MOLECULAR AND CELLULAR BIOLOGY, Oct. 1981, p. 939-948
0270-7306/81/100939-10$02.00/0

Vol. 1, No. 10

Effects of Free Fatty Acids on the Organization of Cytoskeletal Elements in Lymphocytes

R. L. HOOVER,[1]* K. FUJIWARA,[2] R. D. KLAUSNER,[1]† D. K. BHALLA,[1]‡ R. TUCKER,[3]§ AND M. J. KARNOVSKY[1]

Department of Pathology[1] and Department of Anatomy,[2] Harvard Medical School, and Sidney Farber Cancer Institute,[3] Boston, Massachusetts 02115

Received 27 March 1981/Accepted 15 July 1981

Treatment of mouse lymphocytes with *cis*-unsaturated free fatty acids produced alterations in the immunofluorescence patterns of the cytoskeleton and contractile proteins. Saturated free fatty acids and *trans*-unsaturated free fatty acids had no effect. In untreated cells, the microtubular pattern exhibited radiation from an organizing center, resembling the spokes of an umbrella. The addition of linoleic acid produced a polarized submembranous aggregate. Under control conditions, staining for actin revealed a diffuse pattern over the entire cell, but the addition of linoleic acid caused the formation of a single large patch, or polarized submembranous aggregate. The pattern for α-actinin normally revealed intense perinuclear staining on a diffuse background. Linoleic acid caused the loss of this pattern and the formation of a polarized submembranous aggregate. Linoleic acid treatment also caused the pattern for myosin to change from diffuse to uniform submembranous patching around the periphery of the cell. For all of these proteins, calcium (8 mM), but not magnesium, partially reversed the effects of linoleic acid. Sodium azide had little effect on the normal distribution of actin, tubulin, and α-actinin; however, myosin staining revealed prominent patch formation. Colchicine treatment caused diffuse staining, some polarized submembranous aggregate formation of tubulin, and some patching of myosin, but not as extensively as did treatment with linoleic acid. Actin and α-actinin were unaffected. These results, in view of the previously shown facts that pretreatment of cells with linoleic acid followed by anti-immunoglobulin inhibits capping of surface immunoglobulin (Klausner et al., Proc. Natl. Acad. Sci. U.S.A. **77**:437-441, 1980) and that free fatty acids partition into the surface membrane (Klausner et al., J. Biol. Chem. **255**:1286-1295, 1980), suggest that the perturbation of the plasma membrane with unsaturated free fatty acids alters the interaction of surface receptors with the cytoskeleton, which in turn affects cytoplasmic distribution of the proteins.

Recent advances in biochemical, ultrastructural, and immunocytochemical approaches have established the presence of cytoplasmic contractile proteins and microtubules as components of the cytoskeleton in eucaryotic cells. It is believed that these cytoskeletal elements are involved in many aspects of cellular function (2, 16, 17, 20). One feature of the cytoskeleton that is of particular interest is its possible relationship to the cell membrane. It is probably involved in many membrane-mediated events, such as receptor movement, membrane protein mobility, and internalization of surface molecules. Unanswered questions concerning cytoskeleton-membrane interactions include how the cytoskeleton attaches to the membrane, how perturbations in the membrane affect the cytoskeleton, and which of the cytoskeletal elements are responsible for membrane-initiated cytoskeletal events (22).

We have used low concentrations of free fatty acids to perturb membrane structure. Previous work from this laboratory has shown that free fatty acids can be divided into two classes according to their structure and their influence upon the organization of biological membranes (15). The group A free fatty acids (*cis*-unsaturated) disorder the acyl chain interior, whereas the group B free fatty acids (saturated and *trans*-unsaturated) do not. Furthermore, the

† Present address: Laboratory of Theoretical Biology, National Institutes of Health, Bethesda, MD 20205.

‡ Present address: Cell Biology Section (151E), Veterans Administration Hospital, San Francisco, CA 94121.

§ Present address: Department of Medical Oncology, Johns Hopkins Oncology Center, Baltimore, MD 21205.

group A free fatty acids partition preferentially into fluid lipid domains, whereas the group B free fatty acids preferentially partition into less fluid lipid domains. We have also documented the existence of lipid domains in plasma membranes by using the technique of fluorescent lifetime heterogeneity analysis (15). We have since extended these structural studies and shown that the group A free fatty acids inhibit surface receptor capping in lymphocytes, whereas the group B free fatty acids have no such effect (13, 14), and we have derived a model (14) in which we hypothesize that the group A free fatty acids, by perturbing membrane lipid structure, cause a disruption of some form of linkage between the cytoskeleton and the membrane. Because of the postulated role of the cytoskeleton in receptor capping, we examined in this paper the effects of a group A free fatty acid (linoleic acid [LA]) and a group B free fatty acid (stearic acid [SA]) upon the structure of the lymphocyte cytoskeleton. We found that group A, but not group B, free fatty acids have a marked effect upon the structure of the lymphocyte cytoskeleton. This effect was partially reversed by Ca^{2+}, but not Mg^{2+}, and the dose response and the ionic sensitivity paralleled previous findings concerning the reversal by Ca^{2+} of group A free fatty acid inhibition of surface immunoglobulin capping (14).

MATERIALS AND METHODS

Lymphocyte isolation. Lymphocytes were isolated from spleens of A/st mice obtained from West Seneca Laboratories (Buffalo, N.Y.) and A/J mice obtained from Jackson Laboratories (Bar Harbor, Maine). An initial lymphocyte preparation was obtained by teasing the spleens in Hanks balanced salt solution buffered with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4 (HH). The cells were then washed twice with HH, and the erythrocytes were lysed with the addition of distilled water for 20 s. The final pellet was suspended at a concentration of 5×10^6 to 10×10^6 cells per ml of HH.

Adhesion assay. The adhesion of lymphocytes to plastic and polylysine-coated plastic was measured essentially by the methods of Walther et al. (21). Lymphocytes were labeled with ^{51}Cr (as sodium chromate in normal saline, 200 to 500 Ci/g of chromium; New England Nuclear Corp., Boston, Mass.) for 1 h, washed once with HH, and suspended at a concentration of 5×10^6 to 10×10^6 cells per ml of HH. A 0.5-ml amount of this suspension was added to each well of a Costar cluster dish which had been treated with polylysine (1 mg/ml of distilled water) for 15 min before the addition of the lymphocytes. After 30 min of incubation at 37°C, the unattached cells were washed off, and the remaining attached cells were lysed with 0.5 ml of 1 N NH_4OH. This was mixed with 9.5 ml of Biofluor (New England Nuclear Corp.) and counted on a Beckman liquid scintillation counter (model LS-333). The percentage of cells adhering was calculated by comparing counts obtained after the 30-min incubation with those for unincubated samples.

Antibodies. Rabbit antisera against sea urchin cytoplasmic tubulin (9), human platelet myosin (8) and chicken gizzard α-actinin (10) have already been characterized. The antiactin used in the study was a gift from Keith Burridge, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Absorbed antisera were prepared by incubating each antiserum (0.5 ml) overnight (22°C) with 1 mg of the appropriate antigen (sea urchin tubulin, human platelet myosin, chicken gizzard α-actinin, or human platelet actin). The mixture was then precipitated with cold acetone, fixed with 10% Formalin in phosphate-buffered saline (PBS), pH 7.2, and centrifuged at $3,000 \times g$ for 15 min. The supernatant was collected and used as absorbed antiserum.

Indirect immunofluorescence. Mouse lymphocytes, both normal and appropriately treated, were allowed to attach to microscope cover slips coated with polylysine in a moist chamber for 30 min at 37°C. The cells were fixed with 10% Formalin (Mallinckrodt Inc., St. Louis, Mo.) in PBS for 10 min at 22°C and washed in PBS for 15 min with several changes. The cells were then permeabilized with acetone at −20°C for 5 min. After air drying, the lymphocytes were treated with primary antibodies diluted 50 to 100 times with PBS for 45 min at 37°C. The cells were washed in PBS for 15 min and stained with either fluorescein-labeled (Miles Laboratories, Inc., Elkhart, Ind.) or rhodamine-labeled (Cappel Laboratories, Downington, Pa.) goat anti-rabbit immunoglubulin G diluted 250 to 500 times with PBS for 45 min at 37°C. After 15 min of PBS wash, they were mounted with 90% glycerol in PBS. The fluorescence was observed with a Leitz Orthoplan microscope equipped with xenon (for fluorescein excitation) and mercury (for rhodamine excitation) lamps, a vertical illuminator with K2 (fluorescein observation) and N2 (rhodamine observation) filter blocks, and a Zeiss 63× planapo phase objective lens (numerical aperture, 1.4). Fluorescent images were recorded with a Leitz Orthomat microscope with Tri-X film (Eastman Kodak Co., Rochester, N.Y.).

The staining controls included omission of the primary antibody, substitution of the primary antibody with the preimmune serum, and substitution of the primary antibody with absorbed antiserum.

Fatty acid addition. Fatty acids were prepared in stock solution at a concentration of 10 mg/ml of absolute alcohol. These were then stored at −20°C under N_2. All experiments were carried out at a concentration of 10 μg/ml; therefore, 1 μl of the stock solution was added per ml of cell suspension (2×10^6 to 3×10^6/ml). Cells were treated at room temperature for 30 min before fixation and subsequent staining with the fluorescent antibodies. Samples containing equal amounts of alcohol and no fatty acids and samples containing fatty acids and the antioxidant α-tocopherol were examined periodically with the fluorescent antibodies. The patterns produced by these two samples were similar to those for alcohol-free controls and LA-treated cells, respectively.

The effects of divalent cations were examined by

first incubating the cells with the fatty acids for 30 min at room temperature. The cells were then rinsed once in calcium-magnesium-free Hanks balanced salt solution, pH 7.4, and suspended in the same solution with either Ca^{2+} or Mg^{2+}. The lymphocytes were incubated in the latter medium for 15 min before fixation and staining.

Electron microscopy. Lymphocytes were fixed for 1 h by the addition of 2.5% glutaraldehyde (Taab Laboratories, Reading, United Kingdom) in PBS, pH 7.3, postfixed in 1% aqueous osmium tetroxide, rinsed with PBS, and dehydrated through a series of alcohols. The cells were then treated with 0.05 M hafnium chloride (Alfa Products, Danvers, Mass.) in 100% acetone for 1 h. This treatment enhances the contrast of the microtubules (M. J. Karnovsky, unpublished data). Cells were rinsed three times with 100% acetone and embedded in Epon. Ultrathin sections were cut, stained with lead citrate, and examined in a Philips 200 electron microscope.

Cells prepared for scanning electron microscopy were fixed in 2% glutaraldehyde in PBS for 30 min, rinsed several times in the same buffer, attached to polylysine-coated cover slips, and postfixed in 2% osmium tetroxide for 60 min. The samples were dehydrated in a graded series of acetone and brought to the critical drying point out of liquid CO_2 in a Samdri PV-2 critical-point dryer (Tousimis Research, Rockville, Md.). The cells were coated with gold-palladium in a sputtering device (Technics, Alexandria, Va.) and examined in an ETEC Autoscan electron microscope.

RESULTS

Microtubules. The distribution of staining with antitubulin antibodies in untreated cells was quite distinctive (Fig. 1A). Virtually all cells exhibited a fine network, resembling umbrella spokes, emanating from an organizing center, similar to that described by Yahara and Kakimoto-Sameshima (23). Because the lymphocytes remained rounded, even on the cover slips, the ubiquity of this pattern was only appreciated by focusing through several planes in the field. This pattern was found when the cells were in a Ca^{2+}-free buffer or with up to 8 mM Ca^{2+} or 8 mM Mg^{2+} present in the medium. The addition of SA produced no detectable change in the microtubular structure; however, LA treatment resulted in a dramatic loss of the umbrella spoke pattern (Fig. 1B). Many of the LA-treated cells appeared to exhibit a polarized submembranous aggregate (PSA) of antitubulin fluorescence (Fig. 1B). We have adopted the term "polarized submembranous aggregate" to indicate that the cytoplasmic elements were polarized at one end of the cell under the surface membrane. This is to avoid confusion with the term "cap," which refers to a single aggregation of receptors on the cell surface. The effect of LA was enhanced in Ca^{2+}-free buffer. The addition of 8 mM Mg^{2+} had no effect upon the LA-induced changes, but 8 mM Ca^{2+} showed a partial reversal towards a diffuse pattern (Fig. 1C), somewhat resembling the original unperturbed pattern.

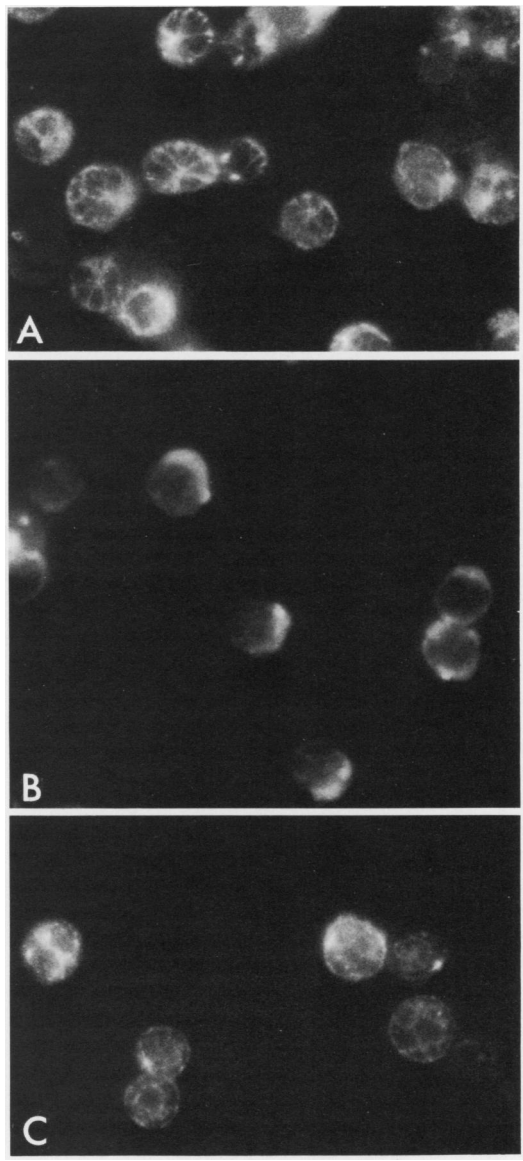

FIG. 1. *Fluorescence pattern of antitubulin in mouse lymphocytes. (A) Control; (B) LA (10 μg/ml); (C) LA posttreated with 8 mM Ca^{2+}. ×700.*

The percentage of fatty acid-treated cells exhibiting fluorescence patterns similar to controls was calculated and then compared with controls (Tables 1 and 2). For example, antitubulin staining showed that 74% of the control cells exhibited an umbrella spoke array; treatment with LA decreased the percentage to 4.1%, representing a 94% decrease from control values. Control patterns for staining of the cytoskeletal elements

TABLE 1. *Lymphocyte perturbations and effects on cytoskeleton*

Conditions	% of cells in normal patterns[a]			
	Tubulin	Actin	Myosin	α-Actinin
Control	74.0	90.6	95.2	94.3
SA (10 μg/ml)	78.1	77.1	95.9	95.7
Ca^{2+} (8 mM)	80.2	93.1	89.2	93.2
Colchicine (10^{-6} M)	3.2	79.1	49.8	84.3
Sodium azide (10^{-3} M)	91.2	95.8	6.4	39.1
LA (10 μg/ml)	4.1	17.0	16.1	8.8
Posttreated with Ca^{2+} (8 mM)	38.1	70.1	68.5	83.9
Pretreated with sodium azide (10^{-3} M)	1.8	40.7	0	0

[a] Percentages of cells in a "normal" configuration, i.e., the predominant pattern in untreated cells.

TABLE 2. *Description of the predominant fluorescence patterns of the cytoskeletal and contractile elements of lymphocytes under control and experimental conditions*

Conditions	Patterns			
	Tubulin	Actin	Myosin	α-Actinin
Control	Umbrella spoke	Diffuse	Diffuse	Diffuse
SA (10 μg/ml)	Umbrella spoke	Diffuse	Diffuse	Diffuse
Ca^{2+} (8 mM)	Umbrella spoke	Diffuse	Diffuse	Diffuse
Colchicine (10^{-6} M)	Diffuse, few PSAs	Diffuse	Patched, few PSAs	Diffuse
Sodium azide (10^{-3} M)	Umbrella spoke	Diffuse, few patched	Patched	Patched
LA (10 μg/ml)	PSA	PSA	Patched	PSA
Posttreated with Ca^{2+} (8 mM)	Diffuse, few PSAs	Diffuse, few PSAs	Diffuse	Diffuse, few PSAs
Pretreated with sodium azide (10^{-3} M)	Patched	Patched	Patched	Patched, few PSAs

were taken as the most prominent pattern observed in the untreated cells. Because the measurements were taken from photographs, the percentages cannot be taken as absolute, as the definition of the pattern was defined at the level of focus. This was a particular problem with antitubulin staining because of the umbrella spoke pattern and the very limited focus plane, which does not reveal the entire pattern.

Actin. The immunofluorescence pattern of actin in normal cells revealed a diffuse staining (Fig. 2A). This was not affected by SA, 8 mM Mg^{2+}, or 8 mM Ca^{2+} (Tables 1 and 2). The addition of LA produced significant patching and evidence of a PSA of the actin (Fig. 2B; Tables 1 and 2). This was partially reversed with high external calcium (Tables 1 and 2).

Myosin. Cell staining with antimyosin antibodies revealed a diffuse pattern of myosin within the cell and often a more intense staining around the periphery (Fig. 3A; Tables 1 and 2). Ca^{2+}-free medium produced a slightly beaded or patched pattern in some cells, but the majority remained diffuse. LA treatment led to virtually uniform myosin patching around the periphery and the loss of the intracellular diffuse staining (Fig. 3B; Tables 1 and 2). SA had no effect, and 8 mM Mg^{2+} did not affect either the control cells or the LA-treated cells. However, 8 mM Ca^{2+} partially reversed the effect of LA, such that many of the cells regained diffuse intracellular staining similar to the controls (Tables 1 and 2; Fig. 3C). A diffuse staining also reappeared in those cells that retained the patched pattern. Untreated, control cells were unaffected by 8 mM Ca^{2+} (Tables 1 and 2).

α-Actinin. The distribution of α-actinin in the lymphocyte is shown in Fig. 4A as diffuse staining containing occasional projections emanating from a ring of intense staining around the nucleus. This pattern was not altered by the absence of Ca^{2+} in the buffer. Once again, SA was without effect, whereas LA produced a loss of the intracellular structure, including the perinuclear staining and formation of a PSA (Fig. 4B; Tables 1 and 2). The difference between control cells and LA-treated cells was much more apparent in Ca^{2+}-free medium, where the LA-induced PSA was even more prominent (Fig. 4B). The addition of high Mg^{2+} had no effect on the control cells or the LA-treated cells. Ca^{2+} (8 mM) added to otherwise untreated cells had no effect upon the α-actinin distribution, but it essentially reversed the LA-induced changes (Tables 1 and 2).

Cross-linking of surface receptors—relation to PSA formation. Experiments were also carried out to determine whether LA treatment could inhibit capping even after the putative linkage between the surface receptor and the cytoskeleton had been formed. After incubation (4°C, 20 min) with fluorescein-labeled

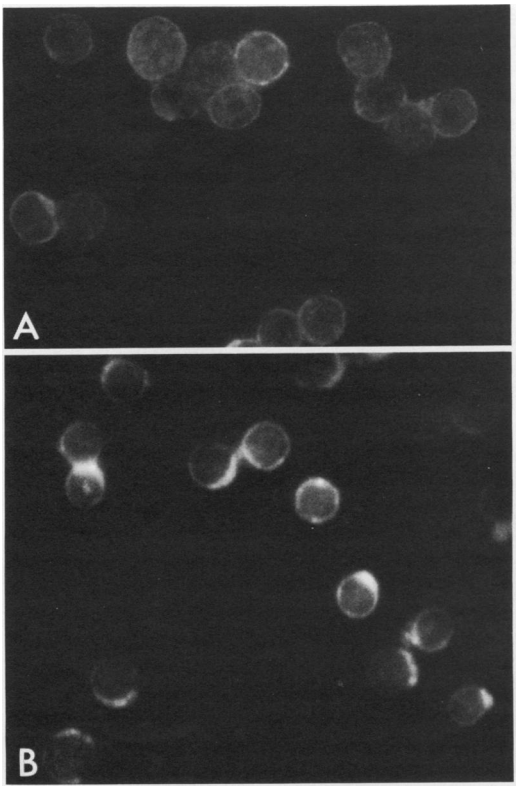

FIG. 2. *Immunofluorescence staining of actin in mouse lymphocytes. (A) Control; (B) LA (10 μg/ml). ×600.*

rabbit anti-mouse immunoglobulin (100 μg per 10^7 cells per 2 ml), the lymphocytes were treated with LA (10 μg/ml) for 30 min at 4°C. The cells were then washed and brought up to room temperature (30 min) to induce capping. At the end of this incubation, some of the cells were fixed and observed for immunofluorescence, scoring the number of cells having caps, and some cells were put onto polylysine-coated cover slips, fixed, and stained for actin. Under these conditions, LA inhibited capping to the same extent as it did when it was added before incubation with anti-surface immunoglobulin—from 69% in B-cell controls to 23% in LA-treated B-cells.

We also looked at the distribution of the cytoskeleton and surface immunoglobulins under control conditions and under those conditions in which PSAs were produced. Lymphocytes were pretreated with LA for 30 min, rinsed, and stained with fluorescein-labeled goat anti-mouse surface immunoglobulin as described above. The cells were then allowed to attach to polylysine-coated cover slips, fixed, permeabilized, and stained with antiactin by using the indirect labeling technique with rhodamine-labeled antibodies. In control cells, the antiactin distribution followed the staining of the surface immunoglobulin, as previously demonstrated by Bourguignon et al. (1). In contrast, in the LA-treated cells, cytoskeletal and surface immunoglobulin staining did not coincide. Antiactin exhibited a PSA, as in Fig. 2B, whereas the anti-surface immunoglobulin demonstrated a patchy distribution, as we had previously found (14). These results suggest that the link between the cytoskeleton and surface receptors had been affected.

Sodium azide—energy requirement for PSA formation. We tested whether any of the alterations in the cytoskeletal organization required metabolic energy. Sodium azide (10^{-3} M)

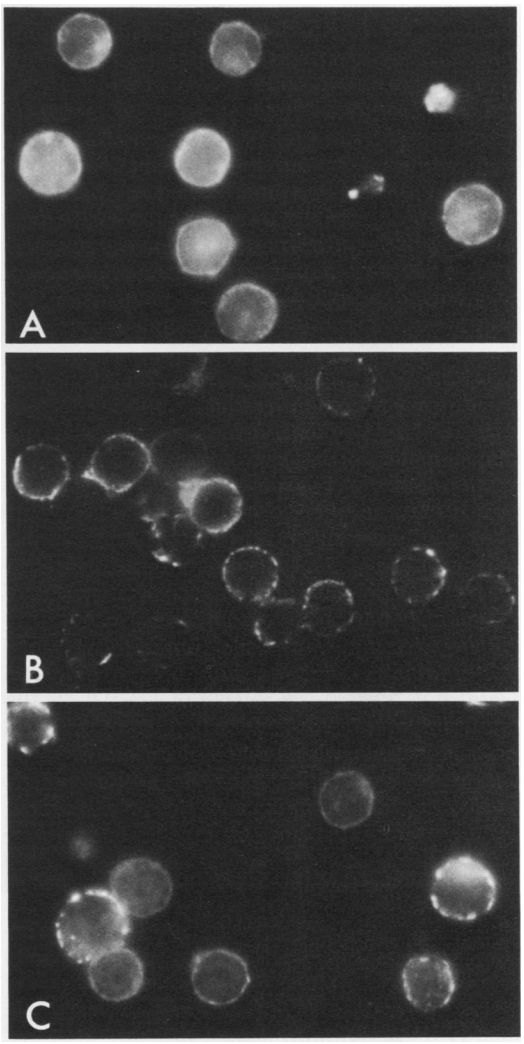

FIG. 3. *Distribution of myosin in lymphocytes. (A) Control; (B) LA (10 μg/ml); (C) LA posttreated with 8 mM Ca^{2+}. ×700.*

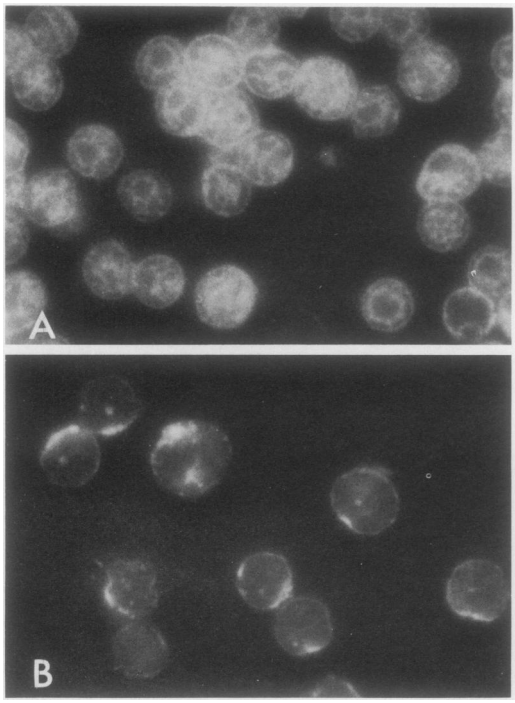

FIG. 4. *Fluorescence staining of α-actinin in the lymphocyte. (A) Control; (B) LA (10 μg/ml). ×700.*

added to untreated cells had no effect upon tubulin and actin, but produced patching in cells stained for α-actinin and dramatically altered the distribution of myosin, leading to prominent patch formation (Fig. 5A). Sodium azide did affect the ability of LA to induce PSAs of tubulin, actin, and α-actinin. When LA was added to cells pretreated with sodium azide (10^{-3} M, 30 min), they became predominantly patched, but few PSAs were observed (Tables 1 and 2). The patching of myosin induced by LA was unaffected by sodium azide. Sodium azide, however, could not reverse the effects of LA; i.e., if the cells were treated with sodium azide after LA treatment, PSA formation was not affected and the PSAs were not disrupted.

Colchicine—relation of microtubular organization to other cytoskeletal and contractile elements. Although LA was able to alter the structure of each of the cytoskeletal elements examined, we had no way of knowing whether LA was affecting each element separately or whether a disruption of one could account for the alterations in the others. In these experiments, we examined the effects of colchicine (10^{-6} M, 30 min), which disrupts microtubules, on the distribution of the non-tubulin-containing cytoskeletal elements. There was a tendency for rearrangement, but not as dramatic as in the LA-treated cells. Figure 5B shows that colchicine produced some PSAs and general staining for tubulin (Tables 1 and 2). Myosin was altered as well, being patched with occasional PSAs (Fig. 5C), but was not as dramatically altered as with LA (Tables 1 and 2). Actin and α-actinin, on the other hand, were essentially not perturbed by colchicine (Tables 1 and 2). None of the changes induced by colchicine treatment could be reversed with high external calcium.

Adhesion studies. One concern that we had in these studies was that we were seeing a particular subpopulation of cells in the LA-treated samples. Using the methods of Walther et al.

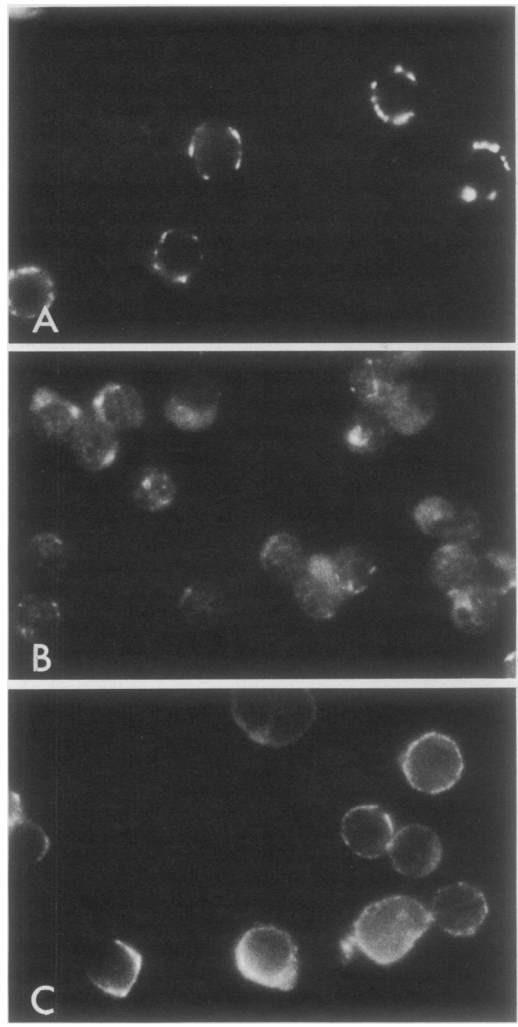

FIG. 5. *Immunofluorescence patterns. (A) Myosin pattern after treatment with sodium azide (10^{-3} M); (B and C) tubulin and myosin patterns, respectively, after colchicine treatment (10^{-6} M). ×700.*

(21), we measured cell adhesion to cover slips and found that only 30% of the LA-treated lymphocytes, as compared with the controls, attached to the cover slips. This could have been a source of a selection artifact if the LA treatment were preventing the attachment of cells with "normal" cytoskeletal configurations only. To test this, we counted the percentage of control cells that looked like LA-treated cells when stained for each of the cytoskeletal elements. Doing this, we found that 3% of the tubulin-, 0.9% of the actin-, 1.1% of the myosin-, and 1.9% of the α-actin-stained control cells had patterns similar to those in LA-treated cells, i.e., PSAs for tubulin, actin, and α-actinin and patching for myosin. These numbers were incompatible with a 30% selection artifact due to differential adhesive properties.

Electron microscopic studies. Since we had seen the tubulin aggregated at one end of the cell in our immunofluorescence studies, we wanted to determine whether the tubulin array had been depolymerized or concentrated at one end of the cell. Electron micrographs of the control and LA-treated cells revealed very little difference between the two (Fig. 6 and 7); however, there was some indication that the microtubules in the LA-treated cells were closer to the surface membrane than those in the untreated cells. Although the micrographs indicate no differences in the number of microtubules between the two groups, we do not know how much depolymerization may have occurred.

Scanning electron micrographs of control and LA-treated cells fixed in suspension and then attached to polylysine-coated cover slips revealed no differences between control and LA-treated cells with regard to overall shape, number, and length of microvilli (Fig. 8A and B). Phase microscopy of the same cells also showed no differences between the cells. We did find that the number of amoeboid, presumably motile forms was reduced from 40 to 5% after treatment with *cis*-unsaturated fatty acids.

DISCUSSION

In this paper, we report our observations on the structural effects of free fatty acids upon the organization of the cytoskeleton in murine lymphocytes. Only *cis*-unsaturated free fatty acids produce perturbations in the cytoskeleton detectable by immunofluorescence techniques. We concentrated on LA and SA in these studies, but we have found that other *cis*-unsaturated free fatty acids (e.g., oleic acid) act like LA, whereas either *trans*-unsaturated (e.g., elaidic acid) or saturated (e.g., palmitic acid) free fatty acids act like SA. The question that confronts us is: What is the mechanism(s) for these dramatic effects?

We can consider three broad potential mechanisms: (i) direct effects on the cytoskeletal elements, (ii) indirect effects on the cytoskeleton via perturbations in cellular physiology, and (iii) alteration of the cytoskeleton via the perturbation of other cellular structures, such as the cell membrane. We do not know whether free fatty acids either bind to or alter cytoskeletal elements directly. Furthermore, we would have to postulate that this direct interaction is extremely sensitive to subtle alterations in the structure of the free fatty acids; however, there is no evidence at this time for the direct interaction of free fatty

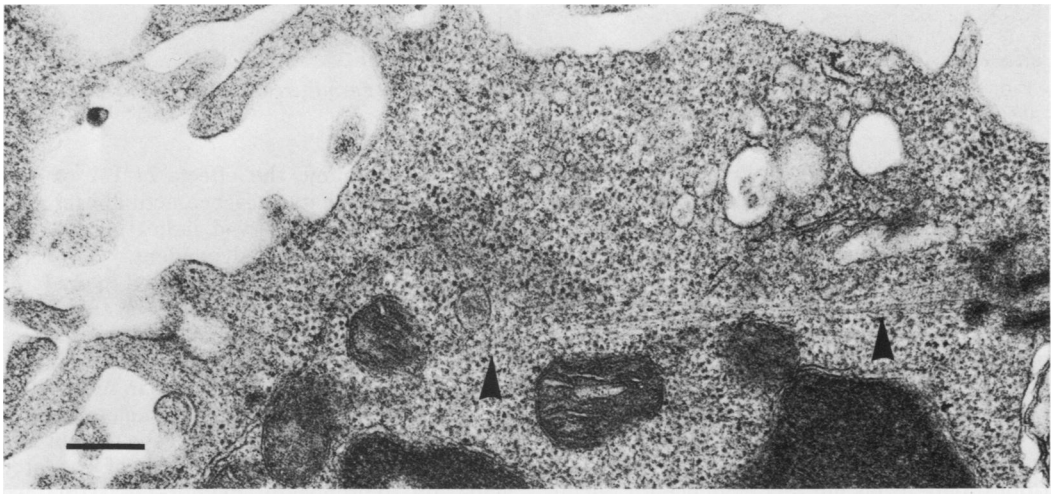

FIG. 6. *Electron micrograph of untreated lymphocytes. Note the presence of microtubules (arrowheads). Bar = 0.25 μm.*

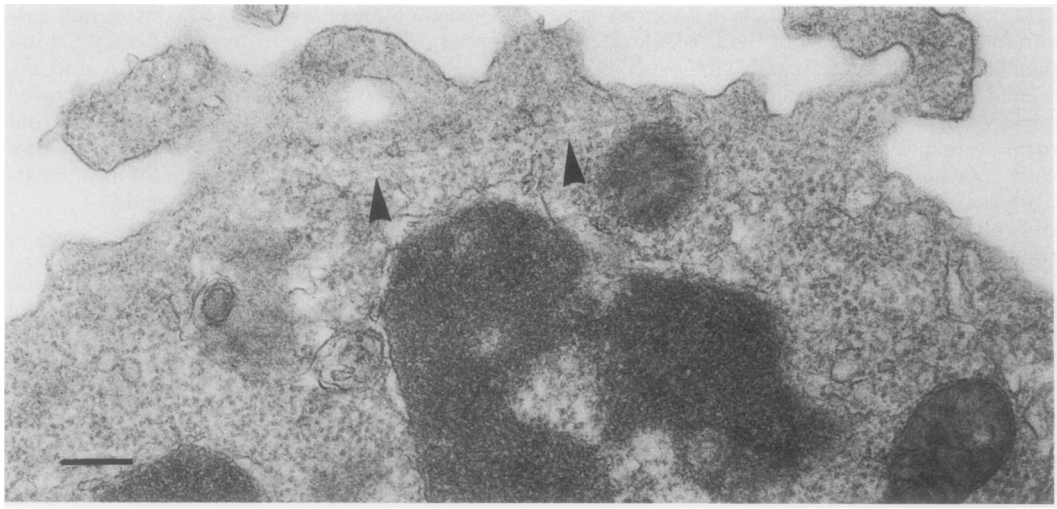

FIG. 7. *Electron micrograph of LA (10 μg/ml)-treated lymphocytes. Note the presence of microtubules (arrowheads). Bar = 0.25 μm.*

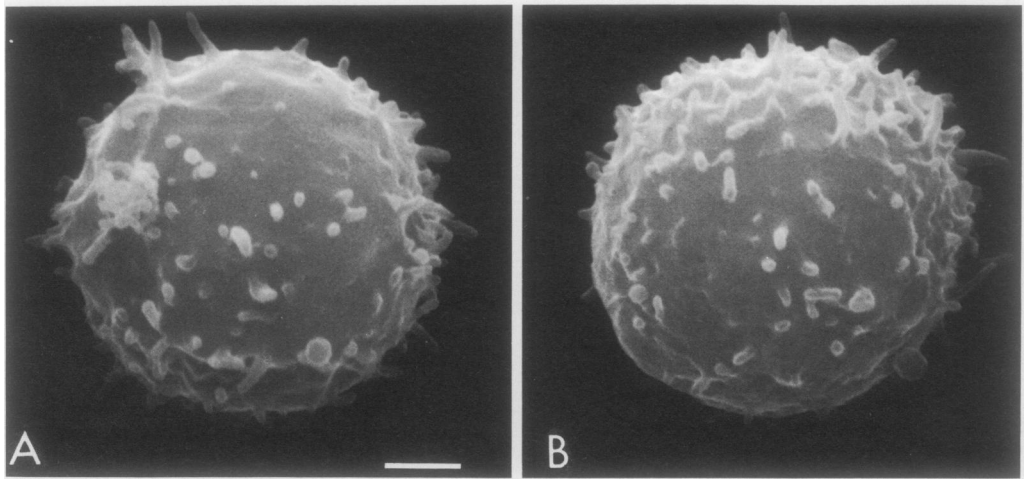

FIG. 8. *Scanning electron micrograph of untreated (A) and LA (10 μg/ml)-treated (B) lymphocytes. Bar = 2 μm.*

acids with the cytoskeleton. Free fatty acids can affect cellular physiology and lipid metabolism, producing, in some systems, an inhibition of receptor function, e.g., adenyl cyclase coupling (6).

In a recent paper by Corps et al. (3), it was claimed that LA acts as a metabolic uncoupler, resulting in a reduction in adenosine triphosphate levels and a concomitant increase in O_2 consumption. We, however, under the conditions of our experiments and using their methods, have found neither any decrease in adenosine triphosphate levels nor any evidence for uncoupling. These results will be reported in detail elsewhere. Furthermore, not only does sodium azide fail to mimic the effects of LA on the cytoskeleton, but its presence actually inhibits some of the effects observed, indicating that the results of LA are energy dependent.

By reviewing the effects produced in the lymphocyte cytoskeleton by LA, we see that all of the changes may well relate to the plasma membrane. Thus, free fatty acids, which are primarily confined to the plasma membrane in these studies, are likely to be altering cytoskeleton-membrane interactions at the level of the membrane. In our previous study on the effects of free fatty acids on lymphocyte membranes (15), we showed that (i) free fatty acids markedly perturb the packing of the lipid in the bilayer, as reported

by fluorescence depolarization of hydrophobic fluorescence probes and (ii) *cis*-unsaturated free fatty acids have very different structural effects from other free fatty acids. We are currently examining how these structural effects on membrane lipid organization could produce changes in membrane-cytoskeleton interactions, but to date, precise mechanisms are not established. In a model previously put forward by us (14), we ascribe the inhibition of capping by *cis*-unsaturated free fatty acids to specific perturbations of membrane lipid structure and related membrane protein conformational changes, with a consequent failure of linkage of the cytoskeleton to the membrane or its activation or both.

One would thus expect that the ability of LA to alter membrane-cytoskeleton interactions would be reflected in perturbations of cellular events that involve these structures. The role of the cytoskeleton in the patching, capping, and internalization of membrane immunoglobulin in the lymphocyte has been studied extensively (for a review, see reference 22). Although colchicine does not inhibit capping, cytochalasin B can (4). Furthermore, tubulin, myosin, actin, and α-actinin have been demonstrated to cocap with immunoglobulin (7, 11, 12, 18, 23). It is believed that this association, at least in part, reflects their participation in the active mechanisms responsible for the cap formation. Clearly, for the cytoskeleton to engineer successfully the movement of surface molecules, its structural relationship with the membrane must be maintained. We have found (13–15; D. K. Bhalla, R. Hoover, M. Inbar, and M. Karnovsky, J. Cell Biol. **79**:2, 1978) that LA and other *cis*-unsaturated free fatty acids, at exactly the same doses used to produce cytoskeletal structural perturbations, inhibit surface receptor capping and internalization. Dragsten et al. (5) have shown that once surface receptors are patched, they do not exhibit any free diffusion in the plane of the membrane. However, the movement of the patches into a polar cap is an energy-dependent, nondiffusional process. It is in this part of the capping process that the cytoskeleton is likely to be involved. It is precisely this process, and not the free diffusion of receptors in the membrane, that is altered by LA (14). Thus, our previous studies on the inhibition of lymphocyte surface receptor capping by LA provide an excellent functional correlate of the alteration in cytoskeleton-membrane interactions reported here. Consistent with this correlation is the effect of extracellular calcium. Calcium, but not Mg^{2+}, is able to reverse the inhibition of capping produced by LA and produce a definite, but partial, reversal of the cytoskeletal changes induced by LA. This reversal is seen at the same doses as the reversal of the inhibition of capping. In our earlier paper (14), we postulated that Ca^{2+} was bound to an anchoring protein which is necessary for the cross-linking of immobilized patches of surface receptors to the contractile elements in the cytoplasm, which, when completed, results in the capping phenomenon. We also proposed that when a group A free fatty acid is incubated with the cells, the lipid environment around this protein is altered and the bound Ca^{2+} is released, inhibiting this cross-linking phenomenon. The addition of external Ca^{2+} reverses this inhibition by altering the lipid environment, which in turn allows the anchoring protein to return to a state that binds Ca^{2+} and permits capping. The present results support this proposal that Ca^{2+} may be affecting the interaction of the cytoskeleton with the receptors on the surface membrane. It should also be pointed out that Schreiner and Unanue (19) proposed a similar role for calcium, based on experiments in which chlorpromazine inhibited surface immunoglobulin capping.

The nature of the interaction between the plasma membrane and the cytoskeleton is an important unanswered question in biology. The ability to alter reversibly the overall structure of the cytoskeleton and its interactions with the membrane provides a useful tool with which to unravel some of these problems. The specific reasons responsible for the differences between the effects of the two groups of free fatty acids may give insight into the molecular mechanisms involved in the dramatic distribution of the cytoskeletal elements.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Wendy Haering and Betsy Renshaw and the assistance of Robert Rubin in the preparation of the photographic material. We also acknowledge Susan Byers for her assistance in the preparation of the manuscript.

This work was supported by Public Health Service grants GM 25637 and AI 10677 from the National Institutes of Health. R. T. was supported by grants from the American Cancer Society, Massachusetts, and the Medical Foundation, Inc., Boston, Mass.

LITERATURE CITED

1. **Bourguignon, L., K. Tokuyasu, and S. J. Singer.** 1978. The capping of lymphocytes and other cells, studied by an improved method for immunofluorescence staining of frozen sections. J. Cell. Physiol. **95**:239–258.
2. **Clarke, M., and J. A. Spudich.** 1977. Nonmuscle contractile proteins: the role of actin and myosin in cell motility and shape determination. Annu. Rev. Biochem. **46**:797–822.
3. **Corps, A., T. Pozzan, T. R. Hesketh, and J. Metcalfe.** 1980. *cis*-Unsaturated fatty acids inhibit cap formation on lymphocytes by depleting cellular ATP. J. Biol. Chem. **255**:10566–10568.

4. **DePetris, S.** 1974. Inhibition and reversal of capping by cytochalasin B, vinblastine and colchicine. Nature (London) **250**:54–56.
5. **Dragsten, P., P. Henkart, R. Blumenthal, J. Weinstein, and J. Schlessinger.** 1979. Lateral diffusion of surface immunoglobulin, Thy-1 antigen, and a lipid probe in lymphocyte plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **76**:5163–5167.
6. **Engelhard, V. H., J. Esko, D. Storm, and M. Glaser.** 1976. Modification of adenylate cyclase activity in LM cells by manipulation of the membrane phospholipid composition *in vivo*. Proc. Natl. Acad. Sci. U.S.A. **73**: 4482–4486.
7. **Flanagan, J., and G. Koch.** 1978. Cross-linked surface Ig attaches to actin. Nature (London) **273**:278–281.
8. **Fujiwara, K., and T. D. Pollard.** 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow and mitotic spindle of human cells. J. Cell Biol. **71**:848–875.
9. **Fujiwara, K., and T. D. Pollard.** 1978. Simultaneous localization of myosin and tubulin in human tissue culture cells by double antibody staining. J. Cell Biol. **77**:182–195.
10. **Fujiwara, K., M. E. Porter, and T. D. Pollard.** 1978. Alpha-actinin localization in the cleavage furrow during cytokinesis. J. Cell Biol. **79**:268–275.
11. **Gabbiani, G., C. Chaponnier, A. Zumbe, and P. Vassalli.** 1977. Actin and tubulin co-cap with surface immunoglobulins in murine B lymphocytes. Nature (London) **269**:697–698.
12. **Geiger, B., and S. J. Singer.** 1979. The participation of α-actinin in the capping of cell membrane components. Cell **16**:213–222.
13. **Hoover, R. L., D. K. Bhalla, S. Yanovich, M. Inbar, and M. J. Karnovsky.** 1980. Effects of linoleic acid on capping, lectin mediated mitogenesis, surface antigen expression, and fluorescent polarization in lymphocytes and BHK cells. J. Cell. Physiol. **103**:399–406.
14. **Klausner, R. D., D. K. Bhalla, P. Dragsten, R. L. Hoover, and M. J. Karnovsky.** 1980. Model for capping derived from inhibition of surface receptor capping by free fatty acids. Proc. Natl. Acad. Sci. U.S.A. **77**: 437–441.
15. **Klausner, R. D., A. M. Kleinfeld, R. L. Hoover, and M. J. Karnovsky.** 1980. Lipid domains in membranes. Evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. J. Biol. Chem. **255**:1286–1295.
16. **Korn, E. D.** 1978. Biochemistry of actomyosin-dependent cell motility. Proc. Natl. Acad. Sci. U.S.A. **75**:588–599.
17. **Pollard, T. D., and R. Weihing.** 1974. Actin and myosin and cell movement. Crit. Rev. Biochem. **2**:1–65.
18. **Schreiner, G., K. Fujiwara, T. D. Pollard, and E. Unanue.** 1977. Redistribution of myosin accompanying capping of surface Ig. J. Exp. Med. **145**:1393–1398.
19. **Schreiner, G., and E. R. Unanue.** 1976. Membrane and cytoplasmic changes in B lymphocytes induced by ligand-surface immunoglobulin interaction. Adv. Immunol. **24**:37–165.
20. **Stephens, R. E., and K. T. Edds.** 1976. Microtubules: structure, chemistry and function. Physiol. Rev. **56**: 709–777.
21. **Walther, B. T., R. Ohman, and S. Roseman.** 1973. A quantitative assay for intercellular adhesion. Proc. Natl. Acad. Sci. U.S.A. **70**:1569–1573.
22. **Weihing, R. R.** 1979. The cytoskeleton and plasma membrane, p. 42–109. *In* G. Gabbiani (ed.), Methods and achievements in experimental biology, vol. 8. S. Kargard Co., New York.
23. **Yahara, I., and F. Kakimoto-Sameshima.** 1978. Microtubule organization of lymphocytes and its modulation by patch and cap formation. Cell **15**:251–259.