like microvilli (Fig. 21) were also observed in unstimulated cultures after six to thirteen days.

DISCUSSION

The most striking feature observed in the present study in mitogen-activated lymphocytes is the abundance of dense, very fine, short microvilli which replace the longer and thicker microvilli present on normal unstimulated lymphocytes. A transition between these two forms is evident in the cells at 24 hours. Such a surface structure has not been reported in previous studies of mitogen-stimulated lymphocytes. In the studies performed prior to 1972 (Biberfield, 1971; Clarke *et al.* 1971; Gormley & Ross, 1972), the activated lymphocytes were mostly smooth-surfaced as a result of the air-drying preparative procedure. Polliack *et al.* (1975) found that most of the activated 'blast' cells in phytohaemagglutinin or pokeweed mitogen-stimulated cultures possess finger-like or conical microvilli and occasional round blebs. Several smooth cells also present are possibly artefactual, caused by aspiration filtration of the living cells (Alexander & Wetzel, 1975). Hoffmann *et al.* (1977) found a great increase in length of the microvilli three days after phytohaemagglutinin activation but a subsequent decrease in length by the fifth day. Newell & Roath (1978) distinguished activated spherical cells on which microvilli are slightly longer but less dense than normal, and large irregular 'blast' cells with smooth areas scattered among stub-like or conical villi, blebs and ruffles. Mouse splenic lymphocytes, activated with concanavalin A or lipopolysaccharide, show a complex villous

surface (Criswell *et al.* 1975). A possible explanation for these differences in the surface structure of activated lymphocytes may be that the culture conditions differed in all these studies. The type of medium, the concentrations of phytohaemagglutinin, fetal calf serum and antibiotics, cell density and even the type of container may affect the cellular activity and rate of proliferation (Ling & Kay, 1975). It is possible that such conditions may also affect the surface structure of the cells. Microvilli are dynamic features of the cell surface which may be produced or retracted in response to changes of the cellular environment (Catovsky, Frisch & Van Noorden, 1975), temperature (Lin, Wallach & Tsai, 1973), cell density (Porter, Prescott & Frye, 1973), cellular interactions *in vitro* (Lin, Cooper & Wortis, 1973; Rubin & Everhart, 1973; Kay *et al.* 1974), the phases of the mitotic cycle (Porter *et al.* 1973) or the transformation of cells by viruses (Hatanaka & Hanafusa, 1970). In spite of the differences in detail, most of the studies on activated lymphocytes have demonstrated some increase in number, length or complexity of the microvilli. Such an increase may be associated with enhanced transport of substances across the cell membrane, as suggested by Willoch (1967).

For scanning electron microscopy, individual cell size is an important criterion for distinguishing between normal and activated lymphocytes since the characteristic morphological changes in the nucleus and cytoplasm cannot be observed. Shrinkage of cells occurs during dehydration and critical-point drying procedures for electron microscopy (Boyde *et al.* 1972). Thus the cell diameters recorded in this study may not be a faithful representation of the actual dimensions of the living cells, but it is likely that they reflect the actual changes in cell size.

The histograms show a general increase in cell size accompanying activation. This is followed after the sixth day by a progressive increase in the proportion of small cells, and by Day 13 about 65 % of the cells are again within the size range of normal lymphocytes. However, a morphologically distinct population of large cells is also present.

The observations on the later stages of culture shed light on the fate of the activated lymphocytes. The small lymphocytes observed at 9 and 13 days have a uniformly villous surface structure similar in all respects to that of normal, unstimulated lymphocytes. It is unlikely that these cells are persistent, unstimulated lymphocytes because they are infrequent at 3 days and their number increases progressively in the later stage. Furthermore, in the unstimulated controls, lymphocytes rapidly lose their normal villous surface structure and show signs of degeneration. These results suggest that, after proliferation ceases, the activated cells revert to small lymphocytes even in the continued presence of mitogen. Reversible 'transformation' of lymphocytes in culture has been previously demonstrated following brief exposure to mitogen (Yamamoto, 1966; Polgar, Kibrick & Foster, 1968) in which case the resulting small lymphocytes could be restimulated.

These results also indicate the presence of a population of large cells with very heterogeneous surface features. The large cells with a predominance of ridges and ruffles are suggestive of monocytes, whereas the cells with a predominance of villi resemble more closely the villous cells which predominate on day 6 and could be of lymphocyte origin. However, macrophages with a predominantly villous surface have been described (Parakkal, Pintu & Hanifin, 1974). Besides, a sharp distinction between the two cell types cannot be made. It is, therefore, not possible to conclude whether large cells are all of the same origin and whether they are derived from monocytes or activated lymphocytes.

It is, however, noteworthy that in unstimulated cultures the vast majority of large cells are undoubtedly monocytes since they always have a typical ruffled surface and, in contrast to the large cells in phytohaemagglutinin cultures, they lack villi or blebs. Their relative prominence is obviously due to a great decrease in the number of lymphocytes and the very small size and featureless surfaces of the remaining few. The large, uniformly villous cells which are occasionally seen in the later stages of unstimulated cultures probably represent the small proportion of activated cells which are normally present in peripheral blood and which undergo 'blast-like' changes in culture in the absence of mitogens.

The present results using pokeweed mitogen and phytohaemagglutinin fail to show obvious differences which could be attributed to the preferential stimulation of B or T lymphocytes. The observed changes with the two mitogens differ only in degree and are partly attributable to the toxicity of pokeweed mitogen which, in the dosage used, causes marked cellular degeneration especially in the later stages of culture.

SUMMARY

Human peripheral blood lymphocytes were cultured with phytohaemagglutinin or pokeweed mitogen for various intervals up to 17 days and studied by scanning electron microscopy.

Activated lymphocytes in 3 day cultures were large irregular cells characterised mainly by an abundance of very fine microvilli, which were much thinner, shorter and more densely packed than the microvilli on uncultured lymphocytes. Cells intermediate in size and surface morphology between these and unstimulated lymphocytes were numerous in 1 day cultures. Some motile cells and large cells with finger-like or conical microvilli were also present. Cell counts showed that after 6 days in phytohaemagglutinin culture small villous cells resembling normal healthy lymphocytes were progressively more numerous, suggesting that most of the activated cells reverted to small lymphocytes. Very large cells were also present and displayed a heterogeneous surface morphology of villi, ridges, blebs and ruffles. A few of these cells had typical monocytoid features but others were predominantly villous.

In pokeweed mitogen cultures there was extensive cellular degeneration affecting all cell types after 6 days. In unstimulated cultures the lymphocytes had fewer microvilli or were smooth-surfaced. There was extensive lymphocyte degeneration after 3 days and eventually typical monocytes were the predominant cells. Large, villous cells, which could be activated lymphocytes, were occasionally encountered in unstimulated cultures.

We thank Mr Basim A. Kharbat for his skilful technical assistance. This study was supported by grant MA 007 from the College of Graduate Studies, Kuwait University.

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