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LIPOSOME MODULATION OF SURFACE IMMUNOGLOBULINS ON RABBIT SPLEEN CELLS[1, 2]

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Treatment of rabbit spleen cells with lecithin liposomes caused a substantial decrease in the number of cells bearing surface immunoglobulin (Ig) as assessed by rosetting and immunofluorescent techniques. Immunofluorescent studies demonstrated that the decrease of surface Ig was the result of a capping phenomenon. A comparison of liposome *versus* antibody-induced capping was made by monitoring the respective rates of recovery of displaced surface Ig on spleen cells. Cells treated with specific antibody recovered their surface Ig 6.5 hr after antibody-cell incubation, whereas cells treated with liposomes required 24 hr for a comparable recovery. Thus, the two phenomena appeared to be mechanistically distinct. This conclusion was supported by the fact that disruption of cytoskeletal function with lidocaine completely inhibited antibody-induced capping, whereas capping stimulated by liposomes was unaffected. The data indicate that liposome-induced capping may be modulated by the cholesterol/phospholipid ratio in the lymphocyte membrane. Data in support of this evaluation are as follows. 1) Capping was inhibited by incremental addition of cholesterol to the liposomal membrane. 2) Stimulation of the rate of cholesterol biosynthesis in spleen cells with dextran sulfate before the addition of liposomes reduced the time needed for the recovery of surface Ig on liposome-treated cells from approximately 24 to 8 hr. 3) Inhibition of cholesterol biosynthesis by 25-hydroxycholesterol resulted in the failure of surface Ig to recover. Our data suggest that there is a relationship between the expression of lymphocyte surface Ig and cholesterol biosynthesis. Furthermore, the failure of lidocaine to inhibit liposome-induced capping suggests that surface Ig, under normal conditions, exists free in the lipid bilayer rather than anchored to the lymphocyte cytoskeleton.

The phenomenon of redistribution of lymphocyte surface immunoglobulins (surface Ig) induced by cross-linking with anti-Ig Ab (capping) was first described by Taylor *et al.* in 1971 (1). Since that time, capping of numerous intrinsic membrane proteins on the membranes of a wide variety of cell types has been demonstrated (for a review see Reference 2). Although most methods of inducing capping have involved the cross-linking of specific receptors with antibodies (3–8) or lectins (6, 7, 9–11), other techniques have been described. Yahara and Kakimoto-Sameshima (12) have shown that capping of immunoglobulins, H-2 antigens, Thy-1,2 antigens, and Con A receptors can be stimulated by suspending murine lymphocytes and thymocytes in hypertonic medium. Stanbridge and Weiss (13) have demonstrated that mycoplasma, bound to murine lymphoma cells, will spontaneously form into a cap structure independent of specific antibody. Capping of surface receptors has been shown to occur spontaneously in B lymphocytes during translatory motion (14) and has also been induced by the use of microfilament disruptive agents (15, 16).

The mechanism of cap formation by cross-linking agents has been the subject of considerable controversy, although the consensus is that the process involves an initial energy-independent patching of cross-linked receptors involving their attachment to the mechanochemical proteins comprising the microfilament layer of the cell (4). This is followed by an energy-dependent movement of the patched receptors into a cap (4). Whether all capping induced by cross-linking occurs in this manner is still subject to speculation, although, regardless of the cytoskeletal proteins thought to be involved in the process, for capping to occur, the specific receptor must be able to move within the lipid bilayer of the cell membrane. Since this movement can be affected by the fluidity of the plasma membrane (17), a characteristic that has been shown to be inversely proportional to the cholesterol/phospholipid (c/p)[4] ratio within the lipid bilayer (18–21), it becomes important to further elucidate the role of cell membrane fluidity in the capping process.

The relationship between membrane fluidity and a variety of cellular functions has been studied by the artificial manipulation of membrane c/p ratios with liposomes. Lecithin liposomes have been shown to substantially increase membrane fluidity by both reducing cholesterol levels via a lipid exchange mechanism as well as by increasing the membrane lecithin content by liposome-cell fusion (22). Conversely, liposome-induced decreases in membrane fluidity have been accomplished by using vesicles with relatively high c/p ratios (20, 23, 24). The liposome modulation of cell membrane fluidity has been used to study the relationship between membrane lipid composition and tumor development (21, 24), and has also been employed to elucidate the role of lipids in the response of lymphocytes to

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[4] Abbreviations used in this paper: L, egg yolk lecithin; C, cholesterol; D, dicetylphosphate; HEPES, *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; Ab-E, antibody-coated erythrocytes; RFC, rosette-forming cells; IF, immunofluorescence; C/P, cholesterol to phospholipid ratio.

mitogens (22, 23, 25), the ion flux across erythrocyte membranes (26), and the vertical displacement of intrinsic erythrocyte membrane proteins (18).

In this paper we report the observation of a new phenomenon—the capping of lymphocyte surface Ig induced by cholesterol-free, lecithin liposomes. We present a comparison of liposome-induced capping with Ab-induced capping and also present data that relate the lymphocyte membrane lipid composition to the expression of surface Ig.

MATERIALS AND METHODS

Preparation of liposomes. Large unilamellar liposomes were prepared by the ether infusion technique (27), as adapted by Ostro *et al.* (28). Lipids were solubilized in petroleum ether at a concentration of 4 μmoles lipid/ml solvent. Liposomes were composed of egg yolk lecithin (L) and cholesterol (C), both obtained from Grand Island Biological Company, Grand Island, N. Y., and dicetylphosphate (D), purchased from Sigma Chemical Co., St. Louis, Mo., in the following molar ratios: 8L:2D; 7L:1C:2D; 6L:2C:2D; 5L:3C;2D; 4L:4C:2D. Ten milliliters of the ether-lipid solution were withdrawn into a 20 ml glass syringe and mounted on a vertical infusion pump. An aqueous buffer phase consisting of 2 ml of 5 mM *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, containing NaCl-KCl at a final concentration of 0.145 M, were placed in a stoppered Leibig condenser and heated to 55°C with a circulating water bath. The ether-lipid phase was injected into the aqueous solution through a 22-gauge needle that had been inserted through a rubber stopper at the bottom of the condenser. The injection rate was maintained at 0.5 ml/min. During the injection period, sterile nitrogen was gently bubbled through the aqueous phase in order to promote mixing and reduce lipid oxidation. After ether infusion, the milky-white liposome suspension was removed and gel-filtered on a Sepharose 4B column. Liposome suspensions were adjusted with HEPES buffer to give a standard A_{650} of between 0.5 and 0.8. The lipid concentration of the liposome preparations was shown to be proportional to the A_{650} reading so that an A_{650} of 0.6 was equivalent to approximately 12 μmoles of lipid in 1 ml of liposomes.

Spleen cell preparations. Spleens were excised from rabbits exsanguinated by cardiac puncture. Single cell suspensions were prepared by pressing the spleens through stainless steel screens and filtering the cells through cotton gauze to remove clumps. The cells were then washed four times in Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin (BSA).

Co-culture of liposomes and spleen cells. Spleen cells (2×10^7) were incubated at 37°C with varying amounts of liposomes (0.05 to 1 ml) for periods of up to 1 hr on a tumbledrum apparatus. In most experiments the 8L:2D liposomes were employed, however, in some experiments 7L:1C:2D, 6L:2C:2D, 5L:3C:2D, and 4L:4C:2D liposomes were used. During the incubation period the cells were cultured in RPMI 1640 supplemented with 10% autologous serum. In all experiments the final volume of the liposome-cell co-cultivation was brought up to 5.0 ml with this medium. These standard conditions were used unless otherwise specified. In some experiments, lidocaine (1 mM) was added to the cells 30 min before liposome addition and maintained at this concentration throughout the 1 hr liposome-cell co-cultivation. After liposome-cell cultivation, the cells were washed four times in RPMI 1640 or HBSS to remove nonspecifically adsorbed liposomes. This washing procedure has previously been shown to be effective in the removal of liposomes bound to a variety of cell types (28, see Reference 39). Briefly, this conclusion is based on the results of experiments in which rabbit spleen cells were mixed with liposomes containing *E. coli* [^{3}H] RNA. The [^{3}H] RNA was re-extracted from the washed (4×) cell pellet, and by sucrose density gradient analysis it was found to be degraded. Further, we have shown that the liposome-entrapped RNA remains undegraded (i.e., presence of 16S and 23S RNA) when the liposomes are not mixed with spleen cells. Even after incubation of the liposomes with spleen cells, the RNA remains undegraded in the supernatant containing the liposomes after the first cell wash. If liposomes containing entrapped RNA had nonspecifically adsorbed to the spleen cells, we would have found undegraded RNA in the cell pellet after the washing procedure, and we did not.

Antisera and purified antibody preparations. The anti-allotype antisera (anti-b4 and anti-b5) used in these experiments were prepared in rabbits as previously described (29). The anti-isotype antisera, goat anti-light chain and goat anti-rabbit Ig, were obtained by methods employed in previous studies (30, 31). The antibodies (Ab) were purified and their specificity was established by indirect radioprecipitation (32, 33), by immunofluorescence (IF) and/or by rosetting with antibody-coated erythrocytes (Ab-E).

Rosetting with Ab-E. Purified Ab was coated onto erythrocytes according to the method of Vyas *et al.* (34) as modified by Molinaro and Dray (35). Ab (1 mg/ml in saline) was added to a similar volume of packed sheep red blood cells and the mixture was added to an equal volume of chromium chloride solution (0.01% w/v in saline). The red cell suspension was allowed to incubate at room temperature for 5 min after which the reaction was stopped by adding 50 volumes of saline. The cells were washed four times in saline, and the Ab-E were adjusted so that the final concentration was 2% v/v in saline.

To determine the percentage of cells forming rosettes with Ab-E, the following procedure was used. Equal volumes (0.1 ml) of the lymphocyte (3×10^6 cells/ml) and Ab-E suspensions were mixed and centrifuged. After adding 0.1 ml of 0.1% toluidine blue, the cells were resuspended and a minimum of 300 cells were scored. Lymphocytes surrounded by at least five erythrocytes were counted as rosetted cells. To ensure objectivity in the counting of rosettes, the person doing the counting was not informed as to the nature of the treatment the cells had received (blind experiment).

Membrane immunofluorescence. Specifically purified anti-Fc_γ Ab was conjugated with fluorescein, as described previously (36, 37). After removal of unbound fluorescein on a column of Sephadex G-25-fine, the fluorescein-conjugated Ab was passed through DEAE-cellulose in 0.2 M phosphate, pH 7.5, and eluted with increasing concentrations of NaCl. The fractions eluting with 0.3 M NaCl were used in these studies. For the immunofluorescence (IF) assay, 2×10^7 cells were washed free of liposomes in HBSS containing 10% fetal calf serum (FCS) and 0.02% azide and resuspended in 100 μl of this medium. Twenty-five microliters of the washed cells were mixed with 25 μl of specifically purified anti-b4 Ab (1 mg/ml in saline) and incubated at 4°C for 30 min. The cells were then washed three times in HBSS containing 10% FCS and 0.02% azide, resuspended in 25 μl of this medium, and mixed with 25 μl of fluorescein-conjugated anti-Fc_γ under the conditions described above. After washing, the cells were examined with a Zeiss Universal fluorescent microscope equipped with the Epi-Condenser III RS. A minimum of 300 cells were examined. All experiments were done blind. In some experiments lymphocytes were labeled with rhodamine-conjugated anti-light chain Ab as previously described (36, 37).

Evaluation of Ab-induced capping. Rabbit spleen cells (6 ×

10^6) were incubated with 50 μg of anti-b4 Ab in RPM1 1640 supplemented with 20% FCS for 2 hr at 37°C under 5% CO_2. After the incubation period, the cells were washed three times in HBSS containing 10% FCS and 0.02% sodium azide. These cells were assessed for surface Ig by rosetting with goat anti-rabbit Ig Ab-E as described above.

Recovery of surface Ig capped with either Ab or liposomes. Rabbit spleen cells were treated with liposomes, anti-b4 Ab, or HEPES buffer for 1 hr as described above, washed four times with HBSS containing 0.1% BSA and immediately assessed for the presence of surface Ig by rosetting with Ab-E. The percentage of cells bearing surface Ig directly after the 1-hr incubation period was taken as the 0-time value. Liposomes, Ab, and Hepes-treated cells were each subsequently divided into seven groups containing 2×10^6 cells and cultured at 37°C in 2.0 ml of RPMI 1640 medium supplemented with 10% autologous serum and 50 μg/ml of dextran sulfate, a B cell mitogen. At 2, 6, 8, 18, 20, 22, and 24 hr after the initiation of the culture period, cells from each of the three groups were removed, washed, and analyzed for the presence of surface Ig by rosetting. Cell viability at each time point was determined by trypan blue exclusion. In some experiments, spleen cells were preincubated for 10 hr with 50 μg/ml of dextran sulfate before capping with liposomes. In other experiments 50 μg/ml dextran sulfate was added 10 hr before liposome addition followed by 1 μg/ml of 25-hydroxycholesterol at the time of liposome-cell cocultivation (Steraloids, Wilton, N. H.). It is important to note that extended treatment of spleen cells with 25-hydroxycholesterol leads to reduced viability, which is why the drug is not added during the preincubation period. After liposome-induced capping, the evaluation of the surface Ig recovery rate was done as described above. All drugs were maintained at the stated levels during the entire recovery period.

Determination of the rate of cholesterol biosynthesis in rabbit spleen cells in the presence of dextran sulfate. Spleen cells were suspended in 5 ml of RPMI 1640 medium supplemented with 10% autologous serum and incubated at 37°C under 5% CO_2 with either 50 μg/ml dextran sulfate or an equal volume of HEPES buffer. At designated time intervals after the initiation of the culture period, cells were pulsed for 2 hr with 25 μCi of [^{14}C]acetate, washed, and the cholesterol extracted by the method of Kandutsch and Saucier (38). Briefly, 5 ml of cells, suspended in RPMI 1640 medium, were autoclaved for 1 hr in the presence of 0.6 ml of 95% KOH (lipid saponification), mixed with 3 ml of ethanol and extracted with 15 ml of petroleum ether. The extracted lipids were evaporated to dryness with N_2 and solubilized in 2 ml of acetone-petroleum ether (1:1) containing 0.05 ml of 10% acetic acid, 0.25 mg of nonradioactive cholesterol, and 2.5 mg of digitonin. The mixture was allowed to set at room temperature overnight to allow the cholesterol to precipitate as a digitonide. The following day, the precipitate was collected, washed, and assessed for radioactivity.

Determination of the inhibition of cholesterol biosynthesis by 25-hydroxycholesterol. The effect of 25-hydroxycholesterol on cholesterol biosynthesis was established by incubation of 2×10^7 spleen cells with 1 μg/ml of drug and 50 μg/ml of dextran sulfate for 10 hr before a 2-hr pulse with [^{14}C]acetate. The quantity of radioactivity in the cholesterol extracted from the cells treated with both 25-hydroxycholesterol and dextran sulfate was compared to the cholesterol-associated radioactivity derived from cells treated with only dextran sulfate. Significant reduction in the radioactivity of the cholesterol extracted from 25-hydroxycholesterol-treated cells in relation to the radioactivity associated with cholesterol derived from cells treated with dextran sulfate alone, was taken as evidence for inhibition of endogenous cholesterol biosynthesis. As an added control, cells treated with 25-hydroxycholesterol as described above, were pulsed with 50 μCi of [^{3}H]amino acids for 2 hr in place of the [^{14}C]acetate in order to determine if drug treatment significantly altered the rate of protein synthesis. In these experiments pulsed cells were washed, precipitated with 10% trichloroacetic acid, and assessed for radioactivity.

Effect of energy inhibition on liposome-induced capping. Spleen cells (2×10^7) were treated with either liposomes or HEPES buffer as described above in the presence or absence of 0.02% sodium azide or 0.05 M deoxyglucose. After liposome-cell co-cultivation, the cells were washed and assessed for surface Ig by rosetting with anti-light chain coated Ab-E.

Effect of liposomes on protein synthesis. Rabbit spleen cells were aliquoted into 14 tubes, each containing 2×10^7 cells suspended in 5 ml of RPMI 1640 medium supplemented with 10% autologous serum and 50 μg/ml dextran sulfate. One-half of the cells were incubated for 1 hr at 37°C with 1 ml of liposomes; the second half were treated in a similar fashion with 1 ml of HEPES buffer. During the 1-hr co-cultivation period, one tube of cells from each group was incubated with 50 μCi of [^{3}H]amino acids and used to establish a 0-time value for the rate of incorporation of amino acids into protein. The remaining cells were washed and pulse-labeled with 50 μCi of [^{3}H]amino acid 2 hr before harvesting at 2, 6, 8, 12, and 24 hr. Harvested cells were washed, precipitated with trichloroacetic acid, and assessed for radioactivity.

RESULTS

Liposome-mediated loss of spleen cell surface Ig. Rabbit spleen cells were treated with 1 ml of 8L:2D liposomes for 1 hr at 37°C and assayed for the percentage of Ig-bearing cells by rosetting with either purified anti-light chain allotype Ab-E (anti-b4 or anti-b5), goat anti-light chain Ab-E, or goat anti-rabbit Ig isotype Ab-E (Table I). After liposome treatment, the cells were found to be 95% viable. Although the percentage of rosette-forming cells (RFC) in both control (spleen cells + HEPES buffer) and liposome-treated groups fluctuated, there was a substantial decrease in the percentage of Ig-bearing cells after liposome treatment ranging from 50 to 96%. When spleen cells obtained from a single animal were treated with varying doses of liposomes (0.05 to 1.00 ml), a linear dose-response curve was obtained (Fig. 1). Although the maximum dose of 1 ml of liposomes did not result in total inhibition of rosetting,

TABLE I

Liposome-induced loss of surface Ig

Rabbit No.	Ab-E Specificity	% RFC at Time 0	% RFC after Liposome Incubation[b]	% Inhibition of Rosetting
			1 hr	
1	b5[a]	49	2	96
2	b4[c]	30	7	77
3	Light-chain	37	4	86
4	Light-chain	47	24	50
5	Light-chain	62	16	75
6	Light-chain	55	5	91
7	Light-chain	53	19	65
8	Light-chain	55	17	70
9	Ig	60	15	75

[a, c] Spleen cells isolated from $a^1a^1b^5b^5$ and $a^2a^2b^4b^4$ homozygous rabbits were mixed with anti-b5 or anti-b4 Ab-E (Ab to κ light chain allotypes) and the % RFC was determined.

[b] O.D.$_{650}$ of 8L:2D liposomes ranged between 0.50 and 0.80.

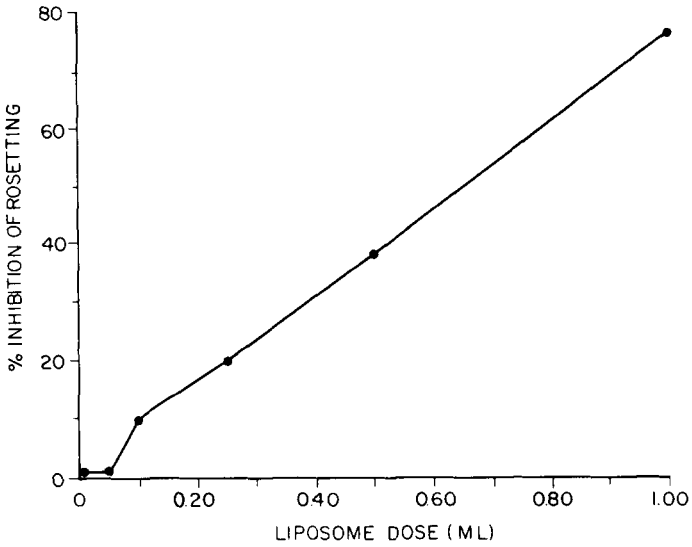

Figure 1. Dose dependency of the liposome-induced loss of rabbit spleen cell surface Ig. Spleen cells (2×10^7) were mixed with varying doses of 8L:2D liposomes ($O.D._{650} = 0.70$) for 1 hr at 37°C, washed free of liposomes, and assayed for surface Ig by rosetting with anti-light chain Ab-E. In the control (no liposomes added), 57% of the spleen cells rosetted with Ab-E.

higher doses were not employed due to the development of cytotoxicity. It is important to note that the loss of surface Ig was probably not due to the impairment of the rosetting assay by nonspecifically adsorbed liposomes. This conclusion is based upon the fact that the washing procedure employed in this study has been shown to effectively eliminate adsorbed liposomes from the lymphocyte surface (28, 39).

Liposome-mediated capping of spleen cell surface Ig. The possibility that the liposome-mediated loss of spleen cell surface Ig was due to capping was investigated by monitoring the movement of surface Ig by IF (Fig. 2 and Table II). Spleen cells were treated for either 5, 15, 30, or 60 min with liposomes under standard conditions. Cells at each time interval were washed with RPMI-azide and assayed for surface Ig by either rosetting with anti-light chain Ab-E or by IF by using either rhodamine-conjugated anti-light chain Ab or fluorescein-conjugated anti-Fc_γ after treatment of the cells with anti-b4 Ab. The original percentage of Ig-bearing cells was assessed by rosetting and IF before liposome incubation and was determined to be similar to the percentage of RFC obtained when spleen cells were incubated with HEPES buffer at 37°C for 1 hr. It was found (Fig. 2) that after 5 min of liposome-cell incubation, 95% of the original number of Ig-bearing cells exhibited membrane fluorescence with only 4% of the cells appearing capped. After 30 min of liposome-cell cultivation, only 30% of the cells exhibited membrane fluorescence. This loss (65%) in membrane fluorescence was associated with an increase in the percentage of capped cells from 4 to 50%, and nonfluorescent cells from 1 to 20%. After 60 min of incubation, the percentage of membrane fluorescent cells remained constant, whereas the percentage of capped cells decreased from 50 to 20%, and the percentage of nonfluorescent cells increased from 20 to 50%. The percentage of the cells detected with RFC at 5, 30, and 60 min was similar to the percentage of Ig-bearing cells that exhibited general membrane fluorescence (5 min:88% RFC, 95% MF; 30 min: 20% RFC, 30% MF; 60 min:25% RFC, 30% MF) suggesting that the capped cells do not form rosettes. Whether the loss of both membrane fluorescent and capped cells represents a shedding of surface Ig or endocytosis has not yet been determined. The kinetics of capping shown in Figure 2 was supported by IF data obtained by the more sensitive indirect IF technique (Table II) described in the *Methods* section. It should be noted that the values reported for the number of nonfluorescent cells were determined by counting the number of cells that did not exhibit any fluorescence at each time point and subtracting from that value the number of cells that were determined not to have surface Ig by rosetting and IF at time 0. This extrapolation was considered valid since at all time points the cell viability was greater than 95%, precluding the possibility of selective killing of T cells.

Figure 3 shows a representative sample of labeled membrane

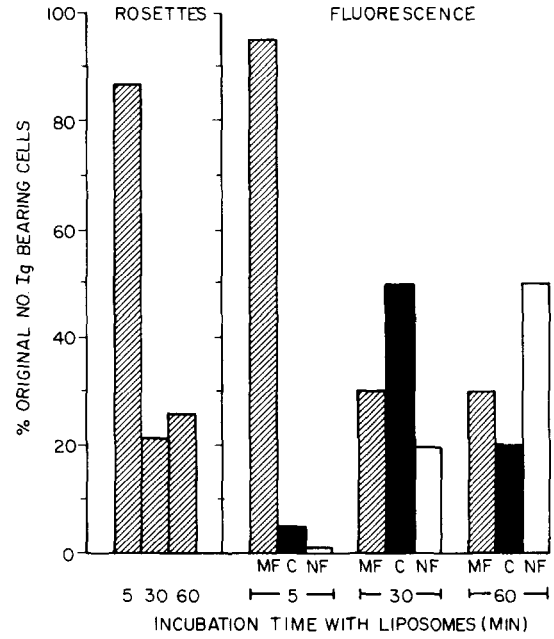

Figure 2. Time-dependent study of the liposome-induced loss of rabbit spleen cells surface Ig. Spleen cells (2×10^7) were mixed with 1 ml of 8L:2D liposomes ($O.D._{650} = 0.75$) for either 5, 30, or 60 min at 37°C, washed with 0.02% azide, and assessed for surface Ig by both rosetting with anti-light chain Ab-E and IF with rhodamine-conjugated anti-light chain Ab. MF, membrane fluorescence, C, capped cells, NF, nonfluorescent cells.

TABLE II

Kinetics of liposome-induced loss of surface Ig

Time of Liposome-cell Incubation	% of Original Number of Ig-Bearing Cells[a]			
	RFC[b]	Membrane fluorescent cells[c]	Capped cells[c]	Nonfluorescent cells[d]
min				
5	88	88	11	1
15	61	55	41	3
30	47	40	50	10

[a] Original percentage of Ig-bearing cells was determined by rosetting and indirect immunofluorescence before the addition of liposomes and was found to be 51% and 53%, respectively. All subsequent calculations were based on a valve of 52% (average of two determinations).

[b] RFC's were determined by rosetting with anti-b4 Ab-E.

[c] Fluorescent cells were determined by the indirect immunofluorescent technique described in detail in *Materials and Methods.* Labeled Ab was fluorescein-conjugated anti-rabbit Fcγ.

[d] Nonfluorescent cells were determined by counting the number of cells that did not exhibit any fluorescence at each time point and substracting from that valve the number of cells determined not to have surface Ig at time 0 (48%).

fluorescent and capped cells with fluorescent anti-light chain Ab after liposome incubation. Although the cells with intensely stained polar areas have been designated as capped, one must eliminate the possibility that these brilliant regions of fluorescent label are due to cytoplasmic staining resulting from labeled antibody nonspecifically inserted into the cells by liposome carriers. To address this problem, liposome-treated cells were incubated with fluorescein-conjugated anti-rabbit Fc_γ antibody and assessed for the presence of fluorescent label. Of the 300 cells that were examined, none exhibited detectable amounts of fluorescence. This would indicate that the cells that appeared to be capped were in fact the result of a membrane phenomenon. Morphologically, the caps shown in Figure 3 could not be distinguished from those induced in our laboratory by cross-linking Ab or from those originally published by Taylor *et al.* (1).

Since the rosetting and IF data correlated quite well, all subsequent assessment of surface Ig was done by using the rosetting technique. (It should be noted that a close correlation between rosetting data and data obtained with ^{125}I-labeled Fab fragments has also been found (40)). The rosetting procedure was used for two reasons: 1) the methodology allows for more rapid assays when large numbers of cells are employed, and 2) it has been shown that the rosetting technique is more sensitive than IF (41).

Recovery kinetics of liposome and Ab-capped surface Ig. Figure 4 represents the kinetics of recovery of surface Ig on spleen cells treated with either 8L:2D liposomes or anti-b4 Ab. Immediately after spleen cells were incubated with either liposomes or Ab, the cells were washed and put into culture as described in the Methods section. Aliquots of cells were removed from the culture over a 24-hr period, washed with azide, and rosetted with anti-Ig Ab-E. Loss of surface Ig was induced by both the liposome and Ab treatment. The Ab-treated cells recovered 90% of their surface Ig after 6.5 hr, the time of protein turnover (42, 43), whereas substantial recovery of the surface Ig on liposome-treated cells was not complete until 24 hr, thus implying that the two phenomena are mechanistically distinct.

The suggestion that diverse mechanisms are associated with liposome and Ab-induced loss of surface Ig is supported by data obtained on the effect of lidocaine (xylocaine) on the ability of lymphocytes to form rosettes. Lidocaine has been shown to disrupt the microfilament and microtubule components of the lymphocyte cytoskeleton, thus inhibiting Ab-induced capping, a process that, at the very least, is dependent upon microfilament integrity (44). When 1×10^{-3} M lidocaine was added to 2×10^7 spleen cells 30 min before the addition of either cross-linking Ab (anti-b4) or liposomes, the Ab-induced loss of RFC was completely inhibited, whereas the RFC loss stimulated by 8L:2D liposomes was uneffected (Table III). In view of the previous data presented on the ability of liposomes to induce capping of surface Ig, it appears likely that the liposome-induced loss of RFC in the presence of lidocaine is due to a capping phenomenon. Viability of cells after lidocaine treatment was found to be greater than 90%.

Effect of liposomes on protein synthesis. The slow recovery of surface Ig on lymphocytes after liposome-induced capping (24 hr) may be due to the inhibition of protein synthesis by

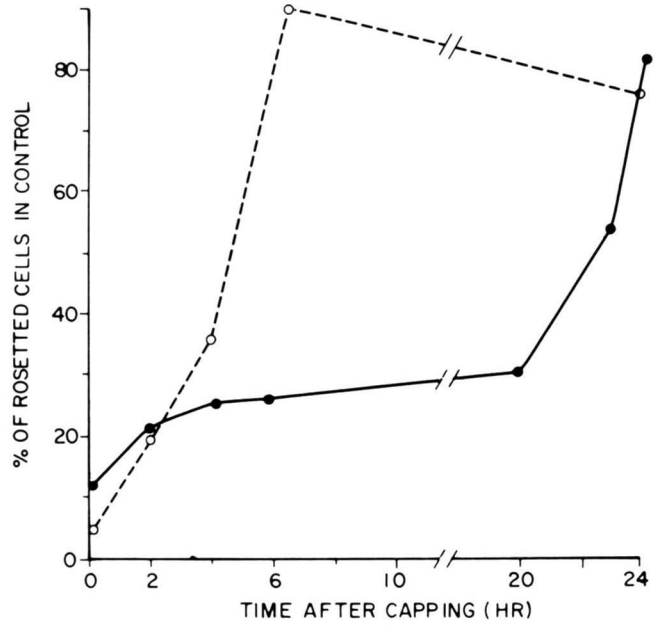

Figure 4. Comparison of the kinetics of recovery of rabbit spleen cell surface Ig after treatment with either 8L:2D liposomes (●——●) or anti-b4 Ab (○----○). Spleen cells (2×10^7) were treated with 1 ml of 8L:2D liposomes ($O.D._{650}$ = 0.7) for 1 hr at 37°C, washed free of liposomes, and assessed for surface Ig by rosetting with anti-light chain Ab-E immediately after incubation with liposomes and after culturing in RPMI for periods up to 24 hr. Simultaneously, spleen cells (6×10^7) were incubated with anti-b4 Ab (50 μg) for 2 hr at 37°C, washed, and assessed for surface Ig by rosetting with anti-Ig Ab-E.

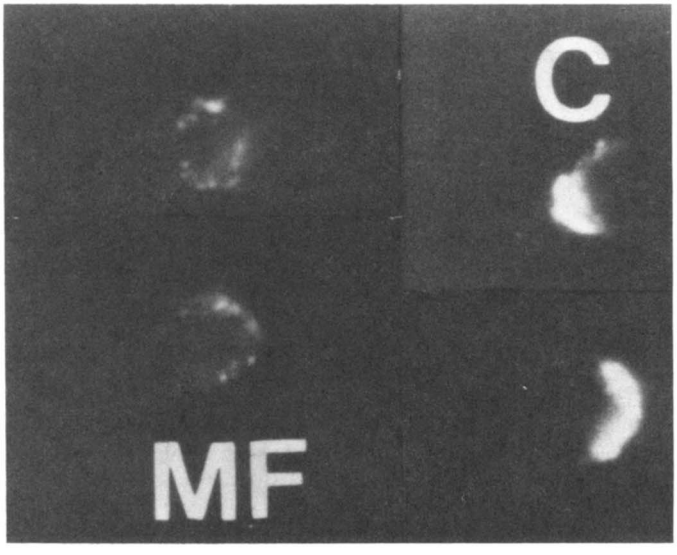

Figure 3. Fluorescent micrograph of rabbit spleen cells incubated with 8L:2D liposomes at 37°C. Spleen cells were washed free of liposomes with 0.02% azide and treated with rhodamine-conjugated anti-light chain Ab for 30 min at 4°C. MF, membrane fluorescent cells, C, capped cells.

TABLE III

Effect of lidocaine on capping induced by liposomes and antibody

Cell Treatment	% RFC after 1 Hr of Incubation		
	Expt. 1	Expt. 2	Expt. 3
Cells + hepes buffer	47	50	54
Cells + lidocaine (1 mM)	44(94)[a]		
Cells + liposomes[b]	14(28)	10(38)	19(35)
Cells + antibody[c]	10(21)	13(26)	19(35)
Cells + lidocaine + liposomes[b]	13(28)	20(40)	24(44)
Cells + lidocaine + antibody[c]	47(100)	50(100)	48(88)

[a] Numbers in parentheses represent the % of the buffer control.
[b] $O.D._{650}$ of 8L:2D liposomes was 0.75; 2×10^7 cells were used.
[c] Fifty micrograms of anti-b4 antibody was added to 6×10^6 cells.

liposomes. This possibility was investigated by monitoring the rate of incorporation of [^{3}H]amino acids into acid precipitable protein in cells that had been treated with liposomes, and comparing these values with those obtained from control cells that were incubated with HEPES buffer (Fig. 5). Dextran sulfate was added to each group of cells to maintain a high percentage of viability. Over a 24-hr period, the level of protein synthesis in liposome *versus* HEPES-treated cells was not found to be significantly different, indicating that the slow recovery of liposome-capped surface Ig is probably not due to a liposome-induced reduction in the rate of protein synthesis.

Energy requirements for liposome-induced capping. The effect of inhibition of glycolysis (deoxyglucose) and oxidative phosphorylation (sodium azide) on liposome-induced capping is shown in Table IV. When spleen cells were mixed with liposomes in the absence of energy inhibitors, there was a substantial loss (75%) in the number of Ig-bearing cells as assessed by rosetting. However, when the liposome-cell co-cultivation was done in the presence of sodium azide, the amount of loss of surface Ig was insignificant (9%). To determine if the observed inhibition of capping was in fact due to a depletion of the energy supply, a second inhibitor, deoxyglucose, was used. In this instance, there was a significant amount of loss of surface Ig, from 41% RFC in the control (HEPES buffer and deoxyglucose) to 22% RFC when liposomes were added. Correct interpretation of these data is difficult in view of the failure to inhibit liposome-induced capping by cytoskeletal disruption (Table III). If capping of surface Ig by liposomes is independent of the mechanochemical proteins that comprise the cytoskeleton, then one would predict that the process should be energy independent and not inhibited by azide.

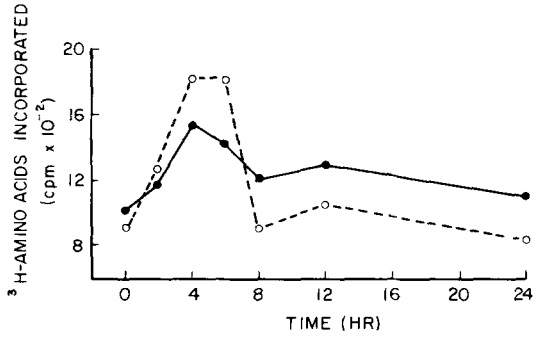

Figure 5. Effect of liposomes on spleen cell protein synthesis. Spleen cells (2×10^7) were incubated for 1 hr at 37°C with either 8L:2D liposomes (○——○) or Hepes buffer (●——●), washed, and cultured in RPMI-1640 medium containing 50 μg/ml of dextran sulfate and 10% autologous serum. At the designated time periods, the cells were pulsed for 2 hr with ^{3}H-amino acids, washed, and assessed for protein-associated radioactivity by precipitation of whole cells by trichloroacetic acid.

TABLE IV

Effect of azide and deoxyglucose on liposome-induced loss of spleen cell surface Ig

Cell Treatment	% RFC[a]
Hepes buffer	55
Liposomes[b]	10
Azide (0.02%)	55
Azide + liposomes[b]	50
Deoxyglucose (0.05 M)	41
Deoxyglucose + liposomes	22

[a] RFC was determined after 1 hr of incubation at 37°C under the conditions described as cell treatment.

[b] O.D.$_{650}$ of 8L:2D liposomes was 0.56.

However, although azide totally inhibited capping, deoxyglucose had only a marginal effect, which might imply that the inhibition of capping by azide is due to a secondary effect and not the result of energy inhibition. This problem is currently under investigation.

Dependence of liposome-induced capping on membrane cholesterol and phospholipid content. Since capping of surface Ig by liposomes appeared to be independent of cytoskeletal function, it was hypothesized that the observed capping was caused by a decrease in the lymphocyte c/p ratio resulting from lipid exchange between cholesterol-free liposomes and cells as well as fusion of the vesicle bilayer with the lymphocyte membrane, and that the recovery rate of liposome-capped surface Ig was dependent upon the restoration of a normal membrane c/p ratio. In fact, preliminary data indicate that after 1 hr of liposome-cell co-cultivation, the c/p ratio (standardized per mg protein) of the purified lymphocyte plasma membrane drops from 0.70 to 0.18, a shift contributed to by both a loss of cholesterol and an increase in lecithin in the cell membrane. It was therefore predicted that the liposome-induced capping should be abrogated if the cholesterol content of the liposome was increased to a level at which the ability of the vesicle to affect a net reduction in the c/p ratio of the lymphocyte membrane was impaired. To that end, spleen cells were treated with liposomes of increasing cholesterol content, and the loss of surface Ig was monitored by rosetting (Fig. 6). The addition of 8L:2D liposomes resulted in a decrease in the number of RFC from 53% in the HEPES-treated control to 17%. As the mole-percent of cholesterol in the liposomes was increased, there was a concomitant increase in the percent RFC until the control level was reached. Although these data are consistent with the above hypothesis, it is conceivable that the cholesterol-dependent elimination of capping is due to a reduced tendency of cholesterol-containing liposomes to fuse with lymphocytes. However, this interpretation does not appear likely since the difference between the ability of 8L:2D and 4L:4C:2D liposomes to fuse with rabbit lymphocytes has been found to be minimal (39).

The effect of cell membrane cholesterol levels on the capping and recovery of surface Ig was further investigated by studying the consequence of altering the rate of endogenous cholesterol biosynthesis on the rate of recovery of liposome-capped surface Ig. It was found that cholesterol biosynthesis could be stimu-

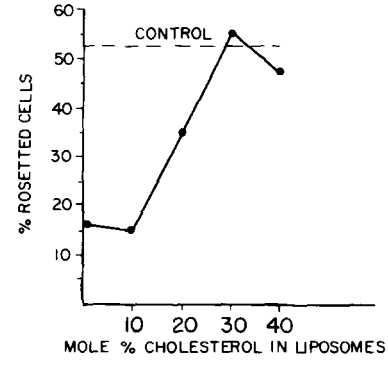

Figure 6. Determination of rabbit spleen cell surface Ig as a function of liposome cholesterol content. Spleen cells (2×10^7) were treated for 1 hr at 37°C with either 8L:2D, 7L:1C:2D, 6L:2C:2D, 5L:3C:2D, or 4L:4C:2D liposomes normalized to an O.D.$_{650}$ of 0.55. Following liposome-cell incubation, the spleen cells were washed free of liposomes and assessed for surface Ig by rosetting with anti-light chain Ab-E. Control consisted of spleen cells(2×10^7) treated with 1 ml of Hepes buffer for 1 hr at 37°C.

lated 3- to 4-fold by dextran sulfate and that this stimulation could be specifically abrogated by the addition of 1 μg/ml of 25-hydroxycholesterol to the mixture (Fig. 7). [25-hydroxycholesterol has been shown to specifically inhibit cholesterol biosynthesis by preventing the production of 3-hydroxy-3-methylglutaryl CoA reductase, a regulatory enzyme required for the production of mevalonic acid (45).] By using this information, the effect of altered cholesterol biosynthesis on the recovery of liposome-capped surface Ig was assessed. Spleen cells were pretreated with dextran sulfate 10 hr before liposome addition, and the rate of recovery of capped surface Ig was compared to that observed when mitogen pretreatment was eliminated. It was found that cells that had been pretreated with dextran sulfate recovered their surface Ig in approximately 8 hr compared to the normal 24-hr recovery rate (Fig. 8).

The observed increase in the recovery rate induced by dextran sulfate pretreatment could be due to an unassayed effect of the mitogen on the lymphocytes. Since 25-hydroxycholesterol inhibits the dextran sulfate stimulation of cholesterol biosynthesis (Fig. 7), the enhanced recovery rate of surface Ig in the presence of dextran sulfate should be eliminated when 25-hydroxycholesterol is added to the cells. The results of this experiment can be seen in Figure 8. Although cells pretreated with dextran sulfate before liposome capping recovered their surface Ig in 8 hr, the inclusion of 25-hydroxycholesterol at the time of liposome addition prevented the recovery of capped surface Ig up to 21 hr. Cells treated with 25-hydroxycholesterol were found to be greater than 90% viable after the 21 hr of

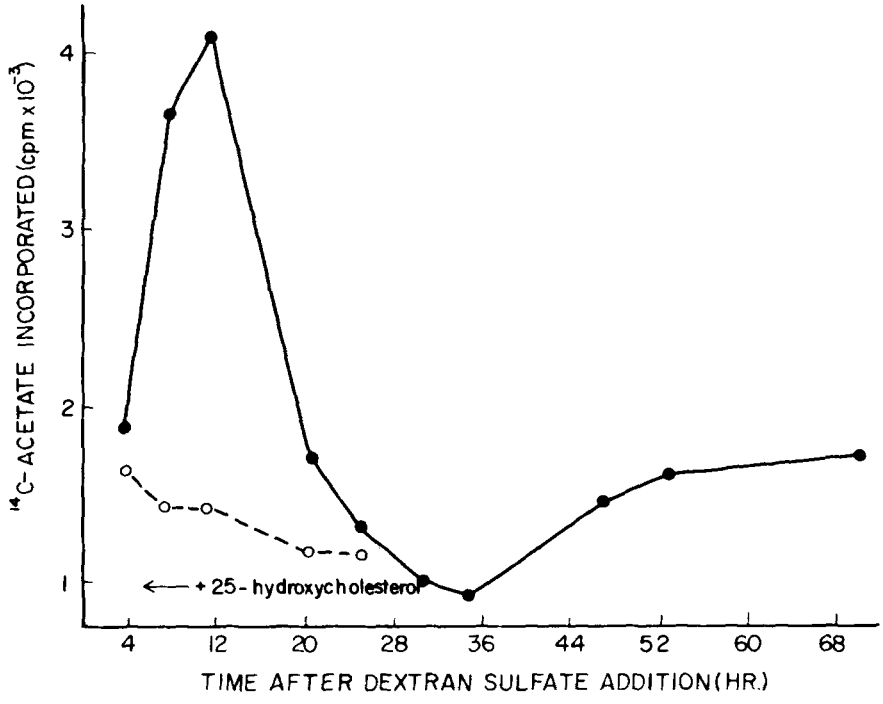

Figure 7. Evaluation of the rate of cholesterol biosynthesis after treatment of spleen cells with 50 μg/ml dextran sulfate (●——●), Hepes buffer (○-----○), or dextran sulfate plus 1 μg/ml 25-hydroxycholesterol. Cells were maintained in RPMI-1640 medium supplemented with 10% autologous serum and at time intervals pulsed for 2 hr with ^{14}C-acetate. Cholesterol was extracted as a digitonide and assessed for radioactivity. The arrow indicates the level of cholesterol biosynthesis after 10 hr of incubation of spleen cells with dextran sulfate and 25-hydroxycholesterol.

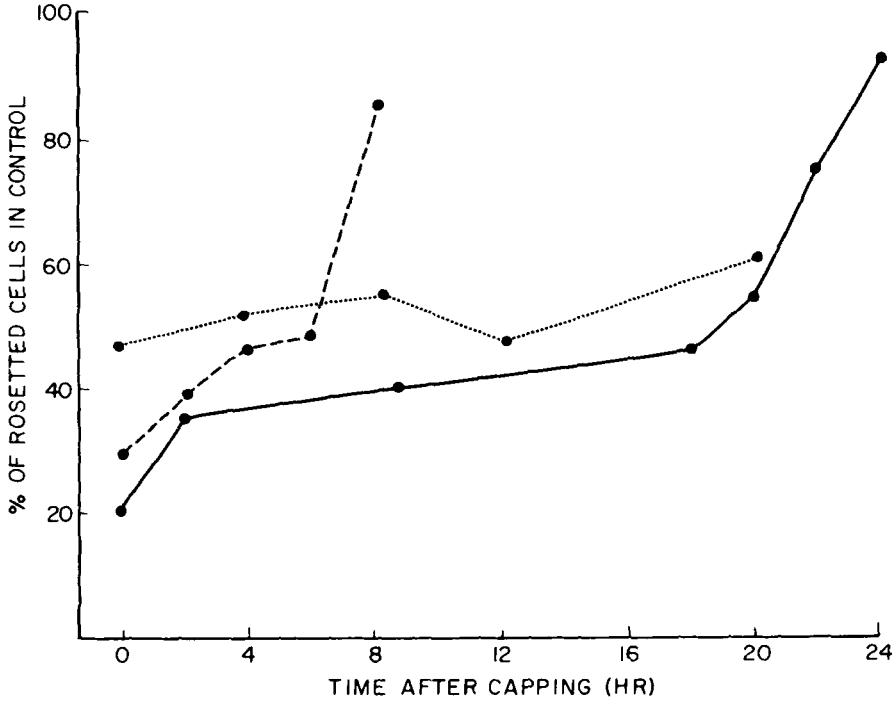

Figure 8. Kinetics of recovery of 8L:2D liposome-capped surface Ig on spleen cells after a 10-hr preincubation of cells with Hepes buffer (●——●), 50 μg/ml dextran sulfate (●— —●), or 50 μg/ml dextran sulfate followed by addition of 1 μg/ml 25-hydroxycholesterol at the time of liposome-cell co-cultivation (●-----●). Following 1 hr of liposome-cell incubation, the cells were washed, cultured in RPMI-1640 medium, supplemented with the additives described above for preincubation, and assessed for surface Ig over time by rosetting with anti-light chain Ab-E.

incubation. The 25-hydroxycholesterol was not added to the cells during the 10-hr dextran sulfate preincubation period in order to minimize the cytotoxicity that results from prolonged exposure to the drug. As an added control, it was found that 25-hydroxycholesterol had no appreciable effect on protein synthesis as determined by comparing the amount of [^{3}H]amino acids incorporated into TCA precipitable material in 25-hydroxycholesterol-treated and untreated spleen cells (data not shown).

DISCUSSION

We have demonstrated that treatment of rabbit spleen cells with 8L:2D liposomes results in a substantial decrease in Ig-bearing cells when assayed by either rosetting or IF. This loss of surface Ig has been shown to be directly proportional to the dose of the liposomes and inhibited by sodium azide but not deoxyglucose. When the liposome-induced loss of surface Ig was assessed as a function of time by IF, it was apparent that the disappearance of the membrane Ig was due to its lateral displacement and followed a pattern consistent with what would be predicted for the capping of lymphocyte surface Ig. The liposome-induced capping was shown to be distinct from capping by anti-b4 Ab by the following criteria: 1) Cells capped with Ab recovered their surface Ig in approximately 6 hr, whereas liposome-capped cells failed to show significant recovery until 24 hr post-liposome-cell cultivation. The slow recovery of surface Ig after liposome-capping was shown not to be the result of impaired protein synthesis. 2) Capping of surface Ig with Ab was totally inhibited by cytoskeletal disruption (lidocaine), whereas liposome capping was unaffected.

The ability of liposomes to cap surface Ig and the subsequent recovery of these displaced receptors seems to be dependent upon the cell membrane c/p ratio, which has been shown to be substantially reduced (increased fluidity) by the addition of cholesterol-free liposomes (23). This conclusion is based on 3 observations. 1) Capping of surface Ig by liposomes is abrogated by increasing concentrations of cholesterol in the liposome membrane, that would tend to diminish the possibility of a net change in the lymphocyte membrane c/p ratio. 2) Stimulation of cholesterol biosynthesis by dextran sulfate prior to liposome-cell cocultivation results in a marked enhancement in the recovery rate of capped surface Ig from 24 to 8 hr. 3) Elimination of the dextran sulfate stimulation of cholesterol biosynthesis by 25-hydroxycholesterol delays the rate of surface Ig recovery without affecting protein synthesis.

The capping of surface Ig by enhancing membrane fluidity would imply that surface Ig is not anchored to the cell's cytoskeleton under noncross-linking conditions. This evaluation is buttressed by the fact that liposome-induced capping of surface Ig is not inhibited by lidocaine and is consistent with recent data that show that surface Ig is probably not a transbilayer protein (46) and that cross-linking with Ab initiates a process whereby free immunoglobulins become attached to the actin in the microfilaments (47). The lack of association between the surface Ig and any mechanochemical protein during the capping process makes interpretation of the liposome capping data difficult. However, reports on capping of surface receptors independent of cytoskeletal control have been published. Albertini *et al.* (9), in studying the Con A receptor on human leukocytes, have found that disruption of the microtubules, elements of the cytoskeleton that have been shown to have a skeletal rather than a contractile function (48), can lead to a microfilament-independent movement of Con A receptors even though the cap eventually corresponds to the region of microfilament concentration. Schroit and Pagano (49) have demonstrated that cross-linking of haptenated phosphatidylethanolamine, inserted into fibroblast membranes by liposomes, resulted in the formation of patches that eventually coalesced into one or more caps. Finally, Mousa *et al.* (15) and Sundqvist *et al.* (16) have suggested that cytochalasin D and B (microfilament disruptive drugs) induce membrane receptor mobility on the membranes of a variety of transformed cell types.

In contrast to the liposome-mediated capping of surface Ig that we report, Ben-Bassat *et al.* (50) have found that increased membrane fluidity does not increase the mobility of the Con A receptor. Moreover, Hilgers *et al.* (51) have demonstrated that increases in the fluidity of the membranes of murine thymus-derived leukemia cells resulted in the reduced ability of histocompatibility antigens as well as two virus-derived receptors to form caps. The differential response to enhanced membrane fluidity by surface Ig, Con A receptor, and histocompatibility antigens may be a function of the orientation of the proteins in the membrane. As previously indicated, surface Ig does not seem to be a transbilayer protein (46) and does not appear to be attached to the cytoskeleton in the absence of cross-linking Ab (47). Conversely, it has been suggested that both the Con A receptor and the histocompatibility antigens are anchored in either the microtubules or microfilaments of the cell, possibly limiting their ability to move in response to changes in membrane lipid composition. This theory is supported by data from our laboratory that show that 8L:2D liposomes are only able to stimulate capping of the Con A receptor on rabbit spleen lymphocytes in the presence of a cytoskeletal disruptive agent (manuscript in preparation).

Although the mechanism for liposome-induced capping is not as yet clear, it seems likely that liposome enhancement of membrane fluidity would result in an increased lateral mobility of intrinsic membrane proteins in the plane of the membrane (17). One possibility to consider is that this enhanced mobility could facilitate collisions between proteins within the lipid bilayer and, due to putative hydrophilic and hydrophobic interactions between segments of these proteins within the lipid compartment, aggregate into patches. Once receptor proteins have patched, cap formation may be initiated in a manner analogous to that suggested by Gershon (52). Specifically, if the membrane is viewed as a supersaturated solution of receptors in the lipid bilayer, then formation of stable patches of "critical size" may "grow by the precipitation of the remaining dissolved receptors from the supersaturated solution (52)." In fact, recent data by Bakardjieva *et al.* (53) involving the β-receptor indicate that capping in response to fluidity changes, at least for some membrane proteins, probably occurs. These investigators have clearly shown that a liposome-induced increase in the membrane fluidity of Chang liver cells (decrease in membrane c/p ratio) results in a 40 to 60% loss of β-receptor. This loss was attributed to an increase in the lateral mobility of the receptor within the fluid membrane.

Our data indicate that 1) the concentration of surface Ig on lymphocytes may be modulated by alteration of membrane lipid composition; and 2) under normal conditions, the surface Ig on rabbit spleen cell membranes is probably not anchored in the cytoskeleton. The ability to cap surface receptors with liposomes may eventually be a useful tool in elucidating the orientation of other proteins in membranes.

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