Clin. exp. Immunol. (1972) **10,** 555–570.

SURFACE STRUCTURE OF NORMAL AND LEUKAEMIC LYMPHOCYTES

I. EFFECT OF MITOGENS

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(*Received* 4 *October* 1971)

SUMMARY

Scanning electron microscopy has been used to examine the surface structure of normal and leukaemic lymphocytes and the effect of specific and non-specific mitogens on them. Both types of mitogens produce an increase in the surface irregularity of the normal cell but not of the leukaemic cell. The uropod is seen to consist of a fork-like structure the prongs of which are joined together by a fine membrane.

The use of the technique of ionic etching on the surface of the lymphocyte has shown variation in the physical hardness of the surface of the lymphocyte as a result of non-specific mitogen stimulation only. The surface of the leukaemic lymphocyte was considerably more resistant to etching than normal.

These results in conjunction with the results of other workers suggest that non-specific activation picks out the thymic dependent cells because these have weaker cell surface membranes and by corollary the difficulty in stimulating leukaemic lymphocytes is due to the increased resilience of this surface membrane. Thus the differentiation of thymic dependent lymphocytes by non-specific stimulation may be purely fortuitous and not directly related to their physiological function.

INTRODUCTION

The ultrastructure of the lymphocyte as seen in ultra-thin microscopy is well known and also the changes produced by activation (Zucker-Franklin, 1969). However, these thin-layer investigations only show structures in a single plane and do not enable the three-dimensional structure to be well visualized. Scanning electron microscopy allows the surface of the cells to be examined and because of the relatively great depth of focus some idea of three dimensional perspective can be obtained. Using this method, the surfaces of blood cells have been examined (Salsbury & Clarke, 1967; Clarke, Salsbury & Rowland, 1968a; McDonald

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& Hayes, 1969; Lewis & Stuart, 1970; Bain, 1971; Michaelis *et al.*, 1971). The polymorphonuclear neutrophil has an irregular appearance both in contour and surface, which is composed of many ridges and folds. The lymphocyte appears much more rounded and symmetrical with smaller more conical and less ridge-like surface projections but is not always easily distinguished from a polymorphonuclear neutrophil. Activation by chemical irritants and mitogens increase the surface irregularity of the lymphocyte (Clarke, Salsbury & Willoughby, 1968b) and macrophage (Carr, Clark & Salsbury, 1969). However, scanning electron microscopy alone only allows surface structures to be visualized.

By means of ionic bombardment in a gas filled atmosphere (radio-frequency sputtering; Lewis, Osborn & Stuart, 1968) surface structures can be removed layer by layer at a molecular level according to their relative hardness and resilience, a local area of resistance being shown by residual material remaining as an elevation. The new surfaces exposed can then be coated and scanned in the normal way. Thus subsurface structures are exposed and an impression can be obtained of topographical variations in relative compositions of the different areas of the surface layers of the cell. It must be emphasized that this reveals purely physiochemical changes and these may not be related to biological function. This method has been used to examine normal human peripheral blood lymphocytes, cells of chronic lymphocytic leukaemia and cells after activation by mitogens.

It is convenient to divide the mitogens into two main types, the non-specific stimulants such as phytohaemagglutinin (PHA), pokeweed and antilymphocyte sera (ALS) and the specific stimulants such as purified protein derivative of tubercle bacilli (PPD) and candida antigen. This division is justifiable on several grounds. The non-specific stimulants act more quickly, they affect a greater proportion of cells from a greater number of individuals and their dose response is more critical than the specific stimulants, excess concentration often being toxic. For specific stimulants to be effective, an intermediary phagocytic cell is necessary (Hersh & Harris, 1968). The resulting RNA (Cooper & Rubin, 1965) and phospholipid (Lucas, 1971) synthesis is dissimilar and the degree of suppression of transformation by prednisone and azathioprine when PHA or candida are used as the mitogen is different (Folb & Trounce, 1971). Further measles virus prevents mitogenic response to PPD but not to PHA (Smithwick & Berkovich, 1966).

METHODS AND MATERIALS

The lymphocytes were obtained from the peripheral blood of human donors in three different ways. The first method was from whole heparinized blood by sedimentation at 37°C in plastic vials, the supernatants being harvested. Secondly, gelatin separation of defibrinated blood was carried out by the method of Coulson & Chalmers (1964), and thirdly separation was by differential gradient sedimentation using the method of Böyum (1968). In each case the lymphocytes were recovered and fixed immediately, or after culture with the appropriate stimulants. No difference was found in the results using lymphocytes obtained by these three methods. Four normal blood donors and three patients with chronic lymphocytic leukaemia were used.

Mitogens

Mitogens were used as follows: phytohaemagglutinin (PHA, Wellcome); pokeweed (Grand Island Biologicals); purified protein derivative of tubercle bacilli (PPD, Ministry of

Agriculture, Weybridge, Surrey); candida antigens prepared by extraction of boiled candida cultures (Dr I. G. Murray); and antilymphocyte serum (ALS), prepared by repeated injections of human lymphocytes from several donors into rabbits; the ALS was decomplemented by heating at 56°C for 20 min before use.

Culture methods

Cells were cultured at a concentration of 10^6 per ml in a culture medium consisting of 0·6 ml of inactivated autologous serum and 2·4 ml of TC119 media (Wellcome) in a 5-ml bijoux bottle. The cultures were stimulated by either PHA, pokeweed, PPD, ALS, or by homologous lymphocytes. The mitogens were used at predetermined optimum concentrations. The PHA stimulated cultures were harvested at 3 days, pokeweed and ALS at 4 days, and the PPD and mixed lymphocyte reaction at 5 days. The cells were gently centrifuged, washed once in TC119 at 37°C and then resuspended in 2 ml TC119. To this 2 ml of freshly prepared cold 2% isotonic glutaraldehyde (buffered to pH 7·3) was added, and the contents gently mixed, 15 min later the fixed cells were gently centrifuged and then resuspended in 2 ml of distilled water. They were again centrifuged and resuspended in 1 ml of distilled water. A drop of these resuspended cells was placed on a cover glass (Chance No. 1 10-mm round) and allowed to dry in a dust-free atmosphere. The sample was then either coated with gold palladiun or etched on the cover glass in a hydrogen atmosphere by the technique of Lewis, Osborn & Stuart (1968) before coating. The specimens were then scanned in the Cambridge 'Stereoscan' 4 electron microscope.

Certain cultures were allowed to form on cover glasses in the bottom of bijoux bottles and fixed *in situ*. This method gave a rather sparse growth.

RESULTS

Control cultures

The white cells obtained from the peripheral blood were obviously a mixed population of cells, therefore it was only possible to group cells together with certain morphological characteristics. Three main types of cells were present (Figs 1 and 2): firstly, the red cell which is easily distinguished by its smooth membrane, central depression and periphery which stands clear of the background; secondly, the polymorphonuclear neutrophil which although roughly spherical tends to flatten out on the surface of the cover glass, has an uneven surface consisting of irregular folds and ridges and with a suggestion of pseudopodia in certain areas. The third cell is the lymphocyte. This appeared round and symmetrical, but there was some variation in size even before activation. Most of the lymphocytes have a relatively smooth surface with many small uniform symmetrical hump-like projections evenly placed over their surface, and occasional small projecting spinous processes. Rarely, small smooth surface openings were seen and a few cells had developed uropods.

The appearances of etched red cells have been described elsewhere (Lewis & Stuart, 1970). Etching of polymorphonuclear neutrophils did not provide much additional information—a rather coarse pattern is produced and the cells appear very soft and thus are easily etched, producing a homogeneous appearance with one or more large elevations due to the underlying nucleus (Fig. 3). Stimulation by any of the mitogens had no discernible effect on either red cells or polymorphs and they will not be described further.

Light etching of lymphocytes revealed a uniform speckled appearance probably due to

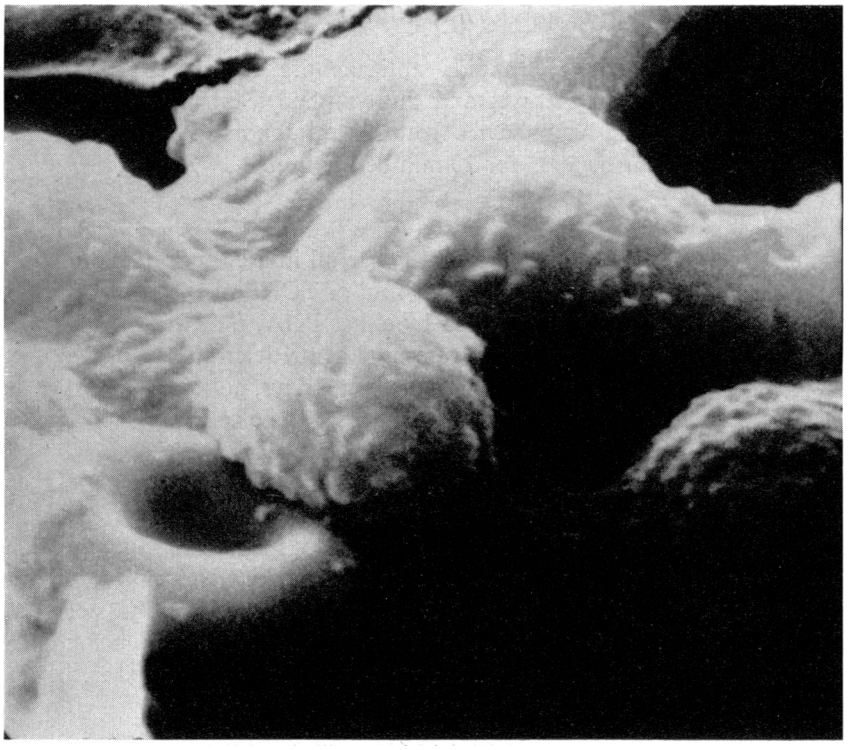

FIG. 1. Unstimulated 3-day culture of normal lymphocytes. A red cell is seen at the bottom of the picture. The other cells are lymphocytes. Cells of two sizes are seen. × 3600.

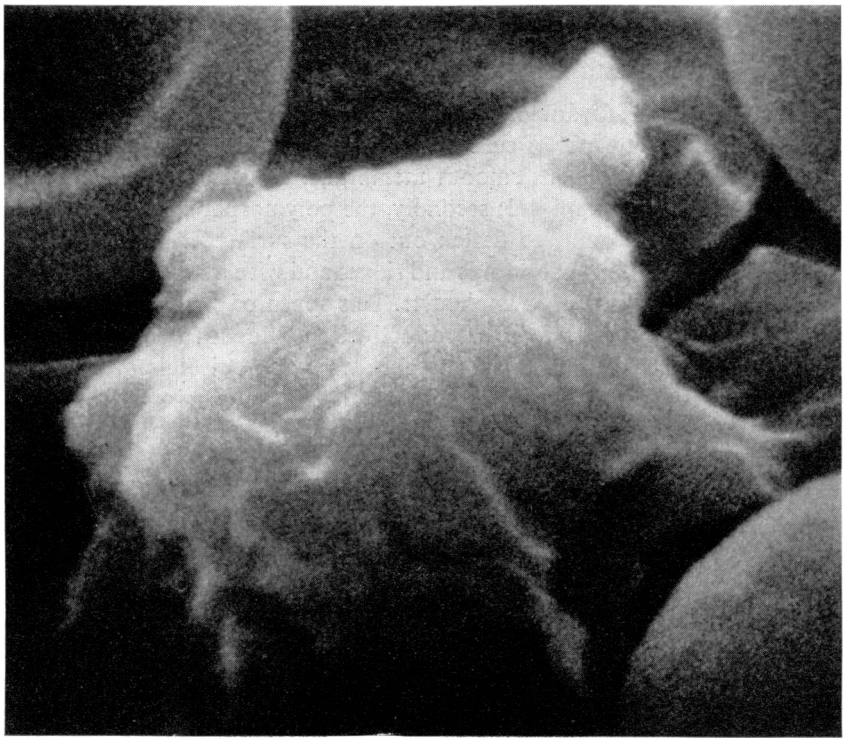

FIG. 2. Normal polymorphonuclear neutrophil, the surrounding cells are red cells. × 8100.

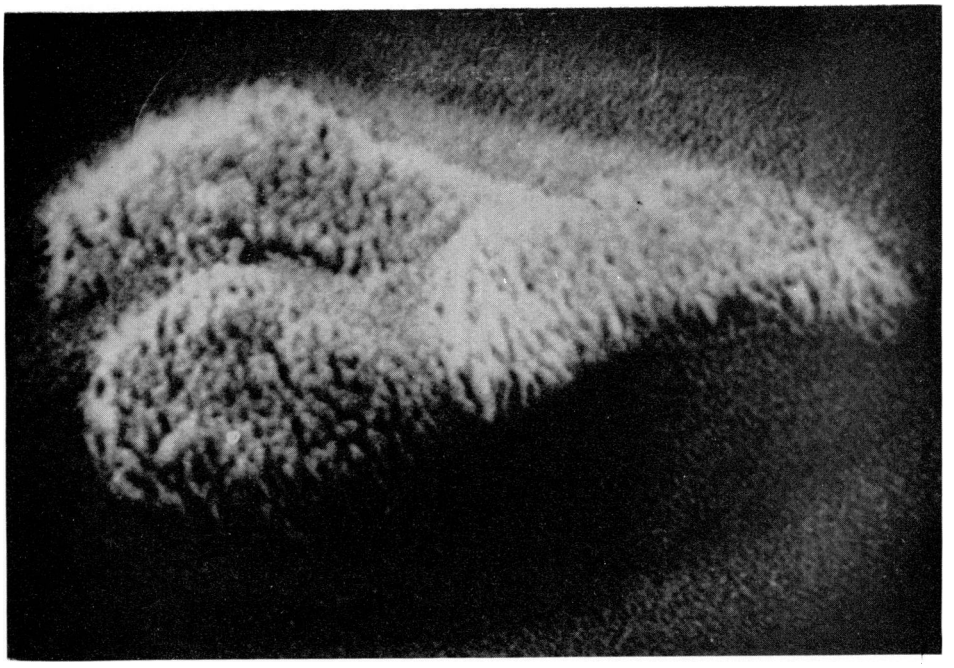

FIG. 3. Medium etching of polymorphonuclear neutrophil to reveal three elevations of underlying nuclei. × 9600.

FIG. 4. Lightly etched unstimulated normal lymphocytes. The cell in lower right-hand corner is probably a polymorphonuclear neutrophil. × 3600.

regularly scattered, superficial areas of relative hardness which protect the underlying structures from etching and thus are thrown into relief by the removal of the intervening softer superficial capsular elements (Fig. 4). In control cultures which had not been stimulated large globular masses which protruded above the remnants of the capsule were demonstrated (Fig. 5). These probably represent the inclusion bodies said to be associated with non-specific stress reactions (Zucker-Franklin, 1969).

Deeper etching did not reveal further structures. This suggests that the lymphocyte cytoplasm and nucleus are probably homogeneous from the point of view of this process, i.e. there are no great variations in physical toughness by contrast with the results obtained by deep etching of the normoblast (Lewis & Stuart, 1970) the neutrophil (Fig. 3) or cultured mouse peritoneal cells (personal observations). Lymphocytes from patients with chronic lymphatic leukaemia had different surface properties. While the untreated surface resembled that of normal lymphocytes, etching was much less effective, less erosion of the surface occurring with a standard degree of etching (Fig. 6). More vigorous etching produced a similar appearance to that found with normal unstimulated lymphocytes (Fig. 7). The number of cells showing changes associated with stimulation were very few and corresponded to the degree of transformation occurring in that population of lymphocytes.

Activated cultures

Activation by either specific or non-specific mitogens leads to aggregation of cells. In the cultures stimulated by PPD, candida or the mixed lymphocyte reaction a proportion of the lymphocytes enlarged and developed small clearly defined ridges and globular projections which produced a nodular surface sharply demarcated from intervening areas of smooth 'normal' surface (Fig. 8). The whole cell surface was scattered with uniform discrete small particles of material. Some of the globular projections had elongated to such a degree that they appeared to be separating. They were most characteristically seen in those cells which developed uropods, this being a sign of a stimulated lymphocyte and they were thus more numerous in activated cultures. The detail of the uropods (foot processes) is well seen in Fig. 9. It consists of several stalk-like prongs each with large globular structures. The prongs are continued as fine nodular structures to meet the corresponding prong of the other side of the uropod. Between the prongs is a fine membrane which in these preparations appears to be a continuous sheet, the whole resembling a horseshoe. The uropods of two lymphocytes occasionally made contact and in these cases the stalks of each uropod were continuous with the other. Whether the uropods in Fig. 9 are from unrelated cells making contact or are the terminal stage of cell division (Marshall & Roberts, 1965; McFarland, 1969; McFarland & Schechter, 1970) cannot be defined in this particular illustration. The surface appearances of non-specifically stimulated lymphocytes were highly characteristic consisting of discrete well-defined nobbly protrusions from the cell surface with relatively normal cell membrane between (Fig. 10). These protrusions were smaller and more numerous than those produced by specific mitogens.

Etching of lymphocytes which had been stimulated by any of the non-specific mitogens revealed large slightly irregular holes in the surface of the lymphocyte (Figs 11–13), these presumably represent areas of localized alteration to the surface structure of the lymphocyte allowing the etching process to be relatively unimpeded. This change was first apparent at 24 hr. No such changes were found in the specifically stimulated lymphocytes and those from the mixed lymphocyte cultures or unstimulated cultures. The delicate uropod is

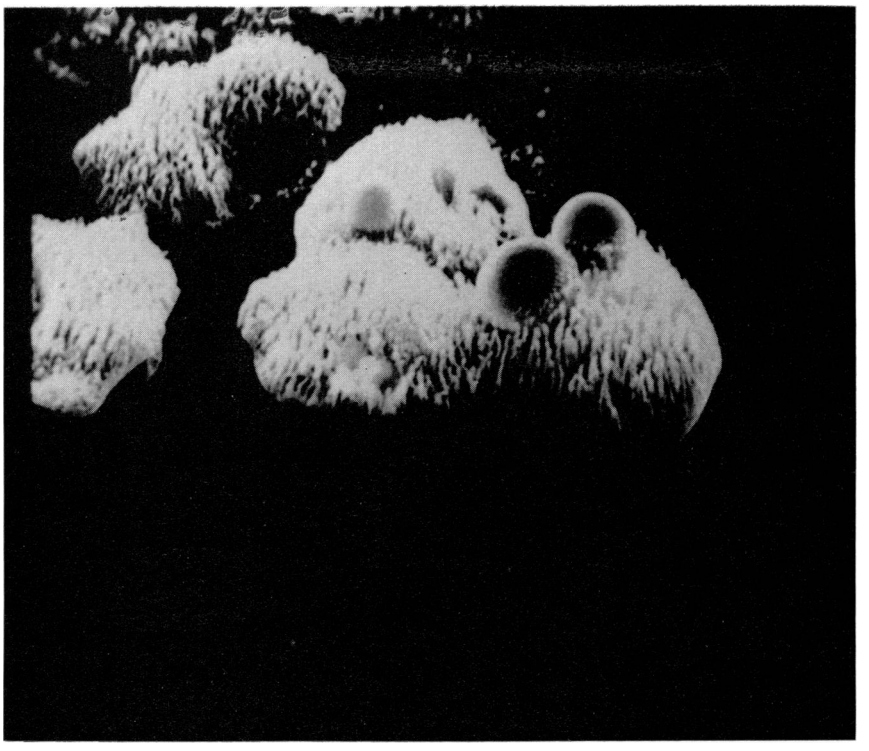

FIG. 5. Unstimulated 3-day culture of normal lymphocytes showing globular structures revealed by etching. × 3600.

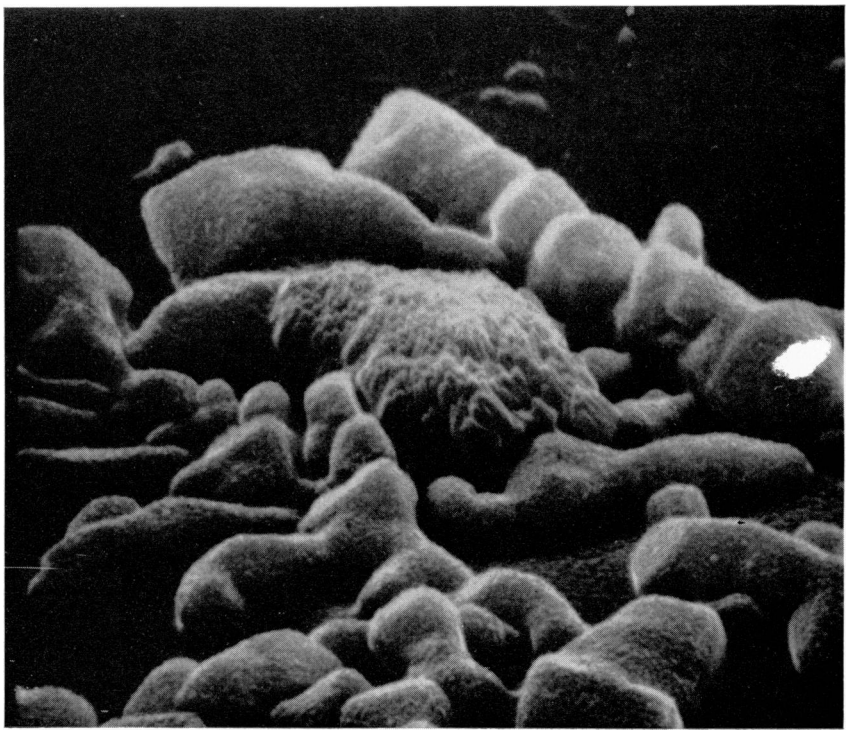

FIG. 6. PHA activated culture from a patient with chronic lymphocytic leukaemia. Light etching has produced much more etching of the large central lymphocyte than of the surrounding lymphocytes. × 3600.

FIG. 7. More vigorous etching of lymphatic leukaemia cells produced the normal pattern found in unstimulated lymphocytes. × 15,750.

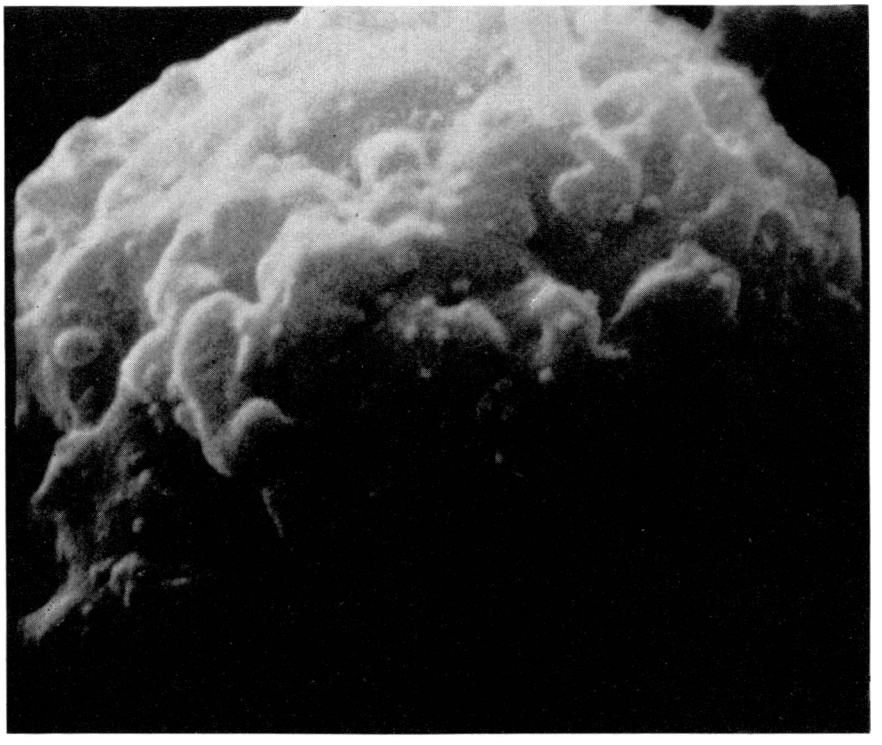

FIG. 8. PPD stimulated lymphocytes showing well demonstrated ridges and nodes arising from a smooth background. The whole surface is irregularly scattered with small uniform particles of material. The uropod is not visible in this view. × 14,400.

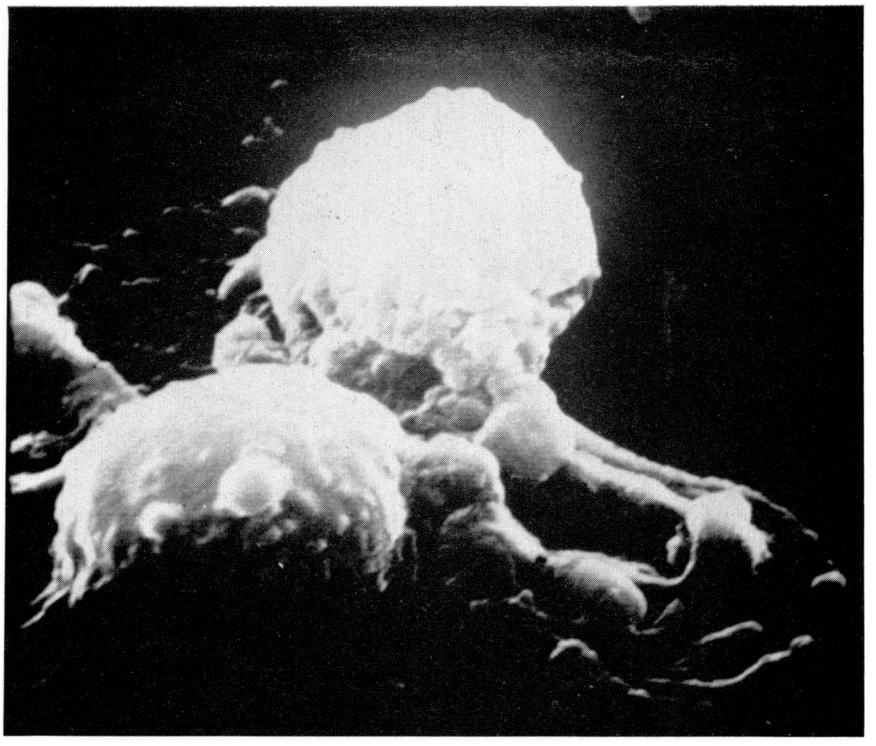

FIG. 9. PPD stimulated normal lymphocyte to show structure of the uropod, probably derived from the top lymphocyte because of altered surface appearance of this lymphocyte. × 7200.

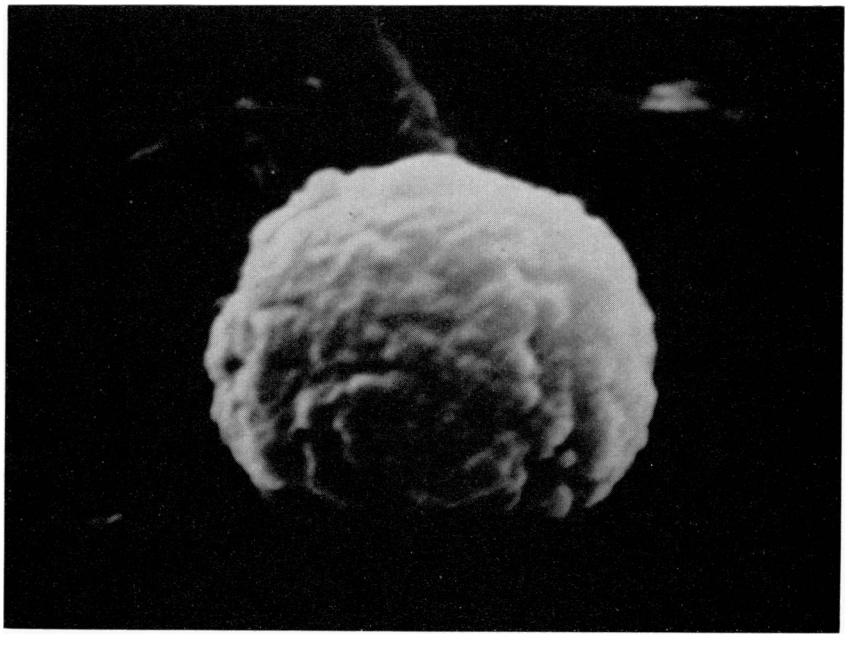

FIG. 10. Normal lymphocyte stimulated by ALS. Note fairly uniform nobbly appearance of surface. × 8000.

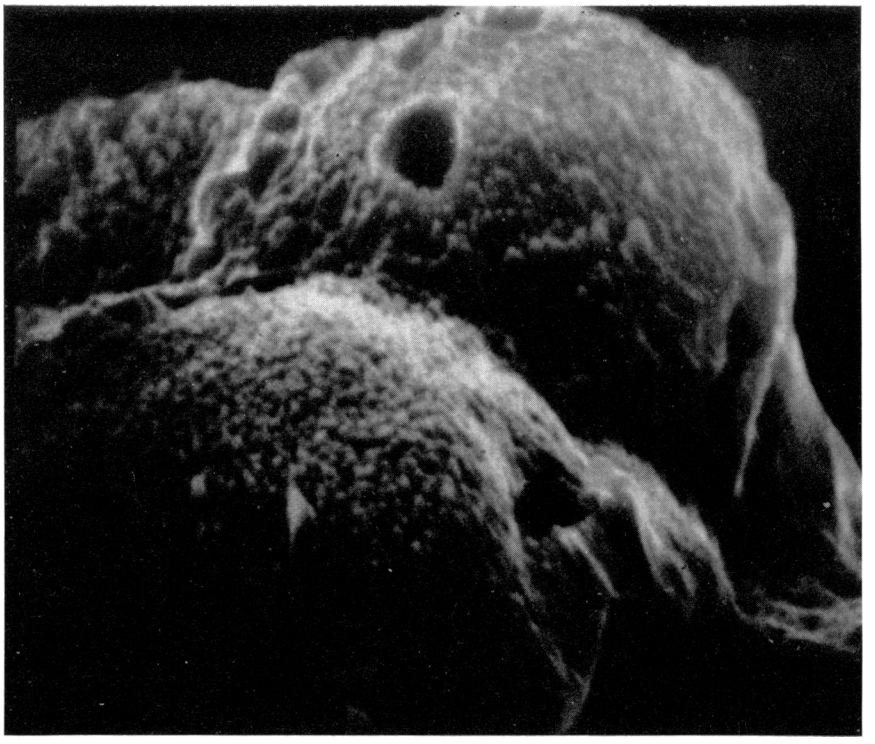

FIG. 11. PHA stimulated cord blood lymphocyte lightly etched to show superficial surface holes. × 10,800.

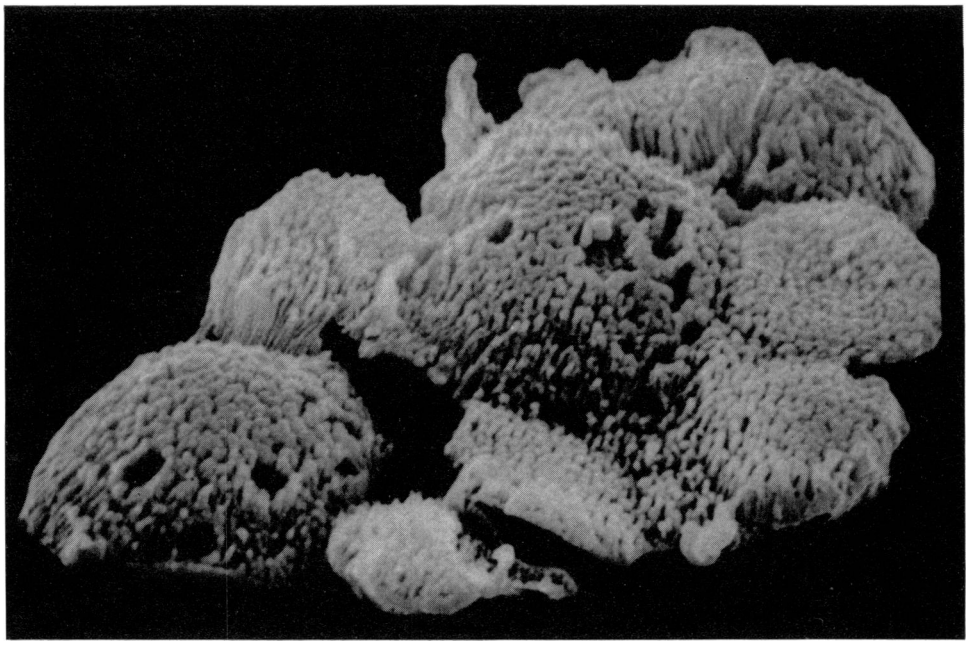

FIG. 12. Four-day pokeweed stimulated culture. The field consists of five unactivated lymphocytes and two larger transforming lymphocytes in whose surface holes produced by etching can be seen. × 4000.

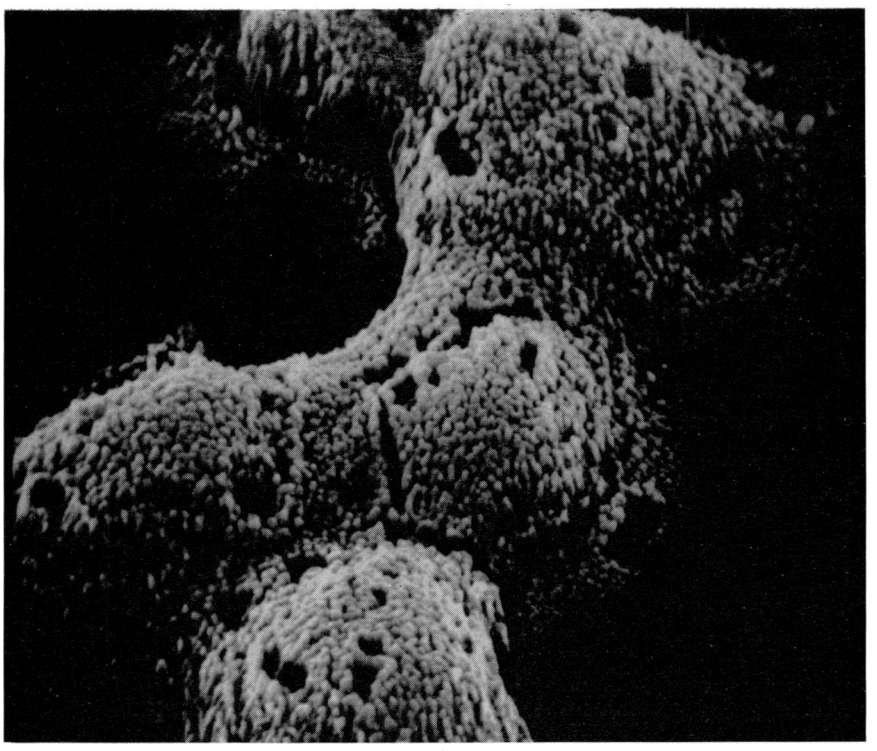

FIG. 13. Four-day ALS stimulated culture of normal lymphocytes, note holes appearing in etched surface of all the lymphocytes. × 3600.

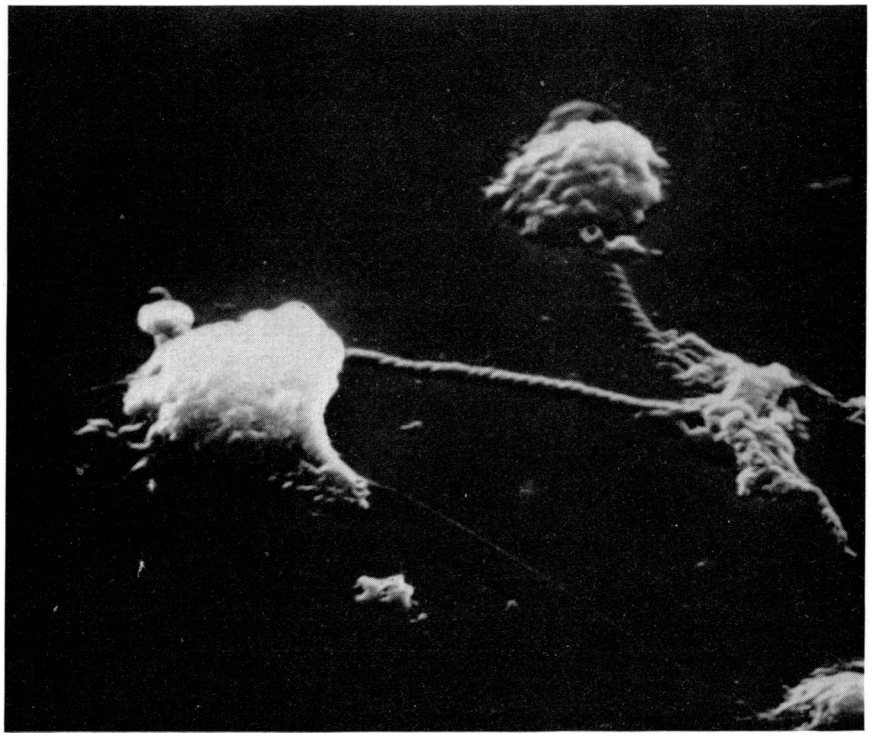

FIG. 14. Culture activated by ALS. A polymorphonuclear neutrophil, joined by a rope-like structure to two lymphocytes. × 3600.

destroyed by even the mildest etching but transforming cells can still be distinguished by their increased size and corresponded in number to the cells developing surface holes on etching.

Certain other phenomena were seen such as connections between lymphocytes and polymorphonuclear neutrophils. These have a definite rope-like structure suggesting they are functional (Fig. 14) and are not artefactual.

DISCUSSION

Relatively little attention has been paid to the surface features of the lymphocyte previously, probably because ultra-thin electron microscopy does not give a very clear impression of these structures. It has been known that the surface of the lymphocyte is uneven since crystae can be demonstrated on the surface by ordinary light microscopy and an undulating appearance of the cell is seen by time lapse and phase microscopy.

The lymphocyte seems to have definite orientation with a relatively rigid uropod (foot process) at one end, by which the cell appears to make contact with its surroundings and a pseudopod at the front end, used in manoeuvring and locomotion of the cell (McFarland, Heilman & Moorhead, 1966).

Previous workers (see above) have shown surface changes corresponding to those shown in our unstimulated lymphocytes. The cells have a regular nobbly surface with little to differentiate them from polymorphonuclear cells except that the latter have a more irregular outline with surface projections which are more elongated and ridge-like.

Activation produces enlargement of the lymphocyte, surface roughening and aggregation (Clarke *et al.*, 1968b) and the development of uropods (Marshall & Roberts, 1965; McFarland *et al.*, 1966). The different appearances obtained using specific and non-specific stimulants are more of degree than definition although the surface changes appear more marked and clear cut in our work. However, etching reveals a distinct difference; surface holes appear to be produced only in cells stimulated non-specifically; these are first apparent at 24 hr and become more numerous later. No such change has been found in polymorphonuclear cells or red cells, but chemical activation with glyceryl triolate is thought to produce flange like folds on the surface of the mouse peritoneal macrophage cell (Carr *et al.*, 1969).

Thus three changes in lymphocyte morphology following mitogenic stimulation have to be considered; the stub-like protrusions, the uropod and the holes produced by etching. We have shown that lymphocytes specifically stimulated with PPD or candida have a different surface appearance from that produced by non-specific stimulation. This presumably represents the effect of the mitogen on the cells or the variable response of the cell to different types of activation. The sites of action of these specific and non-specific mitogens is probably different for the following reasons: most T cells respond to PHA or ALS (Tursi *et al.*, 1969) but only T cells from patients with delayed hypersensitivity respond to the relevant antigen (Oppenheim, 1968), ALS receptors are different from immunoglobulin receptors because of lack of blocking (Klein *et al.*, 1970). Covering immunoglobulin receptors on the cell surface will stop antigenic activation of the lymphocytes but not PHA responses (Greaves, Torrigiani & Roitt, 1971). ALS is an antibody raised against cell constituents and in the presence of complement is cytotoxic. The method of activation of the lymphocyte by specific stimuli such as PPD is not known; however, it is thought that this involves antibody receptor sites on the surface of the lymphocytes, which combine with the antigens, stimulation of the

lymphocyte following (Greaves *et al.*, 1971). The surface stubs seen are quite unlike the antigen–antibody complexes produced on cells (Simonsen & Shklair, 1970) being much larger and more irregular. These complexes are more like the uniform discrete tiny particles seen in our pictures.

Clarke *et al* (1968b) suggest that PPD and ALS can produce altered surface morphology within 15 min which would be compatible with a surface action of the mitogen. Similarly, PHA induces detectable metabolic changes within 15 min (Pogo, Allrey & Mirsky, 1966; Kellander & Rigler, 1969). At this time PHA has not passed further than the cell surface (Michalowski *et al.*, 1965; Conard & Demoise, 1970). We have been unable to show definite changes as early as this as the surface alteration occurred gradually over the first 24 hr.

The development of a uropod by the lymphocyte is thought to represent an activated cell, it was not seen in lymphocytes prepared in suspension but can be demonstrated in cells cultured on flat surfaces. It occasionally occurs in unstimulated cultures but much more frequently in cultures which have been stimulated by mitogens. In view of the much greater mitogenic effect of PHA the number of uropod developing cells produced by PHA seems disproportionately small. Thus it may be that only a subpopulation of PHA responsive cells produce uropods or this change may be readily reversible (McFarland *et al.*, 1966) and perhaps a less persistant feature when non-specific stimulation is used. In the work of McFarland & Schechter (1970) and Biberfeld (1971) the uropod is clearly defined elongated column with buds on the surface and vesicles within. However, Michaelis *et al.* (1971) showed a similar structure as part of a neutrophil. Our work suggests the uropod is a multi-pronged fork-like structure; why there should be a difference between these results is not clear.

The uropod is of great interest, it is thought to be the structure through which activated lymphocytes mediate some of their effects. As shown, it consists of symmetrical stalks which contain globular material whose nature is unknown. However, it is known that lymphocytes during their passage may leave pieces of RNA behind in contact with other cells (Weiss, 1968). The globular material may represent this RNA which is then shed during contact of the uropod with other cells. Other forms of contact have been seen in this work, consisting of rope-like processes between macrophages and lymphocytes. Perhaps these represent two different means of conveying information, programming of the lymphocyte occurring via the bridges and some of the resulting effector mechanisms being mediated via the uropod. However, further mechanisms must exist since cell free fluid can mediate changes at a distance.

The uniformity of the erosion produced by etching of the normal lymphocytes suggests that it is revealing a structural arrangement in the cell membrane. The appearance of the surface holes even with the mildest of etching before the stippled appearance is fully developed (Fig. 11), indicate that the surface alteration is very superficial. If this is so it would appear that the effect of non-specific mitogens is to alter this structure over a localized area allowing etching to occur. The random distribution of the erosions produced imply either a variable extent of the resulting structural alteration or that PHA is attached to the surface of the cell in a random manner. There is no correlation between the number of holes produced in the cell surface by etching and the number of surface stubs induced by mitogens. The mechanism of attachment of the various non-specific mitogens may be different but perhaps the destructive lesion produced is the same. In the case of the non-specific mitogens used, PHA is known to combine with cell surface glycopeptides (Kornfeld

& Kornfeld, 1970) and indeed such attachments seem necessary for its action, possibly to specific receptors (Lindahl-Kiessling & Mattson, 1971). Both PHA and antigens have been shown to increase phospholipid synthesis within minutes of attachment (Pogo *et al.*, 1966). However, the ensuing metabolism is different (Lucas, 1971) only PHA, Concanavalin A and ALS stimulated phosphatidyl inositol production and this correlated with mitogenesis. Thus alteration of phospholipid metabolism may be responsible in part for the observed weakness of the lymphocyte membrane. Similar surface erosions have been produced in Burkitt lymphoma cell lines in tissue culture but not in other types of non-lymphoid cell lines. In the Burkitt lymphoma cell lines these holes are presumably part of the normal activity of the malignant cell line (Holt, Goodall & Pardoe, 1972). These changes are unlikely to be part of the normal immunocyte response since they are not found in specific mitogen induced transformation. Thus we suggest that the site of action is at the level of the cell membrane, producing either a specific change or non-specific micro-injury to the membrane.

Our results indicate that PHA and ALS by producing micro-injury to the superficial layers of the lymphocyte may lead to its activation. This is supported by other workers. Mazzei, Novi & Bazzi (1966) found that mild trypsin, chymotrypsin or papain digestion of the surface membrane could result in lymphocyte transformation. Further, activation by non-specific antigen–antibody complex settling on the cell (Bloch-Shtacher, Hirschorn & Uhr, 1968) or by low concentrations of anti-lymphocyte antibodies is enhanced by complement (Holt, Ling & Stanworth, 1966). A surface action of PHA is also suggested by the alteration of electrophoretic mobility occurring after adding PHA to lymphocytes (Vassar & Culling, 1964). Cell injury produced by micro-wave irradiation (Stodolnik-Baranska, 1967; Turk, Glade & Chessin, 1969) also results in lysosomal changes but not DNA synthesis, producing variable but definitive lymphocyte stimulation. The relative resistance to etching of chronic lymphocytic leukaemia cells indicates increased toughness of the cell membrane and supports the work of Thomson, Robinson & Wetherley-Mein (1966) who showed that CLL cells were more resistant to hypotonic saline shock treatment than normal cells. This alteration in surface structures may also explain the difficulty and delay in activation of CLL cells by PHA (Rubin, Havemann & Dameshek, 1969) if this depends on surface microinjury. The possibility that the PHA responsive cell (T cell) has an intrinsically weaker membrane is suggested by the results of hypotonic saline treatment which leads to bursting of the cell and a reduction in the number of PHA responsive cells (Thomson, Bull & Robinson, 1966).

If PHA responsive lymphocytes represent the long-lived T-cell lymphocyte mediating delayed hypersensitivity, both the observations of Thomson *et al.* (1966) and ourselves might be due to a membrane weakness or defect in thymic-derived cells which results in the selective action of PHA on these cells. Thus the difference between PHA responsive cells and non-responsive cells may depend on different surface structures, which, probably fortuitously, delineates two different functional types of cell. We are unable to propose a physiological reason for these differences.

ACKNOWLEDGMENTS

The assistance of Dr Stuart and Mr Osborn of the National Physics Laboratory, Teddington, and of Roz. Evans and Diane Sherman is gratefully acknowledged. This work was

supported by grants from the Arthritis and Rheumatism Council and the Medical Research Council.

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