LYMPHOCYTE SURFACE AND CYTOPLASMIC
CHANGES ASSOCIATED WITH TRANSLATIONAL
MOTILITY AND SPONTANEOUS CAPPING OF Ig

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SUMMARY
Murine B-lymphocytes during translatory motion undergo a series of changes with respect
to their morphology and distribution of surface immunoglobulins (Ig). The sequence of events
comprising these changes was followed by fluorescence microscopy and a correlated detection
of surface features by scanning microscopy on exactly the same cell. A round, presumably
non-motile lymphocyte exhibited a random distribution of Ig and its surface displayed evenly
distributed microvilli. Formation of a ruffled edge at one pole, accompanied by a decreased
fluorescence at this pole marked the initial events of lymphocyte motility. In the subsequent
stages, the ruffled edge became progressively prominent and displayed a constriction at its base,
while the microvilli were displaced to the opposite pole. Ig in such lymphocytes was localized
at the trailing, microvilli-rich pole. Thin sections of motile lymphocytes revealed Ig, micro-
tubules, microfilaments and coated vesicles as the characteristic features of the trailing end.
These observations may have bearing on the mechanism of lymphocyte motility and spontaneous
capping of Ig.

INTRODUCTION
Binding of a multivalent ligand causes lymphocyte surface receptors to undergo a
lateral redistribution in a process that leads to the 'capping' and often endocytosis of
the ligand-receptor complex (Taylor, Duffus, Raff & DePetris, 1971). Capping, which
is dependent upon temperature and metabolic activity of the cell (Taylor et al. 1971;
Unanue, Karnovsky & Engers, 1973) and is sensitive to the intracellular Ca²⁺ con-
centration (Schreiner & Unanue, 1976a), is often accompanied by cell motility,
although the motility and capping are separable phenomena (Unanue, Ault & Karnovsky,
1974). An intracytoplasmic assembly of microtubules and microfilaments has often
been implicated in the control of receptor mobility (Edelman, Yahara & Wang, 1973;
DePetris, 1975; Unanue & Karnovsky, 1974; Albertini & Clark, 1975; Schreiner &
Unanue, 1976b; Nicolson, 1976; Oliver, 1976). Bourguignon & Singer (1977) have
suggested a direct involvement of actin and myosin in the process of capping. Studies
of Schreiner, Fujiwara, Pollard & Unanue (1977), Gabbian, Chaponnier, Zumbe &
Vasalli (1977) and Toh & Hard (1977) have shown that actin, myosin and tubulin
co-cap with immunoglobulin or concanavalin A caps.
Lymphocytes transformed by Escherichia coli lipopolysaccharide (LPS), a mitogen-
specific for B-lymphocytes, or pretreated with carbamylcholine acquire striking motile
forms upon incubation at 37 °C. Such motile cells undergo spontaneous Ig capping in
the absence of stimulation by anti-Ig (Schreiner, Braun & Unanue, 1976), thus eliminating the prerequisite of ligand cross-linking of receptors. A non-random distribution of \( \theta \), ALS-antigen and Con A receptors on uropod-forming prefixed thymocytes has also been reported by DePetris (1978). Recently, Braun, Fujiwara Pollard & Unanue (1978) have shown that spontaneous capping of Ig, Fc receptors and thymus leukaemia antigen is accompanied by the segregation of cytoplasmic myosin, which can be localized under the cap.

This study was designed to understand further the process of lymphocyte motility that leads to spontaneous displacement and polar distribution of Ig. The technique employed was earlier devised by Karnovsky & Unanue (1978) for a correlated fluorescence and scanning electron-microscopic study of anti-Ig-induced capping of mouse lymphocytes. This allowed us to carry out a sequential and correlated immunofluorescence and scanning electron-microscopic analysis of the cells undergoing spontaneous capping and the results are discussed in terms of surface modifications and a possible involvement of submembranous contractile filaments.

**MATERIALS AND METHODS**

**Lymphocyte preparation**

Spleens from A/St mice (West Senece Laboratories, Buffalo, N.Y.) constituted the source of B-lymphocytes. Spleens were excised and single-cell suspensions were prepared using sterile techniques. The cells \( 10 \times 10^7 \) were washed with 40 ml of RPMI 1640 medium supplemented with 5 % (v/v) heat-inactivated foetal calf serum by centrifugation at 200 g for 8 min. The washing procedure was repeated two more times.

**Lymphocyte culture**

Cultures were set up in 12 x 75 mm culture tubes (Falcon, Oxnard, Ca.), each tube containing \( 1.5 \times 10^7 \) cells in 1 ml of RPMI 1640 medium supplemented with 5 % foetal calf serum. *Escherichia coli* lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) was added to the lymphocyte suspensions to a concentration of 20 \( \mu \)g/ml. After 2 days in culture, the lymphocytes were centrifuged on Ficoll-Hypaque density gradient (Perper, Zee & Michelson, 1968) in order to eliminate dead cells. The lymphocytes were collected from the interface, washed 3 times with Hanks’ balanced salt solution (HBSS) containing 10 mM 2-hydroxyethylpiperezine-N-2-ethanesulphonic acid (HEPES) (Microbiological Associates, Bethesda, Md.) by centrifugation at 200 g for 8 min each time and finally suspended to a concentration of \( 2.5 \times 10^7 \)/ml. Further preparation of cells for fluorescence and scanning electron microscopy was done essentially in the same manner as described earlier (Karnovsky & Unanue, 1978).

**Lymphocyte labelling with anti-Ig and fluorescence microscopy**

Coverslips used for collecting the lymphocytes were precleaned by boiling in 10 % (v/v) of 7X detergent (Flow Laboratories, Hamdon, Ct.) for 30 min, followed by several rinses in double-distilled water and a final rinse in 100 % ethanol. The dried coverslips were lightly etched in an interrupted series of lines by a diamond scorer and placed in 16-mm-diameter multiwell tissue culture dishes (Costar, Cambridge, Mass.). Cells, \( 5 \times 10^6 \), in 0.2 ml of HBSS were layered on each coverslip, allowed to incubate undisturbed, first at 4 °C for 10 min and then at 37 °C for 30 min. At this time, fixation was carried out by adding 2 % formaldehyde. After 30 min of fixation, the coverslips containing cells were rinsed at least 3 times with phosphate-buffered saline (PBS) pH 7.3. PBS contains 8.0 g NaCl, 0.2 g KCl, 0.2 g KH2PO4, and
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0.15 g Na₂HPO₄ in 1 l of distilled water. Cells were then treated with 100 μg/ml of fluorescein-conjugated rabbit anti-mouse Ig (FITC-RAMG) for 30 min. The preparation and specificity of the FITC-RAMG has been described elsewhere (Unanue, Perkins & Karnovsky, 1972). The cells were rinsed again with PBS and examined and photographed in a Leitz Orthoplan microscope with Ploem epi-illumination.

Scanning electron microscopy

The cells, following examination by fluorescence microscopy, were postfixed in 1% aqueous osmium tetroxide for 1 h. Dehydration was carried out through a graded series of acetone, followed by critical-point drying in a Samdri pvt-3 critical point drying apparatus using carbon dioxide. The coverslips containing cells were then mounted by metal-adhesive tapes on aluminium studs and coated with gold-palladium under vacuum.

Micrographs taken by light and fluorescence microscopy were used to locate the same cells by scanning electron microscopy, using the etched lines on the coverslips as landmarks. The cells were photographed in an ETEC scanning electron microscope using an accelerating voltage of 20 kV and a tilt angle of 30°.

Transmission electron microscopy

Lymphocytes incubated on coverslips at 37 °C for 30 min, as described above, were fixed for 1 h by addition of 2.5% glutaraldehyde (Taab Laboratories, Reading, England) in PBS, pH 7.3. The cells were thoroughly rinsed with PBS, treated first with FITC-RAMG and then with anti-FITC antibody (Abbas, Ault, Karnovsky & Unanue, 1975) conjugated with ferritin by the method of Kishida, Olsen, Berg & Procop (1975), postfixed with 1% aqueous osmium tetroxide, rinsed several times with PBS and dehydrated through a graded series of ethanol.

Following absolute ethanol grade, the cells were treated with 0.05 M hafnium chloride (Alfa Products, Danvers, Mass.) in absolute acetone for 1 h. This treatment is believed to enhance the contrast of microtubules (Karnovsky, unpublished observation). Cells were rinsed 3 times with absolute acetone and embedded in Epon by inverting the Epon-filled beam capsules over the coverslips. Following polymerization, the coverslips were separated by immersing the blocks in liquid nitrogen and ultrathin sections of lymphocyte monolayers were cut by a diamond knife using LKB Ultrotome III. Sections were stained with lead citrate and examined in a Philips 200 electron microscope.

RESULTS

Murine splenic B-lymphocytes transformed by LPS over a period of 48 h and then incubated on coverslips at 37 °C for 30 min revealed certain variations with regards to their morphology and distribution of surface immunoglobulins. A round cell presumed to be a non-motile B-lymphocyte exhibited a random distribution of Ig on its surface as judged by a diffuse immunofluorescence staining. Scanning electron microscopy of such lymphocytes revealed numerous evenly distributed microvilli over the cell's entire surface (Fig. 1). With the onset of motility, the lymphocytes underwent a series of changes with regard to the immunofluorescence staining pattern and surface architecture. A sequential analysis of the cells in motility revealed that in the initial stages of movement, the advancing end of the cell membrane stretched out, lost microvilli and exhibited a ruffled edge. A constriction was often noticed at the base of the ruffle (Fig. 2). A simultaneous decrease in the immunofluorescence at the ruffled end and a corresponding increase at the opposite pole was immediately noticed indicating the beginning of Ig mobility. Most of the microvilli by this time were seen
Fig. 1. A round, presumably non-motile cell exhibiting numerous evenly distributed microvilli. The cell is diffusely labelled with FITC-RAMG (inset). ×10500; inset, ×1300.

Fig. 2. A cell showing ruffles at one end. Microvilli still occupy the major part of the cell surface. A close observation of the fluorescent micrograph reveals a slight decrease in the staining intensity of FITC-RAMG at the ruffled end (inset). ×10000; inset, ×1300.

Fig. 3. A cell exhibiting non-uniform distribution of microvilli, which are denser at the trailing end (lower left) and reduced at the advancing end (top right). There is an increased fluorescence intensity at the trailing end and a decreased intensity at the advancing end (inset). ×10500; inset, ×1300.

Fig. 4. A cell showing ruffling activity at 2 opposite poles, while the microvilli occupy the intermediate area. The FITC-RAMG staining seems to be selectively associated with the areas occupied by microvilli but depleted from the ruffled edges (inset). ×10000; inset, ×1300.
Fig. 5. Cell showing a smooth ruffled edge (top). Microvilli are seen only on the bottom half of the cell. Area of the cell surface localized by FITC-RAMG corresponds to the microvilli-rich bottom half (inset). ×10000; inset, ×1300.

Fig. 6. An elongated cell exhibiting a prominent microvilli-free ruffled edge with a constriction at its base and microvilli at the trailing end. Ig-cap is seen at the trailing end of the cell (inset). The area anterior to the constriction is not visible due to the absence of fluorescence in this region of the cell. ×9000; inset, ×1300.

Fig. 7. A cell exhibiting elongated shape and a distinct contractile ring slightly posterior to the middle of the cell body. Microvilli are densely packed at the trailing end. A few elongated microspikes seem to anchor the cell to the substratum. A distinct Ig-cap is seen at the microvilli-rich trailing end, while the anterior parts of the cell margin are not stained by FITC-RAMG (inset). ×9000; inset, ×1300.

Fig. 8. Major part of the cell surface seen in this micrograph is smooth and exhibits ruffled edges. Microvilli are densely packed on a compact protuberance at the trailing end. FITC-RAMG staining is restricted to the trailing end (inset). The remainder of the cell is not visible. ×9000; inset, ×1300.
to have migrated towards the opposite pole (Fig. 3). Occasionally, ruffles developed at the 2 opposite poles of a cell, with microvilli occupying an intermediate area. Such a cell exhibited a bipolar cap associated with the microvilli and the Ig-depleted areas represented by the 2 ruffled poles (Fig. 4). Under normal circumstances of unidirectional motility, however, the ruffled end in the next stage became more pronounced with a distinct constriction at its base. Microvilli and Ig seemed to be selectively displaced from the ruffled edge towards the opposite pole (Fig. 5). Lymphocytes next exhibited an elongated morphology with a constriction in the middle separating a well

Fig. 9. Thin section of the trailing end of a motile lymphocyte exhibiting Ig-cap detected by the ferritin-antibody technique (see text for details). Distinct microfilaments are seen in the microvilli. Note the higher concentration of ferritin particles on the microvilli than on smooth areas of the cell membrane. A number of coated vesicles (arrows) are seen in the cytoplasm at this pole of the cell. × 37700.
developed ruffled end from the opposite pole, which exhibited a dense organization of microvilli and Ig (Fig. 6). The constriction, still quite prominent and appearing to form a contractile ring slightly posterior to the middle of the cell seemed to restrict the microvilli and Ig at the trailing end (Fig. 7). Cellular projections, distinctly larger than microvilli were also seen at this end. Such projections, measuring 0.2 \( \mu \)m in diameter and ranging from 2 to 30 \( \mu \)m in length have been referred to as microspikes, microextensions or filopodia by various authors (Albrecht-Buehler, 1976; Albrecht-Buehler & Goldman, 1976; Taylor & Robbins, 1963; Trelstad, Hay & Revel, 1967). In motile lymphocytes, they appeared to anchor the cell to the substratum, while the ruffles were stretched at the opposite pole, possibly in an effort to develop new adhesive sites on the substratum. The lymphocyte in the final stage exhibited compact microvillous accumulation at the trailing end, which was now represented by a short protuberance, while the majority of the cell’s surface was marked by the presence of well defined ruffles but devoid of microvilli (Fig. 8). Similar changes associated with anti-Ig induced capping have earlier been described by Karnovsky & Unanue (1978).

Motile, capped lymphocytes in thin sections showed that Ig was selectively denuded from the ruffled areas but associated with microvilli at the opposite pole, as revealed by the ferritin-antibody technique. Furthermore, the ferritin particles in motile cells were present at higher concentration on microvilli than on the smooth areas of the cell membrane between them. An accumulation of linearly arranged or a meshwork of cytoplasmic microfilaments was often noticed at the trailing end. Distinct microfilaments could also be seen running through the microvilli. Microtubules in most instances were observed in the deeper areas of the cytoplasm beneath the cap. In addition to microfilaments and microtubules, coated vesicles were constantly seen in the cytoplasm at the capped pole (Fig. 9). They could be localized, both near the surface in association with the microfilaments or deeper in the cytoplasm, intermixed with the microtubules.

**DISCUSSION**

Lymphocyte surface alterations may be related to the cell’s functional state, differentiation stage or the forces in its microenvironment (Van Ewijk, Brons & Rozing, 1975; Roath, Newell, Polliack & Alexander, 1978; Bhalla & Karnovsky, 1978). Lymphocytes during locomotion exhibit various changes in their morphology and they can be distinguished from stationary lymphocytes on the basis of their amoeboid appearance (Lewis, 1931; Unanue et al. 1974; Schreiner & Unanue, 1976). Previous studies had established that 24-48 h culture of B cells with LPS markedly stimulated a locomotory response of the B cells. The motile cells exhibited Ig in a cap. Non-motile round cells showed Ig in a diffuse pattern. Such spontaneous capping was also seen in B cells undergoing locomotion without the previous incubation with any stimuli (Schreiner et al. 1976; Braun et al. 1978). Spontaneous caps did not require detection with a bivalent anti-Ig. These observations excluded that LPS was causing Ig-capping by cross-linking, and also indicate the spontaneous nature of Ig capping in motile cells. In the current study, we have relied on lymphocyte shape and surface
architecture in determining the motility events. A rounded lymphocyte with its surface exhibiting evenly distributed microvilli but devoid of any ruffling activity was characterized as a non-motile cell. Following contact with the substratum, lymphocytes showed certain surface alterations suggestive of motility. The progressive stages of movement were assessed by the relative amount of surface ruffling, displacement of microvilli towards one pole and deviation of the cell form from the rounded appearance.

Abercrombie, Heaysman & Pegrum (1970) have suggested that the lamellipodia at the edge of a moving fibroblast result from the assembly of new surface, which may be favoured by the longitudinal pull applied to the plasma membrane by the cytoplasmic microfilaments (Harris, 1976). Function of microfilaments in cell locomotion and surface ruffling has also been suggested by others (reviewed by Goldman et al. 1973; Korn, 1978). Our scanning electron-microscopic observations indicating a ruffled advancing end and a microvilli-rich trailing end of the motile lymphocytes are consistent with the suggestion of a more stretched anterior region of the plasma membrane and the least stretched region at the posterior end (Harris, 1976). In light of such studies, it seems reasonable to speculate that during the initial stages of motility and in the course of local stretching of plasma membrane in the ruffle, a link between microfilaments and certain membrane proteins is established. In this type of linkage, certain membrane receptors are selectively recognized by the underlying microfilaments without prior crosslinking of the receptors into aggregates, which has been previously proposed to be a requirement for actin or myosin mediated capping (Bourguignon & Singer, 1977). Subsequently, the displacement of microfilaments and the linked proteins may occur in a coordinated manner. According to Borisy & White's (1978) model for cytokinesis in animal cells, a signal emitted from the poles of a mitotic spindle perturbs the filamentous net underlying the plasma membrane such that the tension at the cell surface becomes greater in the equatorial than in the polar regions. As a result of this tension imbalance, the elements of the net become re-distributed and concentrated at the equator, followed by a constriction leading to cleavage of the cell. Such a model may also help explain the formation of a constriction seen behind the ruffled edge in motile lymphocytes. However, the nature of the signal responsible for the formation of the constriction is not clear. Since the local membrane constrictions in a motile lymphocyte appear to proceed from the advancing to the trailing end, they may be envisaged as the cellular specializations favouring the displacement of the complexes of ligand-receptor and locally contracted microfilaments to the trailing end. Microspikes seen at the trailing end of motile lymphocytes might be vital for anchorage and cell motility. Their role in particle transport, cell locomotion and as sensory organs have been discussed by Albrecht-Buehler (1976) and Albrecht-Buehler & Goldman (1976). The selective capping of Ig but not H and H2 may reflect a difference in the nature of insertion of these receptors in the membrane and their interaction with integral membrane proteins and/or with actin and myosin. Presence of a meshwork or linearly arranged microfilaments below the cap probably serves to maintain the cell's motile form and restricts Ig in the cap. Accumulation of microfilaments at the trailing end of the motile lymphocytes and the fact that the density of
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ferritin particles is more on the microvilli, which are clearly rich in microfilaments, further supports the suggestion of an association between microfilaments and receptors during cell motility. Although a physical linkage between Ig and microfilaments during spontaneous capping seems quite likely, the function of microfilaments in balancing the forces created by regional alterations in the physical properties of the capped membrane (Albertini, Berlin & Oliver, 1977) may be equally important.

A directional flow of lipids alone (Bretscher, 1976) or of lipids and proteins (Harris, 1976) of plasma membrane has been hypothesized to explain the capping of surface antigens. Bretscher (1976) has suggested coated vesicles as a means for lipid resorption into the cell. General occurrence of the coated vesicles in the cytoplasm at the trailing end of motile lymphocytes supports the recycling phenomenon; however, it is hard to say if the recycling accompanying motility involves only lipids (Bretscher, 1976) or membrane integral proteins and carbohydrate components also (Harris, 1976). Studies showing an association of coated pits with stress fibres in human fibroblasts (Anderson et al. 1978) and the occurrence of coated vesicles in association with microfilaments and microtubules at the trailing end of motile lymphocytes may suggest a possible role of contractile elements in membrane-recycling.

In conclusion, then, using the correlated immunofluorescence and scanning electron microscopy, this study presents a systematic evaluation of the surface features of the lymphocytes undergoing motility and spontaneous capping of Ig. The observations reported may be relevant to the mechanism underlying motility and capping. The suggestions are supported by the thin-section observations. The study is also consistent with the hypothesis suggested earlier that the ruffles at the anterior edge reflect a degree of membrane flow from that pole posteriorly (Karnovsky & Unanue, 1978).

We thank Dr E. R. Unanue for his advice, Mr Robert Rubin for photographic help and Ms Kay Cosgrove for assistance in manuscript preparation. This work was supported by Grants AI 10677 and GM 01235 from the N.I.H., U.S.P.H.S.

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(Received 2 February 1979)