Role of Cholesterol in the Capping of Surface Immunoglobulin Receptors on Murine Lymphocytes

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ABSTRACT Previously, we have shown that the capping of surface immunoglobulins on murine lymphocytes can be affected by modulating the lipid environment of the surface membrane with free fatty acids. In the present study, murine lymphocytes were depleted of cholesterol by incubation with phospholipid vesicles. As the cellular cholesterol:phospholipid ratio decreased, the capping of the surface immunoglobulin was seen to decrease. This inhibition of capping could not be reversed by calcium and is not accompanied by changes in either the cytoskeletal element α-actinin or cellular ATP levels. Incubation of the cholesterol-depleted cells with cholesterol-containing phospholipid vesicles raised both the cholesterol:phospholipid ratio and capping levels to values close to those of untreated control cells. Remarkably, stearic acid, a saturated fatty acid, could also restore the capping levels in the cholesterol-depleted cells. On the basis of the present data and measurements of the fluorescence polarization of the probe diphenyl hexatriene, we propose a model in which the protein(s) involved in capping is located in a gel-like lipid domain, and that removal of cholesterol makes this domain less gel-like and inhibits capping. Restoration of the gel-like nature of this domain by the addition of either cholesterol or stearic acid enables the protein(s) to function normally.

Previous work from our laboratories (1) has shown that the capping of surface immunoglobulins (IgG) on mouse B lymphocytes can be affected by modulating the lipid environment of the surface membrane. Specifically, pre-incubation with cis-unsaturated fatty acids, but not saturated or trans-unsaturated fatty acids blocks the movement and aggregation of surface receptors to one end of the cell. We have also reported a differential effect of free fatty acids on baby hamster kidney (BHK) fibroblast adhesion and growth (2), human platelet aggregation (3), and the transport of glucose-6-phosphate across rat liver microsomal membranes (4). Cis-unsaturated fatty acids inhibit BHK fibroblast adhesion and growth, platelet aggregation and glucose-6-phosphate transport in microsomes, while saturated and trans-unsaturated fatty acids have no effect. We have proposed (5, 6) that the differential effects of the free fatty acids on protein mediated function are consistent with the presence of lipid domains, composed of either relatively gel-like or fluid lipid, in the membrane. It was further suggested (5) that the preferential partitioning of the fatty acids into particular lipid domains would then perturb the lipid structure in that region and thereby affect a normal protein function.

Measurements of the polarization of the fluorescent probe diphenyl hexatriene (DPH), which reflects the order of the lipid hydrocarbon chains (5), in aqueous dispersions of dimyristoyl phosphatidylcholine (DMPC) indicated that, when the phospholipid is in the gel-state, cis-unsaturated fatty acids have a disordering effect while saturated and trans-unsaturated fatty acids have little or no effect. When the DMPC is in the liquid crystalline or fluid state, the saturated and trans-unsaturated free fatty acids have a slight ordering effect, whereas the cis-unsaturated fatty acids are without effect.

It is well documented (7) that one of the effects of cholesterol in aqueous dispersions of synthetic phospholipids is to affect the order of the phospholipid acyl chains. Furthermore, it is possible to manipulate cholesterol levels of isolated cells in vitro by incubation with lipid vesicles (8). We therefore decided to investigate the effect of the cholesterol content in mouse B lymphocytes on the surface immunoglobulin capping. Our aim was to determine whether the modulation of the lipids in the surface membrane by cis-unsaturated fatty acids, which results in the inhibition of capping, is a general phenomenon.

In the present study, we were able to remove cholesterol

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from the cells and thereby inhibit surface immunoglobulin capping on mouse B lymphocytes. We show that the inhibition is not due to altered ATP levels, changes in the cytoskeleton, or loss of proteins from the membrane. The inhibition of capping was reversed by incubating the cholesterol-depleted cells with either cholesterol-containing phospholipid vesicles or the saturated fatty acid, stearic acid. These results are discussed in terms of a lipid-domain model in which proteins are in either gel-like or fluid regions; regions in which, in order to function properly, these proteins must remain. Alteration of the lipid environment by either incubation with free fatty acids or depletion of cholesterol can have profound effects on cellular functions.

Several reports (9–11) have shown that the addition of excess cholesterol or merely the presence of cholesterol in either cells or reconstituted membranes has an inhibitory effect on protein and cellular function. In marked contrast, studies have also been reported which indicate a requirement of cholesterol for cellular function (12–14). Here we further demonstrate that cholesterol is necessary for the proper functioning of cellular proteins, and strongly suggest that this is due to a modulation of the lipid in the surface membrane rather than a direct cholesterol-protein interaction.

MATERIALS AND METHODS

Lymphocyte Preparation

Lymphocytes were isolated from spleens of A/J mice obtained from Jackson Laboratories (Bar Harbor, ME).[1] The cells were teased from the spleens in Hanks' balanced salt solution, buffered with 15 mM HEPES, pH 7.4 (HH), and washed twice with the same solution. Contaminating erythrocytes were lysed by resuspending the cell pellet in a small volume of distilled water for 20 s, after which isotonicity was restored by the addition of a large volume of HH. The cells were then spun down and resuspended in HH at a concentration of 10^7 cells/ml.

Liposome Preparation

Small unilamellar vesicles (SUVs) of the appropriate lipid composition were prepared using a slight modification of the methods described by Backer and Dawidowicz (15). The lyophilized lipids were dispersed in HH prior to sonication, and the fractionation of the resulting vesicles on a molecular sieve of Sepharose 4B was omitted.

Egg phosphatidyl choline (PC) was obtained from Makor Chemicals (Jerusalem, Israel) or as Type XI-E from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidic acid (PA) was purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Both of these compounds gave a single spot, as visualized by iodine vapors, after thin-layer chromatography on silica gel H using chloroform:methanol:acetic acid:water (25:15:4:2 vol/vol) (16) as the eluting solvent. In addition, minimal oxidation of these compounds was detected by measuring a Klein oxidation index (17) of 0.1. Cholesterol (CH) from Sigma Chemical Co. was recrystallized three times from absolute alcohol prior to use. Both cholesterol and the phospholipids were stored as stock solutions in chloroform at −20°C. No changes in purity or oxidation index were detected during storage.

SUVs were prepared from either a mixture of PC and PA in a molar ratio of 1:0.15 or PC:PA:CH in a molar ratio 1:0.15:1. The lipid concentration was 6 mM in PC for both types of SUVs. In some preparations, a trace amount of cholesteryl [1-^{14}C]oleate (0.6 μCi/ml) (sp act 50 Ci/mol, from New England Nuclear, Boston, MA), a nonexchangeable marker for the vesicles (18), was incorporated into the SUVs to determine the fraction of the vesicles that remained attached to or had fused with the lymphocytes.

[1] Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals at the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education, and Welfare publication No. NIH 78-32; revised 1978).

Liposome Treatment

1 ml of cells (10^7 cells/ml) was incubated at room temperature with 0.5 ml of the SUV suspension (6 mM in PC) composed of either PC/PA or PC/PA/CH. After incubation, the cells were pelleted in a clinical centrifuge, rinsed twice in 15 ml of HH, and resuspended in HH at a concentration of 2–5 × 10^6 cells/ml.

Fatty Acid Incorporation

The uptake of free fatty acids by control and cholesterol-depleted lymphocytes was measured. The ^{14}C-labeled fatty acids (from New England Nuclear, Boston, MA) linoleic acid (sp act 56 Ci/mol) and stearic acid (sp act 51 Ci/mol) were mixed with their unlabeled counterparts (from Nu Chek Prep, Elysian, MN) in a ratio of 1:10 (labeled:unlabeled) to reach a final concentration of 30 μM in HH with 10^7 cells/ml. The lymphocytes were incubated with these solutions of fatty acids for 30 min at room temperature and then rinsed twice in HH. Uptake was calculated by counting the cell pellets in a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA; Model LS-7000). Under these incubation conditions, the free fatty acids are not metabolized by the cells as we have previously shown (5).

Capping Experiments

After incubation of the lymphocytes with the SUVs, treated and untreated cells were incubated with either fluorescein or rhodamine conjugated rabbit anti-mouse IgG (Cappel Laboratories, Westchester, PA) for 20 min at 4°C. The cells were rinsed in cold HH and resuspended in warm (20–25°C) HH. At the end of 30 min, the cells were spun down and fixed in 10% formalin (Fisher Scientific, Medford, MA) in HH for 30 min at room temperature. The cells were then rinsed twice, and the percentage of cells having caps, i.e., those cells in which the fluorescence was restricted to one end of the cell and occupying <25% of the cell surface, was determined by fluorescence microscopy (E. Leitz, Inc., Rockleigh, NJ; Ortholux II). In addition, the state of the cytoskeletal element α-actinin was monitored using immunofluorescence techniques as we have recently described (19). The antiserum against α-actinin was kindly provided by Dr. K. Fujiwara (Harvard Medical School).

Fluorescence Polarization Measurements

Polarization values were determined as outlined by Shinitzky and Inbar (20) by incubating the cells with the aromatic fluorophore, 1,6-diphenyl-1,3,5-hexatriene (DPH), from Sigma Chemical Co. and measuring the depolarization of the fluorescence caused by the rotation of the probe in its lipid environment. High p-values indicate an ordered, gel-like lipid environment and low values, a disordered fluidlike environment (5). DPH was prepared as a stock solution of 2 mM in tetrahydrofuran and added to the cells suspended in HH to a final concentration of 2 μM. The cells were incubated for 20 min at room temperature, rinsed twice in HH and resuspended in 3 ml of HH at a cellular concentration of 2 × 10^6/ml. The p-values were then measured at 25°C on an Elscint microviscometer (Elscint Ltd., Haifa, Israel), model MV-1a.

Analytical Determinations

Lipids were extracted from the whole cells (treated and untreated) with chloroform/methanol (2:1) according to the method of Bligh and Dyer (21). The lipids were dried down under nitrogen and redissolved in a small volume of chloroform. Samples were taken for phospholipid and cholesterol determinations. The phospholipid concentration of the extracted lipids was determined by the method of Bartlett (22). Cholesterol content was measured on a gas chromatograph (Hewlett-Packard Co., Palo Alto, CA; Model 5840A), equipped with a 3% OV-17 column (Applied Sciences, State College, PA). The amount was determined by comparing the areas under the cholesterol peak to those areas obtained from known quantities and plotted as a standard curve. ATP was determined using methods that we have previously described (23).

Ultrastructural Studies

FREEZE-FRACTURE: Following the various treatments cells were washed as described above. Cell pellets were fixed in 3% glutaraldehyde, 0.1 M cacodylate (pH 7.2) with 5% sucrose for 30 min at room temperature. Fixed cells were washed in cacodylate buffer and then treated with filipin (50 μg/ml) in cacodylate for 4 h to overnight as previously described by Robinson and Karnovsky (24). Filipin-treated cells were incubated with 30% glycerol in 0.1 M cacodylate for 2 h and then frozen in Freon 22 near its freezing point. Frozen cells were stored in liquid nitrogen until being fractured in a Balzers BA 360 M freeze-

fracture apparatus (Balzers, Hudson, NH).

THIN-SECTION ELECTRON MICROSCOPY: Lymphocytes were fixed in glutaraldehyde and washed in buffer as above. Cells were then postfixed in 2% OsO_4-0.1 M cacodylate (pH 7.2) for 1 h at room temperature and dehydrated in a graded series of ethanol. During dehydration (70% step), cells were embedded in melted agar. The cells were pelleted through the agar, the agar was solidified, and portions containing cells were cut into small pieces. This enabled us to carry out subsequent steps without centrifugation. The agar blocks were embedded in Epon 812. Sections were cut with a diamond knife and examined in a Philips 200 operated at 60 kV following staining with uranyl acetate and lead citrate.

SCANNING ELECTRON MICROSCOPY: The lymphocytes were also prepared for study by scanning electron microscopy. The cells were fixed as described above for thin-section electron microscopy, rinsed and allowed to attach to polylysine-coated coverslips. They were then postfixed in 2% OsO_4–0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature. After rinsing several times, the samples were dehydrated through a graded series of acetone and brought to the critical drying point out of liquid CO_2 in a Model 11, 120-critical point dryer (Balzers Union Ltd.). The cells were then coated with gold-palladium in a sputtering device (Technics, Alexander, VA) and examined in an ETEC autoscan electron microscope.

RESULTS

Incubation of mouse lymphocytes with an excess of PC/PA SUVs over a period of 2 h decreases the cholesterol content of the cells from a cholesterol:phospholipid ratio of 0.31 to 0.20. Concomitantly, the capping of the surface immunoglobulin decreases from an initial value of 74% to 30% in cells incubated with PC/PA vesicles for 2 h (Fig. 1). Furthermore, the fluorescence depolarization of DPH (p-value) in these treated lymphocytes is inversely related to the inhibition of capping; as the p-value decreases, the inhibition of capping increases (Fig. 2). The changes in p-value are correlated with the amount of cholesterol present in the cells over a certain cholesterol:phospholipid range (Fig. 3). Depletion of cholesterol by incubation with PC/PA SUVs lowers the p-value. Incubation of these cholesterol-depleted lymphocytes for 2 h with an excess of SUVs composed of PC/PA/CH restores the cholesterol levels and the p-value for DPH increases to near its control level. Further incubation with PC/PA/CH SUVs raises the cholesterol level in the cells, but capping remains at the same level as the control values (data not shown). There is a point, however, where the p-value no longer reflects cholesterol content directly; the p-value levels off at a value of 0.285 while the cholesterol:phospholipid ratio in the cells continues to increase. Most of our experiments were carried out at levels of cholesterol in the cells where the direct relationship between p-value and the cholesterol:phospholipid ratio existed, thereby affording us a rapid method to check the cholesterol content of the cells.

A summary of the data obtained from incubating lymphocytes with SUVs monitoring IgG capping and cholesterol:phospholipid is presented in Table I. When the cells are incubated with SUVs that remove cholesterol (PC/PA), capping is inhibited by >50%. The inhibition of capping by cis-unsaturated fatty acids is accompanied by a dramatic redistribution of the cytoskeletal elements, the most pronounced being that of α-actinin which changes from a diffuse distribution to a submembranous aggregate at one end of the cell (19). In marked contrast to inhibition by cis-unsaturated fatty

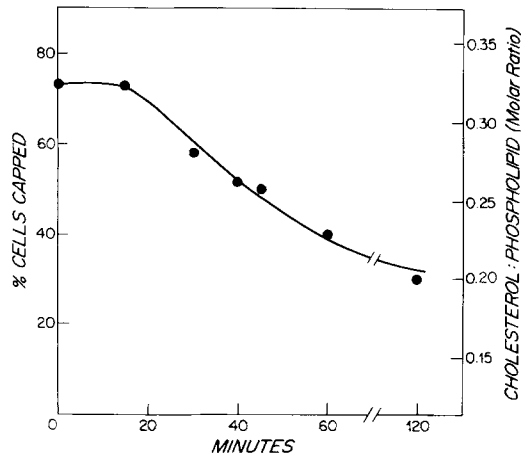

FIGURE 1 The effect of incubating murine lymphocytes with PC/PA SUVs for various times on the cholesterol: phospholipid molar ratio and compared to the percent capping of surface IgG receptors.

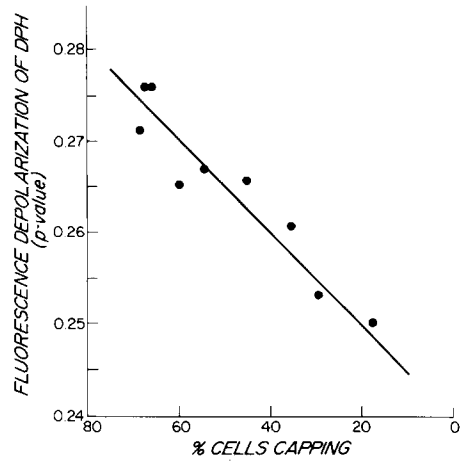

FIGURE 2 The effect of changes in the fluorescence polarization of DPH (p-value), as a result of cholesterol depletion, on the capping of the surface IgG receptors on murine lymphocytes.

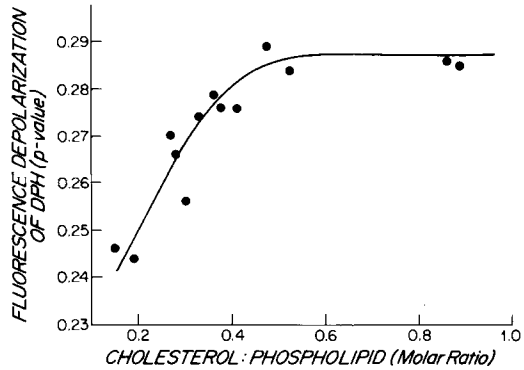

FIGURE 3 The variation in fluorescence polarization of DPH (p-value) with total cellular cholesterol:phospholipid molar ratio.

TABLE I

Relationship between Cholesterol Content and Surface IgG Capping

Treatment	% Capping	cholesterol: phospholipid (molar ratio)
Control, untreated	69 ± 2.5	0.32 ± 0.03
PC/PA 2 h	30 ± 2.0	0.19 ± 0.02
PC/PA/CH 2 h	67 ± 2.5	0.35 ± 0.03
PC/PA 2 h followed by PC/PA/CH 2 h	60 ± 2.5	0.26 ± 0.02

Lymphocytes were treated with SUVs of the appropriate composition, and then assayed for both cholesterol:phospholipid ratio and capping as described in Materials and Methods.

acids, the cytoskeletal element α-actinin is unaffected by cholesterol depletion (data not shown). Lymphocytes incubated with PC/PA/CH SUVs which do not decrease cholesterol content have no effect on the surface IgG capping. Moreover, the process of cholesterol depletion is reversible. If cells that have been depleted of cholesterol are then incubated with SUVs (PC/PA/CH) that can add cholesterol to the cells, cholesterol content and capping are restored to near control values.

Incubation of mammalian cells with phospholipid vesicles or liposomes can also result in sticking, fusion, or endocytosis of the vesicles (25). To minimize these possibilities, a negatively charged phospholipid, phosphatidic acid, was included in all of the SUV preparations used in the present study. Control experiments using PC/PA vesicles containing the nonexchangeable marker [^{14}C]cholesteryl oleate indicated that <1% of the original SUVs remained associated with the lymphocytes after incubation for 2 h. Furthermore, the ultrastructural studies showed no evidence of SUVs attached to the cells.

In a recent report (26), it was suggested that inhibition of capping by free fatty acids is caused by an uncoupling of the respiratory chain and depletion of ATP. This is not the case, as we have shown in a recent paper (23). In the present study we found that lymphocytes, after incubation with SUVs that either deplete cholesterol, replenish cholesterol, or do not affect the cellular cholesterol content, exhibit the same ATP levels as untreated cells (Table II).

Calcium has been implicated by many (27–29) as having a role in regulating the capping phenomenon. We have shown that inhibition of surface IgG capping by cis-unsaturated fatty acids can be reversed by incubating the cells in a high concentration of extracellular calcium (1). It was because of these experiments and those of many others who had investigated the role of calcium that we tested the effects of high extracellular calcium on the capping modulated by cholesterol content. In all instances, untreated, cholesterol-depleted, and cholesterol-repleted cells, we found no effect of incubating with calcium (Table III).

As mentioned above, we have shown that cis-unsaturated free fatty acids but not saturated or trans-unsaturated fatty acids inhibit IgG capping on lymphocytes (1). Also, under these same conditions, the cis-unsaturated fatty acids decrease the p-value of DPH while saturated and trans-unsaturated have little effect. We suggested as part of the explanation for this effect that the fatty acids partition into distinct lipid domains in the membrane, the cis-unsaturated preferentially but not exclusively partitioning into fluid domains, and the saturated and trans-unsaturated partitioning preferentially into gel-like or ordered domains. Several studies (30–34) with synthetic phospholipid membranes containing cholesterol have strongly suggested that the presence of cholesterol induces a lateral phase separation which results in the establishment of lipid domains within the membrane. We therefore felt that depletion of cholesterol from lymphocytes could affect the organization of the lipid domains, and thus we investigated the effects of the free fatty acids on cells in which the cholesterol level had been modulated. Incubating untreated lymphocytes with a cis-unsaturated fatty acid (linoleic acid), we found, as previously, a decrease in p-value and an inhibition of capping. Incubating with a saturated fatty acid (stearic acid) had no effect on either phenomenon. When cholesterol-depleted cells were incubated with fatty acids, the effect was entirely different. Cells in which the cholesterol content had been reduced by half with concomitant decreases in p-values and capping were treated with either linoleic or stearic acids. Linoleic acid incubation further decreased the p-values and amount of capping. Stearic acid, however, reversed the effect, as indicated by a restoration of p-value and capping to levels near control untreated values (Table IV, Fig. 4).

We found further differences in the incorporation of the different free fatty acids into untreated and cholesterol-depleted cells. Equal amounts of stearic and linoleic acids are incorporated into control lymphocytes. In cholesterol-depleted cells, linoleic acid shows a similar amount of incorporation as in the control cells. Stearic acid, however, is incorporated at a level two times higher than its control value and similarly two times higher than the linoleic acid value (Fig. 5).

Ultrastructural studies, both scanning (data not shown) and transmission electron microscopy, revealed no obvious differ-

TABLE II

Levels of ATP in Treated and Untreated Lymphocytes as Determined by the Luciferin/Luciferase Assay

Treatment	ATP
	(mol/cell)
Control, untreated	720
PC/PA 2 h (cholesterol depletion)	735
PC/PA/CH 2 h	765
PC/PA 2 h followed by PC/PA/CH 2 h (cholesterol repletion)	720

Lymphocytes were treated with SUVs and total cellular ATP was measured as described in Materials and Methods.

TABLE III

Effect of Calcium on Capping of Surface IgG after Cholesterol Modulation

Treatment	% Capping	
	1.3 mM Ca^{++}	8.0 mM Ca^{++}
Control, untreated	65 ± 2	63 ± 2.5
PC/PA 2 h (cholesterol depleted)	31 ± 3	38 ± 3
PC/PA/CH 2 h	64 ± 2	60 ± 2
PC/PA 2 h follows by PC/PA/CH 2 h (cholesterol repleted)	64 ± 2	65 ± 2

After treatment with SUVs of the appropriate composition, cells were treated with calcium prior to the capping experiments.

TABLE IV

Effect of Fatty Acid Treatment on Capping and DPH Polarization of Control and Cholesterol-depleted Lymphocytes

	p-value	% Capped
Untreated	0.271 ± 0.003	69 ± 2.5
+SA (30 μM)	0.276 ± 0.002	66 ± 2.0
+LA (30 μM)	0.250 ± 0.003	17 ± 2.5
PC/PA (cholesterol depletion)	0.253 ± 0.003	30 ± 2.0
+SA (30 μM)	0.269 ± 0.002	62 ± 1.0
+LA (30 μM)	0.241 ± 0.002	21 ± 3.0
+PC/PA/CH (cholesterol repletion)	0.265 ± 0.003	60 ± 2.5

Lymphocytes were treated with either SUVs or free fatty acids (*SA*: stearic acid; *LA*: linoleic acid) prior to the determination of both p-value and capping as described in Materials and Methods.

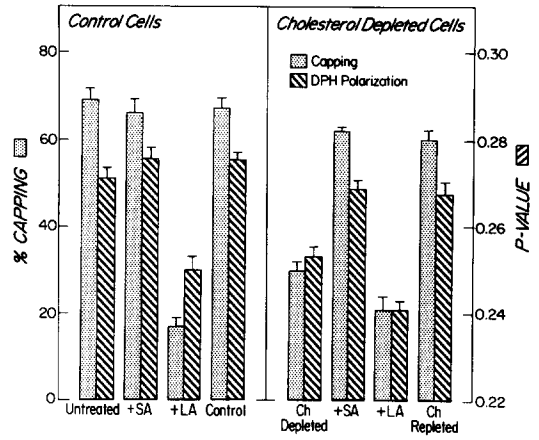

FIGURE 4 The effects of fatty acid treatment (*SA*: stearic acid; *LA*: linoleic acid) on capping and DPH polarization on control and cholesterol (*CH*)-depleted lymphocytes.

ences between the SUV-treated lymphocytes and the untreated cells (Fig. 6). Both had numerous microvilli over the entire surface with no evidence for a change in either number or length. Freeze-fracture studies using filipin to localize and visualize the cholesterol, however, showed dramatic differences (Fig. 7). In the untreated control cells, there were numerous randomly distributed filipin-cholesterol complexes. Lymphocytes treated with PC/PA SUVs showed a dramatic decrease in the density of the filipin-cholesterol complexes which correlated with the cholesterol content. The loss of the complexes appeared to occur in all cells and randomly over the entire surface. On the other hand cells which had first been depleted of cholesterol and then repleted with cholesterol showed not only the reversal in capping and cholesterol content but also an increase in the density of the filipin complexes to the level of the control untreated cells. Cells treated solely with PC/PA/CH SUVs, a condition which does not affect capping or cholesterol content, had a density of filipin-cholesterol complexes similar to that of untreated cells (data not shown).

DISCUSSION

Our results clearly show that a reduction in the cholesterol content of mouse B lymphocytes inhibits the capping of surface immunoglobulins and that the effect can be reversed by replacing the cholesterol. The depletion of cholesterol has been demonstrated by direct chemical measurement and from ultrastructural analysis using filipin. Friend and Bearer (35) have recently shown that the number of filipin-sterol complexes seen in freeze-fracture preparations of *Drosophila melanogaster* cells grown in tissue culture correlated well with the chemically determined sterol content of these cells. The decrease in the number of filipin-cholesterol complexes that we observe after treatment of the cells with SUVs composed of PC/PA (Fig. 7) confirms our chemical measurements. Although it is possible that incubation of the cells with the vesicles could remove surface proteins, the reversibility of inhibition indicates that any such proteins are not involved in the capping process. The inhibitory effect achieved by cholesterol depletion is primarily an alteration of the lipid environment in the surface membrane affecting normal protein function. Unlike our previous studies with cis-unsaturated fatty acids that inhibit capping and cause disruption of the cytoskeleton (19), we find in our present study that cholesterol depletion does not cause rearrangement of α-actinin (data not shown). Also, in contrast to the results in our studies with fatty acids, the inhibition resulting from the removal of cellular cholesterol is not reversed by high concentrations of calcium. Certain similarities do, however, exist between the inhibitory effects observed with cholesterol depletion and with cis-unsaturated free fatty acid treatment. Neither reduces the levels of ATP that would inhibit capping as shown by Pozzan et al. (36). Both treatments lower the polarization values of the fluorescent probe diphenyl hexatriene.

In previous work from our laboratories (5, 6), we proposed that lipid in the surface membrane is organized in the form of domains which are either gel-like or fluid in nature. This suggestion was based on the finding that the fluorescence lifetime for DPH in the surface membrane of the lymphocyte was heterogeneous (5). Furthermore, the differential effect of free fatty acids on membrane function was shown to be consistent with the presence of these lipid domains (5, 6). Using solution theory we have also shown (5) that cis-unsaturated fatty acids partition preferentially but not exclusively into fluidlike lipids, and that saturated and trans-unsaturated fatty acids partition preferentially but not exclusively into gel-like lipid. On the basis of these data, we proposed a model for capping (1, 6) that suggests that the protein(s) necessary for capping is in a lipid domain and that when a cis-unsaturated fatty acid is incubated with the lymphocyte the fatty acid partitions into that domain affecting the cytoskeletal link to the receptor and altering the calcium levels around the protein. As a result, the receptor/antibody complexes aggregate into patches around the cell surface but fail to accumulate into a cap at one end of the cell because the link between receptor and the cytoskeleton has been broken.

We believe that the effect of cholesterol depletion is similar to but less perturbing than treatment with the cis-unsaturated fatty acids. The results of these treatments indicate a disordering of a gel-like lipid domain, as suggested by the lower p-value for DPH, and an inhibition of capping.

In untreated cells, both stearic and linoleic acids are taken up to the same extent. After cholesterol depletion, however, the uptake of linoleic acid is unaltered, whereas the amount of stearic acid incorporated by the cells is almost doubled. Since the binding of linoleic acid would be preferentially in the fluid domains of the membrane, we suggest that these regions would not have been affected by cholesterol removal, thereby leaving uptake of linoleic acid unaltered. We have found (data not shown) that pre-incubation of these control cells with linoleic acid blocks subsequent uptake of ^{14}C-labeled linoleic acid, strongly suggesting that the fluid domains have a finite size.

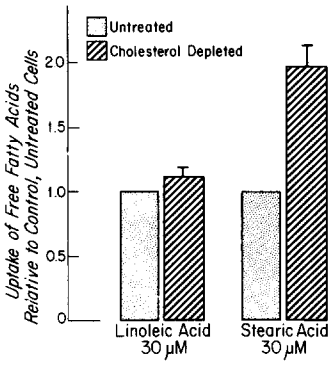

FIGURE 5 The binding of free fatty acids to control and cholesterol-depleted lymphocytes.

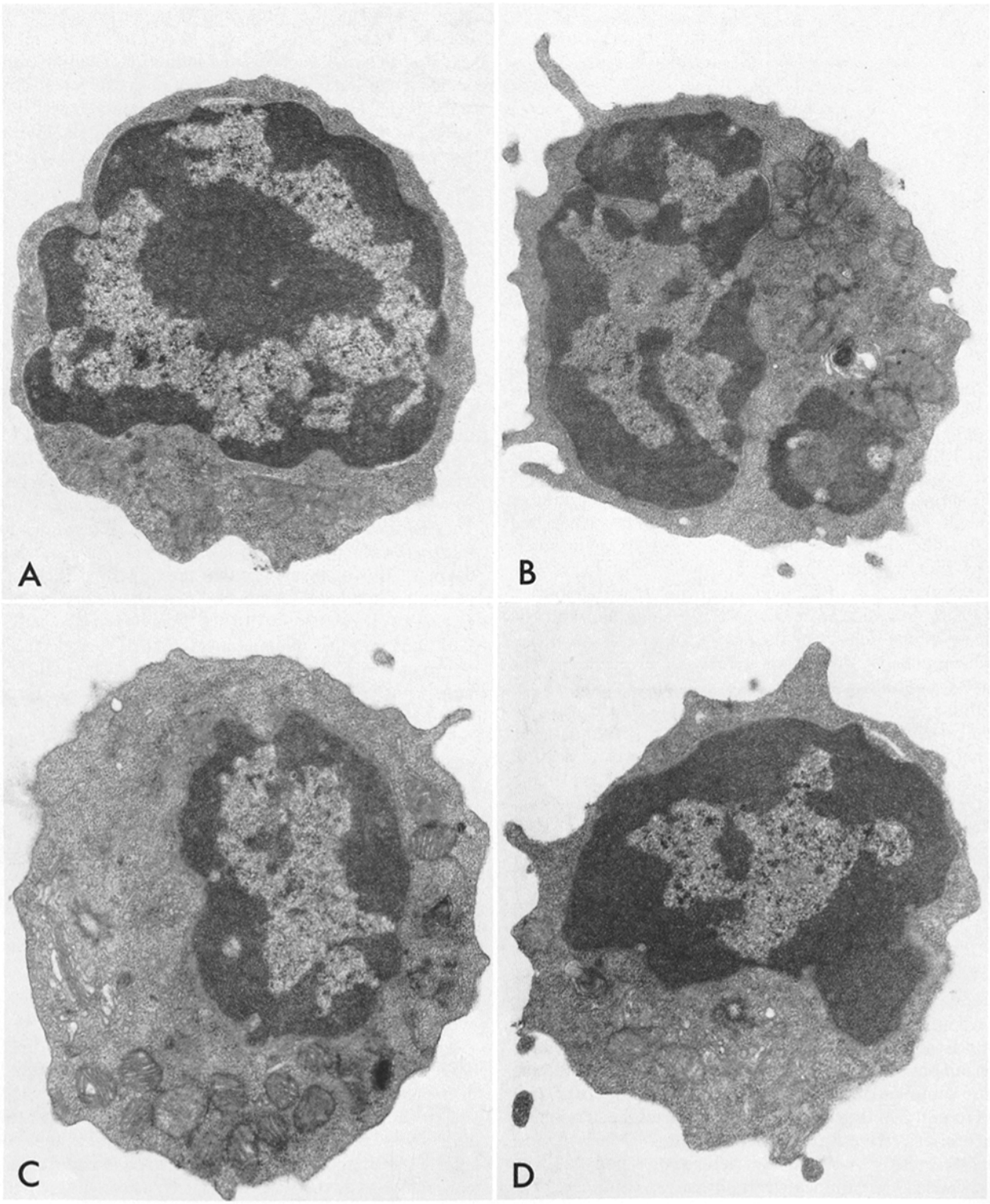

FIGURE 6 Morphology of mouse lymphocytes exposed to SUVs. Cells were treated with SUVs and washed as described in the text prior to fixation. Electron micrographs of mouse lymphocytes. (*A*) Control cell not exposed to SUVs. × 17,000. (*B*) Cell exposed to PC/PA SUVs. × 17,000. (*C*) Cell exposed to PC/PA/CH SUVs. × 17,000. (*D*) Cell exposed to PC/PA and then PC/PA/CH SUVs. × 17,000. Note the healthy appearance of these cells and the absence of SUVs adhering to the surface of treated cells. Stained with uranyl acetate and lead citrate.

We do, however, suggest that cholesterol depletion of lymphocytes would affect the gel-like lipid domains. Our results indicate that cells were depleted by less than half of their total cellular cholesterol. If the gel-like regions in the surface membrane are saturated with respect to cholesterol, then depletion of these regions by one-half would not result in fluid domains. In fact, we have shown that linoleic acid can further lower the p-value for DPH and further inhibit capping in these depleted cells. This we believe is an indication that the protein(s) involved in capping is still in a relatively gel-like lipid domain. The increased binding of stearic acid by cholesterol-depleted cells is consistent with stearic acid partitioning into

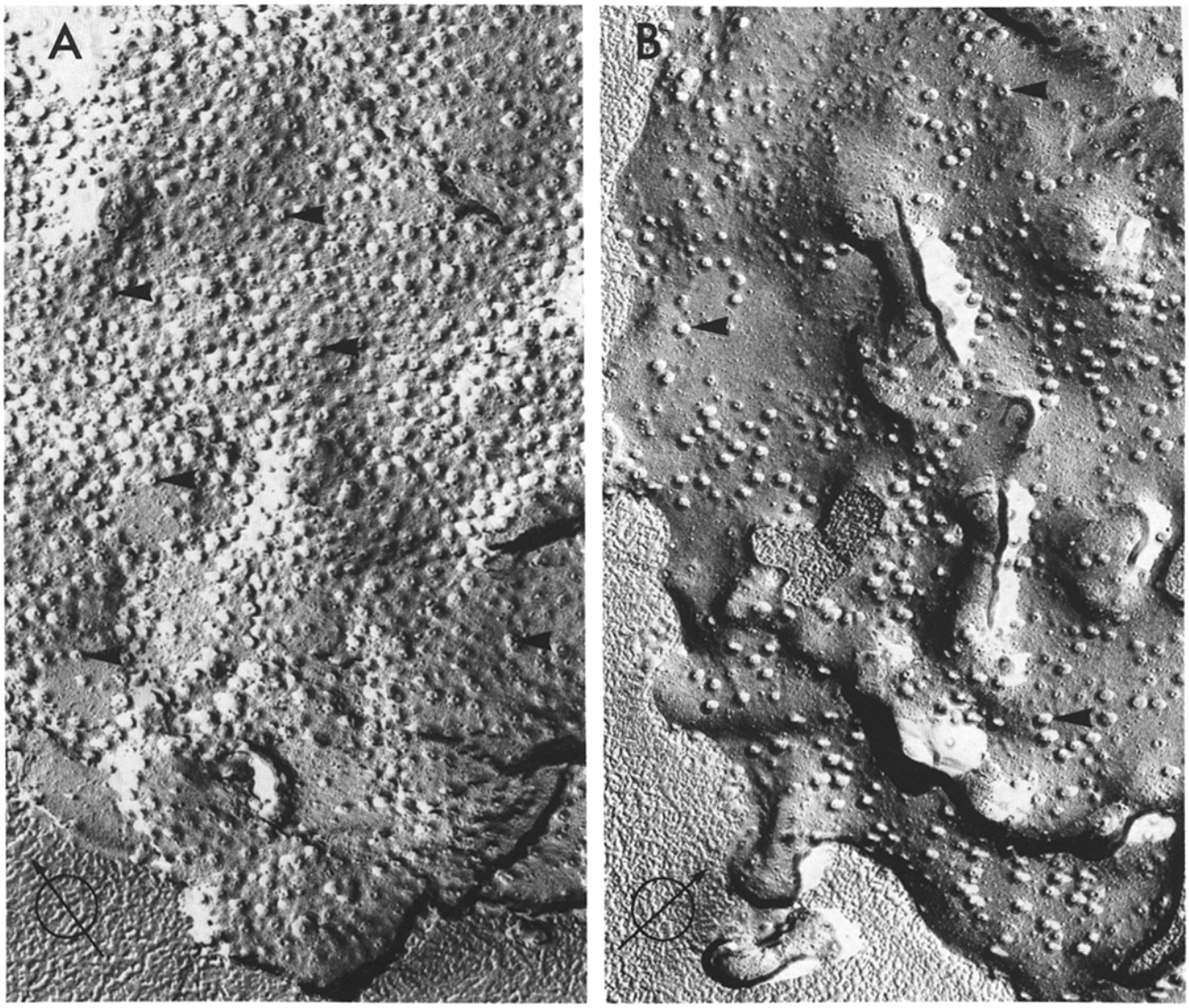

FIGURE 7 Freeze-fracture replicas of filipin-treated mouse lymphocytes. (*A*) Control cell which has numerous filipin-cholesterol (arrowheads) complexes distributed over its plasma membrane. × 30,000. (*B*) Cell exposed to PC/PA SUVs. Note the presence of fewer filipin-cholesterol complexes (arrowheads) than in the control. × 30,000. The direction of shadowing is indicated by the arrows.

the cholesterol-depleted regions to restore their gel-like nature. This, then, might result in a further binding of stearic acid since this saturated fatty acid binds preferentially to gel-like lipid (15). We have found similar effects in control cells. Pretreatment with unlabeled stearic acid enhances the subsequent uptake of ^{14}C-labeled stearic acid over that of cells treated with ^{14}C-labeled stearic acid only (data not shown). We suggest that this effect is possibly a result of stearic acid ordering the fluidlike domain, converting it to a gel-like domain and thereby enhancing further uptake of stearic acid. In support of this proposal, we show that stearic acid increases the p-value for DPH in cholesterol-depleted cells, indicating an increase in the gel-like nature of the lipid. Remarkably, the addition of stearic acid to these depleted cells also reverses the inhibition of capping. It has been shown (7) that most cellular functions of cholesterol are extremely sensitive to the conformation of the hydroxyl group, the planarity of the steroid ring system and the length of the hydrocarbon tail. Stearic acid possesses none of these features, yet it is able to mimic cholesterol in restoring a protein-mediated process initially inhibited by cholesterol removal from the cell.

The results that we have obtained are consistent with our earlier work (5, 6) in which we proposed the existence of lipid domains in surface membranes. As indicated earlier (30–34), the presence of cholesterol in synthetic phospholipid membranes has been shown to bring about phase separations resulting in lipid domains which are gel-like and fluidlike in nature. We wish to suggest that cholesterol in the surface membrane of the lymphocyte plays a similar role with respect to the existence of lipid domains. Removal of cholesterol from the surface membrane modifies the gel-like domain to such an extent that normal protein function is altered. Cornell et al. (14) have also proposed cholesterol-containing domains in the surface membrane of the myoblast which could regulate the aggregation of these cells.

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REFERENCES

1. Klausner, R. D., D. K. Bhalla, P. Dragsten, R. L. Hoover, and M. J. Karnovsky. 1980. A model for capping derived from the inhibition of surface receptor capping by free fatty acids. *Proc. Natl. Acad. Sci. USA* 77:437–441.
2. Hoover, R. L., R. D. Lynch, and M. J. Karnovsky. 1977. Decrease in adhesion of cells cultured in polyunsaturated fatty acids. *Cell.* 12:295–300.
3. MacIntyre, D. E., R. L. Hoover, M. J. Karnovsky, and E. W. Salzman. 1980. Inhibition of platelet aggregation by unsaturated fatty acids. *Circulation.* 62(Pt. II):191. (Abstr.)
4. Hill, D. J., E. A. Dawidowicz, M. L. Andrews, and M. J. Karnovsky. 1983. Modulation of glucose-6-phosphate translocase activity by free fatty acids: implications for lipid domain structure in microsomal membranes. *J. Cell. Physiol.* 115:1–8.
5. 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.
6. Karnovsky, M. J., A. M. Kleinfeld, R. L. Hoover, and R. D. Klausner. 1982. The concept of lipid domains in membranes. *J. Cell Biol.* 94:1–6.
7. Demel, R. A., and B. de Kruyff. 1976. The function of sterols in membranes. *Biochim. Biophys. Acta.* 457:109–132.
8. Bruckdorfer, K. R., P. A. Edwards, and C. Green. 1968. Properties of aqueous dispersions of phospholipid and cholesterol. *Eur. J. Biochem.* 4:506–511.
9. Warren, G. B., M. D. Houslay, J. C. Metcalfe, and N. J. M. Birdsall. 1975. Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. *Nature (Lond.).* 255:684–687.
10. Kimelberg, H. K., and D. Papahadjopoulos. 1974. Effects of phospholipid acyl chain fluidity, phase transitions and cholesterol on ($Na^+ + K^+$)-stimulated adenosine triphosphatase. *J. Biol. Chem.* 249:1071–1080.
11. Klein, I., L. Moore, and I. Pastan. 1978. Effect of liposomes containing cholesterol on adenylate cyclase activity of cultured mammalian fibroblasts. *Biochim. Biophys. Acta.* 506:42–53.
12. Horwitz, A. F., A. Wright, P. Ludwig, and R. Cornell. 1978. Interrelated lipid alterations and their influence on the proliferation and fusion of cultured myogenic cells. *J. Cell Biol.* 77:334–357.
13. Baldassare, J. J., Y. Saito, and D. F. Silbert. 1979. Effect of sterol depletion on LM cell sterol mutants. Changes in the lipid composition on 3-*o*-methylglucose transport. *J. Biol. Chem.* 254:1108–1113.
14. Cornell, R. B., S. M. Nissley, and A. F. Horwitz. 1980. Cholesterol availability modulates myoblast fusion. *J. Cell Biol.* 86:820–824.
15. Backer, J. M., and E. A. Dawidowicz. 1979. The rapid transmembrane movement of cholesterol in small unilamellar vesicles. *Biochim. Biophys. Acta.* 551:260–270.
16. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* 90:374–377.
17. Klein, R. A. 1970. The detection of oxidation in liposome preparations. *Biochim. Biophys. Acta.* 210:486–489.
18. Dawidowicz, E. A., and J. E. Rothman. 1976. Fusion and protein-mediated phospholipid exchange studied with single bilayer phosphatidylcholine vesicles of different density. *Biochim. Biophys. Acta.* 455:621–630.
19. Hoover, R. L., K. Fujiwara, R. D. Klausner, D. K. Bhalla, R. Tucker, and M. J. Karnovsky. 1981. Effects of free fatty acids on the organization of cytoskeletal elements in lymphocytes. *Mol. Cell. Biol.* 1:939–948.
20. Shinitzky, M., and M. Inbar. 1974. Differences in microviscosity induced by different cholesterol levels in the surface membrane lipid layer of normal lymphocytes and malignant lymphoma cells. *J. Mol. Biol.* 85:603–615.
21. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.
22. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.
23. Hoover, R. L., R. D. Klausner, and M. J. Karnovsky. 1982. Inhibition of cap formation on lymphocytes by free fatty acids is not mediated by a depletion of ATP. *J. Biol. Chem.* 257:2151–2154.
24. Robinson, J. M., and M. J. Karnovsky. 1980. Evaluation of the polyene antibiotic filipin as a cytochemical probe for membrane cholesterol. *J. Histochem. Cytochem.* 28:161–168.
25. Pagano, R. E., and J. N. Weinstein. 1978. Interactions of liposomes with mammalian cells. *Annu. Rev. Biophys. Bioenger.* 7:435–468.
26. Corps, A. N., T. Pozzan, T. R. Hesketh, and J. C. Metcalfe. 1980. *cis*-unsaturated fatty acids inhibit cap formation on lymphocytes by depleting cellular ATP. *J. Biol. Chem.* 255:10566–10568.
27. Schreiner, G. F., and E. R. Unanue. 1976. The distribution of immunoglobulin caps by local anesthetics. *Clin. Immunol. Immunopathol.* 6:264–269.
28. Schreiner, G. F., and E. R. Unanue. 1976. Calcium sensitive modulation of Ig capping: evidence supporting a cytoplasmic control of ligand-receptor complexes. *J. Exp. Med.* 143:15–31.
29. Braun, J., R. Sha'afi, and E. R. Unanue. 1979. Crosslinking by ligands to surface immunoglobulin triggers mobilization of intracellular $^{45}Ca^{++}$ in B lymphocytes. *J. Cell Biol.* 82:755–766.
30. Engelman, D. M., and J. E. Rothman. 1972. The planar organization of lecithin-cholesterol bilayers. *J. Biol. Chem.* 247:3694–3697.
31. Demel, R. A., J. W. C. M. Jansen, P. W. M. van Dijck, and L. L. M. van Deenen. 1977. The preferential interaction of cholesterol with different classes of phospholipids. *Biochim. Biophys. Acta.* 465:1–10.
32. Mabrey, S., P. L. Mateo, and J. M. Sturtevant. 1978. High-sensitivity scanning calorimetry of mixtures of cholesterol with dimiristoyl- and dipalmitoylphosphatidylcholines. *Biochemistry.* 17:2464–2468.
33. Rubenstein, J. L. R., J. C. Owicki, and H. M. McConnell. 1980. Dynamic properties of binary mixtures of phosphatidylcholines and cholesterol. *Biochemistry.* 19:569–573.
34. Presti, F. T., and S. I. Chan. 1982. Cholesterol-phospholipid interactions in membranes. I. Cholestane spin-label studies of phase behavior of cholesterol-phospholipid liposomes. *Biochemistry.* 21:3821–3830.
35. Friend, D. S., and E. L. Bearer. 1981. β-hydroxysterol distribution as determined by freeze-fracture cytochemistry. *Histochem. J.* 13:535–546.
36. Pozzan, T., A. N. Corps, C. Montecucco, T. R. Hesketh, and J. C. Metcalfe. 1980. Cap formation by various ligands on lymphocytes shows the same dependence on high cellular ATP levels. *Biochim. Biophys. Acta.* 602:558–566.