# Intramembrane Particles and the Organization of Lymphocyte Membrane Proteins

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ABSTRACT    An experimental system was developed in which the majority of all lymphocyte cell-surface proteins, regardless of antigenic specificity, could be cross-linked and redistributed in the membrane to determine whether this would induce a corresponding redistribution of intramembrane particles (IMP). Mouse spleen cells were treated with p-diazoniumphenyl-$\beta$-D-lactoside (lac) to modify all exposed cell-surface proteins. Extensive azo-coupling was achieved without significantly reducing cell viability or compromising cellular function in mitogen- or antigen-stimulated cultures. When the Lac-modified cell-surface proteins were capped with a sandwich of rabbit antilactoside antibody and fluorescein-goat anti-rabbit Ig, freeze-fracture preparations obtained from these cells revealed no obvious redistribution of IMP on the majority of fracture faces. However, detailed analysis showed a statistically significant 35% decrease ($P < 0.01$) in average IMP density in the E face of the lac-capped spleen cells compared with control cells, whereas a few E-face micrographs showed intense IMP aggregation. In contrast, there was no significant alteration of P-face IMP densities or distribution. Apparently, the majority of E-face IMP and virtually all P-face IMP do not present accessible antigenic sites on the lymphocyte surface and do not associate in a stable manner with surface protein antigens. This finding suggests that IMP, as observed in freeze-fracture analysis, may not comprise a representative reflection of lymphocyte transmembrane protein molecules and complexes because other evidence establishes: (a) that at least some common lymphocyte surface antigens are indeed exposed portions of transmembrane proteins and (b) that the aggregation of molecules of any surface antigen results in altered organization of contractile proteins at the cytoplasmic face of the membrane.

The role of the plasma membrane in the transmission of information can be seen in a number of systems where cells have been shown to be activated by an event at the cell membrane leading to proliferation and/or differentiation (5, 8, 23, 26). Although the mechanism of lymphocyte activation is not known, there is evidence to suggest that the binding of insolubilized antigen or mitogen to membrane receptors may, under some conditions, be sufficient to initiate the cellular activation process (8).

There is considerable evidence for an association between lymphocyte cell-surface proteins and an underlying actin-myosin contractile system (2, 10, 15), which may be critically involved in the transmission of signals to or from the cell interior. However, the role of intramembrane particles (IMP) in this interaction remains unresolved. In erythrocytes, experiments have suggested an interaction of IMP with the underlying peripheral protein, spectrin, as well as with erythrocyte surface proteins (24). In the lymphocyte membrane, associa-

tions between IMP and either cell membrane receptors or an underlying contractile system have not been established.

This research focuses on one aspect of the structure and organization of the lymphocyte plasma membrane by asking whether cross-linking and redistribution of lymphocyte surface proteins alter the distribution of IMP. A number of investigators have explored the consequence of aggregating a portion of surface antigens with either antiimmunoglobulins or lectins. These investigations have failed to detect or establish unambiguously the redistribution of IMP corresponding to the aggregation of the selected surface antigens (13, 16, 17), although the possibility always remains that, had redistribution of the right antigen been achieved, a corresponding redistribution of IMP might have been observed.

To minimize this possibility, a system was devised where the majority of cell-surface proteins could be redistributed to determine whether there was a corresponding redistribution of IMP. Hapten groups were covalently coupled randomly to cell-

surface proteins. After the hapten-modified surface proteins were capped with a sandwich of rabbit antihapten Ab followed by fluorescein (Fl)-goat anti-rabbit Ig, the cells were studied by freeze-fracture. Density and distribution of IMP were analyzed to determine whether a corresponding redistribution of IMP had occurred.

## MATERIALS AND METHODS

### Antisera and Reagents

Rabbit antibodies to $p$-azophenyl-$\beta$-D-lactoside (anti-lac) were obtained by standard procedures (30) and purified by affinity chromatography (29). Polyvalent goat anti-mouse Ig and goat anti-rabbit Ig were purchased from Antibodies, Inc., Davis, Calif.

Fab-goat anti-mouse Ig was prepared essentially according to Fanger et al. (7). Purity of the Fab preparation was established by SDS acrylamide gel electrophoresis.

Fl- or rhodamine (Rh)-labeled preparations of concanavalin A (Con A), antilac, goat anti-mouse Ig, goat anti-rabbit Ig, and Fab-goat anti-mouse Ig were made by dialysis against isothiocyanate reagents according to the procedure of Goldman (11). Fluorescent antisera were used at a final concentration of ~0.5 mg/ml and Fl-Con A at 8 $\mu$g/ml.

Con A was prepared from Jack bean meal (Sigma Chemical Co., St. Louis, Mo.), according to the method of Agrawal and Goldstein (1). Lipopolysaccharide (LPS) and phytohemagglutinin (PHA) were obtained from Difco Laboratories, Detroit, Mich.

Lac was prepared as previously described (24). Lac-ferritin was also prepared as before (28).

### Lac-modification of Spleen Cells

Red cells were removed from BALB/c spleen cell suspensions by hypoosmotic lysis. Briefly, 9 ml of water, pH 7.2, were added to $5 \times 10^7 - 1 \times 10^8$ spleen cells suspended in 1 ml of Hanks' balanced salt solution (BSS) (22). After 15 s, isotonicity was restored with 9 ml of 2 $\times$ BSS. Debris and clumps were removed by passage through loosely packed, prewashed glass wool. Lac hapten groups were coupled to the cells as follows: 2 ml of lac ($1 \times 10^{-5}$ M $- 2 \times 10^{-3}$ M) in BSS + 1 ml 0.2 M PO$_4$ 0.15 M NaCl buffer, pH 7.2, were added to 1 ml of 1 $\times$ $10^8$ spleen cells at 4°C. The reaction was allowed to proceed for 1 h at 4°C on a rotator. The modified cells were washed four times with minimal essential medium and assayed for viability by trypan blue exclusion.

### Functional Assays on Lac-modified Spleen Cells

Lac-spleen cells were tested for their ability to respond to sheep red blood cells (SRBC) in Mishell-Dutton cultures (19). After 5 d, we assayed triplicate cultures for anti-SRBC IgM plaque-forming cells (PFC) (12).

Lac-spleen cells were compared with unmodified cells for response to Con A, PHA, and LPS in 1-ml cultures (18), each containing $2 \times 10^6$ cells. Cells were cultured with 4 $\mu$g/ml Con A, 5 $\mu$g/ml LPS, or PHA 1/2,500, and pulsed after 48 h with 0.1 $\mu$c [$^{14}$C]thymidine (New England Nuclear., Boston, Mass.). After 18 h, the cells were harvested and assayed for $^{14}$C in TCA precipitates.

### Labeling of Cells

Lac-modified spleen cells ($10^8$ in 1 ml) were incubated with rabbit antilac antibody (1 mg in 0.25 ml) for 20 min at 37°C, washed, and then treated under the same conditions with fluorescent goat anti-rabbit Ig to cap surface antigens. To determine the effect of such hapten-capping on the distribution of surface Ig and Con A binding sites, we labeled cells additionally under noncapping conditions (0.2% sodium azide, 4°C) with Fab goat anti-mouse Ig or Con A conjugated with a second fluorochrome. Fluorescence was examined with a Leitz Orthoplan microscope with a 200 W mercury lamp, with BG 38, S 546, BG 36, TK 580/580, and K 610 filters for Rh and BG 38, two KP 490, K 510, and S 525 filters for Fl.

In one experiment, lac-spleen cells were capped with Fl-rabbit antilac followed by lac-ferritin and processed for transmission electron microscopy according to previously described procedures (28).

### Freeze-fracture

After aliquots of labeled cell preparations were examined for capping, remaining cells were processed for freeze-fracture analysis. Cells were fixed in 1% glutaraldehyde or 2% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2, 300 mosM) for 1 h at 4°C, centrifuged, resuspended in 20% glycerol in the phosphate

buffer for 12 h at 4°C, and frozen in liquid Freon 22. Samples were fractured in Balzars apparatus (Balzars AG, Balzars, Leichtenstein) at −115°C. The replicas were recovered after washing in bleach for 1-2 h, mounted on naked grids, and examined in a Siemens Elmskop I. Photographs at magnifications of × 20,000–40,000 were taken of each fracture face observed. Prints were enlarged to × 40,000–100,000. Particle size measurements were determined with a × 10 magnifier having a calibrated micrometer grating (Bausch and Lomb, San Leandro, Calif.) which could be aligned along the interface of the particle and its shadow. The minimum and maximum size limits of particles chosen for counting were 60–120 Å. Particle density measurements were obtained by counting 100 cm$^2$ areas on prints enlarged to × 100,000. In each experiment, 30-50 separate 100 cm$^2$ areas for P- and E-fracture faces were examined, and data from control faces (modified or unmodified) and lac-capped faces were analyzed by the Student's $t$ test. Care was taken to select areas with distinctive shadowing where the particles were measurable; regions where the shadow angle was too great, producing a snowlike or darkened appearance, were not included in counting.

## RESULTS

### Evidence for Modification of Cell Surface Antigens

Lac-spleen cells, modified with concentrations ranging from $1 \times 10^{-5}$ M to $2 \times 10^{-3}$ M diazoniumphenyl lactoside, were labeled with rabbit antilac and Fl-goat anti-rabbit Ig. Fluorescence observation revealed bright staining on cells modified at reagent concentrations of $10^{-4}$ M or higher. In repeated experiments, cells stained with a single layer of Fl-rabbit antilac showed rings and light patching, with no capping. When the second layer was applied, 70-95% of the cells modified with $1 \times 10^{-4}$ M $- 1 \times 10^{-3}$ M diazonium showed clear capping and the remaining cells showed a heavily patched distribution of the fluorescent label. Cells modified with $2 \times 10^{-3}$ M diazonium reagent, although staining brightly with the sandwich procedure, showed a decrease in the percent of capped cells to 40-60%.

Spleen cells modified with $1 \times 10^{-3}$ M diazoniumphenyl lactoside and capped with a sandwich of Fl-rabbit antilac and lac-ferritin were examined by transmission electron microscopy. Fluorescence microscopy of an aliquot of these cells revealed that 91% were capped and the remainder showed a heavily patched distribution of the Fl-marker. Electron micrographs of the lac-capped spleen cells revealed ferritin in a cap on many sections (Fig. 1).

The lac-capping process requires metabolic activity because addition of azide inhibits capping (Table I). The fact that in repeated experiments we found caps on 95% of the labeled lac-modified spleen cells establishes that these cells were viable. Moreover, 90-96% of cells modified with $1 \times 10^{-5}$ M$-1 \times 10^{-3}$ M diazonium reagent excluded trypan blue. Modification with $2 \times 10^{-3}$ M reagent resulted in some loss in cell viability.

Lac-spleen cells modified with $1 \times 10^{-4}$ M or $1 \times 10^{-3}$ M diazoniumphenyl lactoside were cultured with SRBC antigen. Table II shows that the PFC response of modified cells was indistinguishable from that of unmodified cells.

The ability of lac-spleen cells to proliferate in response to Con A, PHA, and LPS is shown in Table III. The responses to the three mitogens of cells modified with $1 \times 10^{-4}$ M or $1 \times 10^{-3}$ M lac reagent were indistinguishable from those of control unmodified spleen cells. Modification at $2 \times 10^{-3}$ M resulted in a depressed response to PHA and LPS.

### Extent and Random Nature of Surface Protein Modification

Our choice of the diazonium reagent for coupling haptens to cell surfaces was made because of its high reactivity with all
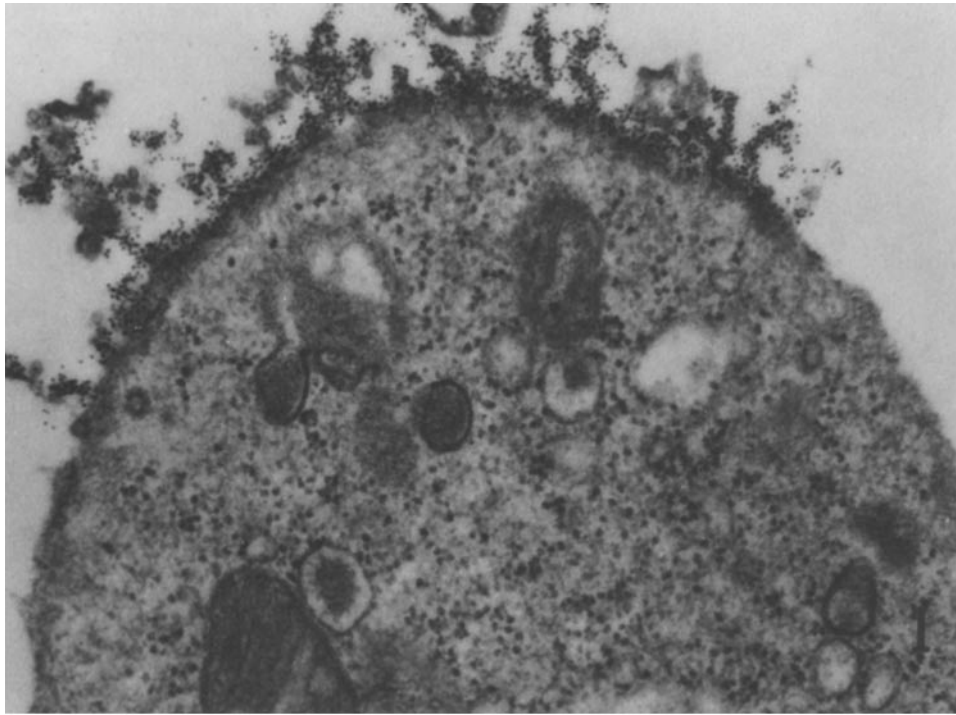
FIGURE 1  Mouse spleen cell capped with FI-rabbit antilac and antiferritin. × 50,000.

proteins. The assumption was that any surface protein with exposed tyrosine, histidine, and/or lysine residues would present a probable target. To test whether the azo-coupling of hapten was indeed extensive and essentially random, we designed cocapping experiments which could establish whether most surface immunoglobulin molecules (characteristic of B lymphocytes) and most Con A binding sites (present on all lymphocytes) had been labeled with hapten.

Lac-modified spleen cells were capped with a sandwich of rabbit antilac and Fl-goat anti-rabbit Ig and then stained at 4°C with Rh-Fab-goat anti-mouse Ig. Table IV presents the results of an experiment in which the capping of lac-modified surface proteins resulted in 94% coincident capping of mouse Ig. This established that most surface Ig molecules on these cells had lac determinants because the monovalent Fab anti-Ig did not itself induce capping.

An analogous experiment yielded comparable results with respect to Con A binding sites (Table V). Lac-modified spleen cells were capped with rabbit antilac and Rh-goat anti-rabbit Ig and then stained with Fl-Con A under noncapping conditions in the presence of azide at 4°C. The results showed 100% coincidence of the Fl-Con A with the Rh-lac caps. Preparations stained with Fl-Con A alone showed 75–78% of the cells with fluorescent rings and the remaining cells with light patches of label.

Although it is, of course, impossible to examine the extent of hapten modification on every individual species of lymphocyte membrane protein, the Ig and Con A results were taken as indicative of the fact that most, if not all, proteins exposed at the cell surface were labeled in our experiments.

## Freeze-fracture Analysis of Lac-capped Spleen Cells

Aliquots of cell preparations referred to in Tables IV and V were subjected to freeze-fracture. We analyzed fracture faces

TABLE I

Effect of Azide on Capping of Lac-modified Surface Proteins of Spleen Cells

| Diazonium concentration for cell modification | NaN₃ 10 mM | Rabbit antilac | Rh-goat anti-rabbit Ig | Capping |
|---|---|---|---|---|
| M | | | | % |
| 1 × 10⁻⁴ | − | − | + | No staining |
| 1 × 10⁻⁴ | − | + | + | 86 |
| 1 × 10⁻⁴ | + | + | + | 6 |
| 1 × 10⁻³ | − | − | + | No staining |
| 1 × 10⁻³ | − | + | + | 84 |
| 1 × 10⁻³ | + | + | + | 10 |
| Unmodified control | − | + | + | No staining |

TABLE II

Primary PFC Response of Lac-Spleen Cells to SRBC

| Diazonium concentration for cell modification | PFC/10⁶ cells | |
|---|---|---|
| | Exp 1 | Exp 2 |
| M | | |
| 0 | 2,207 | 2,128 |
| 1 × 10⁻⁴ | 2,900 | 2,625 |
| 1 × 10⁻³ | 2,218 | 2,184 |

Spleen cells were cultured with SRBC in Mishell-Dutton cultures (19). After 5 d, triplicate cultures were assayed for anti-SRBC plaque-forming cells. The results given are the average of the triplicate cultures expressed as plaques/10⁶ cells.

on the basis of two criteria: first, the degree of aggregation of IMP as indicated by the fraction of faces with particle clusters; and second, the average density of particles per unit area on both P and E faces.

(a) Inspection of freeze-fracture samples of fixed lac-capped (Fig. 2) and uncapped cells revealed no obvious changes in the state of aggregation of IMP on the majority of fracture faces.

TABLE III

Proliferative Responses of Lac-Spleen Cells to Con A, PHA, and LPS

| Diazonium concentration for cell modification | Mitogen | [$^{14}$C]Thymidine | | | |
|---|---|---|---|---|---|
| | | Exp 1 | Exp 2 | Exp 3 | Exp 4 |
| *M* | | *cpm* | | | |
| 0 | PHA | 16,621 | 17,587 | — | 5,806 |
| 1 × 10$^{-4}$ | PHA | 20,230 | 18,878 | — | 4,350 |
| 1 × 10$^{-3}$ | PHA | 16,756 | 14,872 | — | — |
| 2 × 10$^{-3}$ | PHA | — | 6,219 | — | — |
| 0 | LPS | 4,707 | 6,270 | — | — |
| 1 × 10$^{-4}$ | LPS | 6,601 | 7,450 | — | — |
| 1 × 10$^{-3}$ | LPS | 4,932 | 4,077 | — | — |
| 2 × 10$^{-3}$ | LPS | — | 1,152 | — | — |
| 0 | ConA | — | — | 21,321 | 16,556 |
| 1 × 10$^{-4}$ | Con A | — | — | 22,731 | 13,456 |
| 0 | — | 325 | 509 | 570 | 483 |
| 1 × 10$^{-4}$ | — | 302 | 471 | 511 | 460 |
| 1 × 10$^{-3}$ | — | 311 | 553 | — | — |

2 × 10$^6$ spleen cells, either unmodified or modified, were cultured with 4 μg/ml Con A, 5 μg/ml LPS, or 1/2,500 dilution of PHA and pulsed after 48 h with 0.1 μc [$^{14}$C]thymidine. After 18 h, the cultures were harvested and assayed for [$^{14}$C]thymidine in TCA precipitates. Results express the average of triplicate cultures.

TABLE IV

Cocapping of Lac Surface Proteins and Surface Ig

| Rabbit antilac, F1-goat anti-rabbit Ig | Rh-Fab goat anti-mouse Ig | Rh | F1 | Coincident capping |
|---|---|---|---|---|
| + | — | | 95%; Caps | — |
| — | + | 40%; Rings | — | — |
| + | + | 42%; Caps or patches | 92%; Caps | 94% |

Columns three and four record percent cells stained and the predominant staining pattern (i.e., rings, caps, patches). The percent of coincident capping (column five) is based on the fractions of Rh stained cells with an identical F1 staining pattern.

All E faces and 95% of P faces from control uncapped spleen cells fixed in glutaraldehyde had exclusively monodispersed IMP, whereas remaining P faces showed some small clusters of IMP (5–15 particles). Lac-capped spleen cells fixed in glutaraldehyde also had exclusively monodispersed particles on 95% of E faces. There was a slight increase over the controls, from 5 to 15%, of P faces with some small clusters. Large IMP clusters (15–50 particles) were observed in 5% of lac-capped E faces and were entirely absent in controls. Paraformaldehyde fixation resulted in an increase of 10–30% in the number of faces that showed some IMP clusters, but uncapped control and lac-capped spleen cells could not be distinguished on this basis.

(b) A comparison of lac-capped lymphocytes with control modified but uncapped cells, or control unmodified cells, revealed a significant decrease in IMP density on the E face of uncapped cells whereas the P-face IMP density was unchanged (Table VI). The E faces of glutaraldehyde-fixed lac-capped spleen cells showed an average particle density of 201 ± 62 IMP/μm$^2$ compared with 319 ± 100 IMP/μm$^2$ for unmodified

TABLE V

Cocapping of Lac Surface Proteins and Con A Receptors

| Rabbit antilac, Rh-goat anti-rabbit Ig | F1-Con A 40 μg/ml | Rh | F1 | Coincident capping |
|---|---|---|---|---|
| + | — | 100%; Caps or heavy patches | — | — |
| — | + | — | 100%; Rings or light patches | — |
| + | + | 100%; Caps or heavy patches | 100%; Caps or heavy patches | 100% |

Columns three and four record percent cells stained and the predominant staining pattern (i.e., rings, caps, patches). The percent of coincident capping (column five) is based on the fraction of Rh stained cells with an identical F1 staining pattern.

and 325 ± 112 IMP/μm$^2$ for uncapped cell E faces. This represents a statistically significant 37% decrease in IMP density on the E-fracture face ($P < 0.02$) compared with controls. $K_p$ ratios ([number of P-face IMP]/[number of E-face IMP]) calculated for each preparation were 1.87 and 1.86 for controls and 3.07 for the lac-capped preparation (Table VI). Combining P- and E-face densities reveals an overall decrease of 11% on capped cells.

Paraformaldehyde-fixed, lac-capped spleen cells showed a similar decrease in IMP density on the E face compared with paraformaldehyde controls. The paraformaldehyde-fixed, lac-capped spleen cells had 199 ± 78 IMP/μm$^2$ on the E face, whereas controls had 306 ± 105 and 318 ± 98 IMP/μm$^2$ with $K_p$ ratios of 2.42 for the capped cells and 1.80 and 1.77 for the controls (Table VI). The E face decrease of 35% is statistically significant ($P < 0.01$) and in close agreement with the data obtained with glutaraldehyde-fixed cells. Similarly, the combined P- and E-face particle densities decreased by 21% compared to the controls.

Whether the decrease in E-face IMP density is caused by redistribution of a proportion of the IMP into a cap corresponding to the surface protein cap cannot be proven directly. A few E-fracture faces, occurring with a frequency of one in 15–20, do show areas of extreme IMP aggregation suggestive of a capped region (Fig. 3). To see if this was a feasible assumption, we measured the IMP density on these fracture faces to determine whether the observed IMP increase could be accounted for by the average IMP decrease on the remainder of the cell. Assuming that the average diameter of spleen cells is 10 μm, corresponding to an average surface area of 314 μm$^2$, and assuming the average area of a cap is one-eighth of the cell and, therefore, 39 μm$^2$, we calculated the average IMP decrease for the noncapped area. On the basis of IMP density observed on most E faces, the predicted value for IMP density in the capped region was 1,060 IMP/μm$^2$. The observed IMP particle density in the fracture of the presumptive cap was 1,464 IMP/μm$^2$. Thus, it appears that it is a feasible assumption that these fracture faces may represent capped regions.

## DISCUSSION

Capping of selected lymphocyte surface proteins (H-2, Ig, or Con A receptors) with either antiimmunoglobulins or selected lectins has failed to indicate redistribution of IMP (13). This
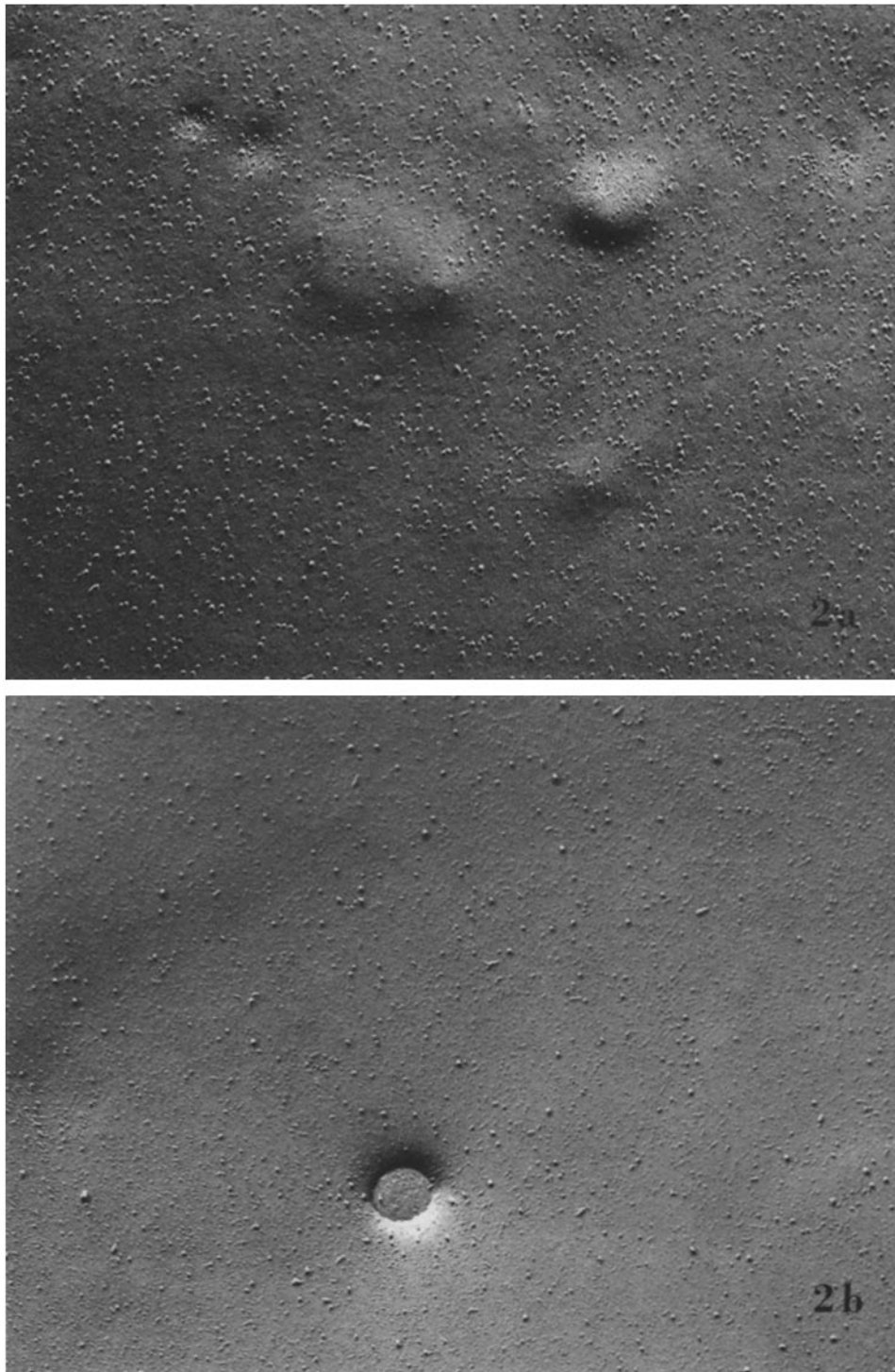
FIGURE 2   Representative replicas of lac-capped, glutaraldehyde-fixed mouse spleen cells. ✕ 50,000. a, P face; b, E face. No significant aggregation of IMP is observed.

study, which achieves aggregation of most surface proteins, demonstrates a resulting change in the distribution and density of a small proportion of lymphocyte IMP.

With the experimental system developed for this study, the majority of cell-surface proteins, regardless of antigenic specificity, are cross-linked and redistributed to determine whether there is a corresponding redistribution of IMP. Cells are chemically modified so as to couple hapten groups covalently to cell-surface proteins. The diazoniumphenyl lactoside reagent reacts by electrophilic attack on reactive protein residues,

principally on the side chains of tyrosyl, histidyl, and lysyl residues. This results in the random coupling, by means of azo bridges, of phenyl lactoside groups to all exposed cell-surface proteins. Thus, these proteins now share a common antigenic specificity regardless of other antigenic differences between them. The lac-modified surface proteins can then be redistributed using a sandwich of rabbit anti-lactoside antibody followed by a layer of Fl-goat anti-rabbit Ig.

To establish the validity of this system, it was necessary to show (a) that the lac coupling reaction is sufficiently extensive

so that a majority of cell-surface proteins are modified, (*b*) that these modified cell-surface proteins can be redistributed in the plane of the membrane, and (*c*) that, under the coupling conditions, the integrity of the cells is conserved as judged by the criteria of viability and cellular functions. The results indicate that this system meets all three requirements. Modification of spleen cells with concentrations ranging from $1 \times 10^{-4}$ M to $1 \times 10^{-3}$ M diazoniumphenyl lactoside resulted in a population of cells that stained brightly with Fl-rabbit antilac, showed a high percent of capping with a sandwich layer of Fl-goat anti-rabbit Ig, and showed no decrease in cell viability by

TABLE VI

*Particle Distribution on the Fracture Face of Lac-capped and Control Spleen Cells*

| Treatment | P Face* | E face* | P + E face | $K_p$ (P/E) |
|---|---|---|---|---|
| Glutaraldehyde/glycerol | 597 ± 123 | 319 ± 100 | 916 | 1.87 |
| Lac modified/uncapped; glutaraldehyde/glycerol | 605 ± 137 | 325 ± 112 | 930 | 1.86 |
| Lac modified/capped; glutaraldehyde/glycerol | 618 ± 158 | 201 ± 62 | 819 | 3.07 |
| Paraformaldehyde/glycerol | 550 ± 163 | 306 ± 105 | 856 | 1.80 |
| Lac modified/uncapped; paraformaldehyde/glycerol | 562 ± 150 | 318 ± 98 | 880 | 1.77 |
| Lac modified/capped; paraformaldehyde/glycerol | 481 ± 64 | 199 ± 78 | 680 | 2.42 |

Particle density measurements for P- and E-fracture faces were obtained by counting 100 cm² areas on prints enlarged to X 100,000. In each experiment, 30-50 separate 100 cm² areas were analyzed. The minimum and maximum size limits of particles chosen for counting were 60 and 120 Å, respectively.
* IMP/μm² ± SD.

trypan blue exclusion. In addition, these lac-modified cells were indistinguishable from unmodified cells in a primary in vitro response to SRBC and in proliferative responses to Con A, PHA, and LPS.

The extent of cell-surface protein modification was demonstrated by cocapping experiments. In these experiments, the lac-modified surface proteins were capped with a fluorescent-labeled sandwich. Then, under noncapping conditions, the distribution of surface Ig or Con A receptors was determined with anti-Ig or Con A labeled with a contrasting fluorochrome. If any substantial fraction of mouse Ig or Con A receptors had not been modified with the lac hapten, they would have been detected outside the cap region. The high percent of coincident capping (94% for Ig and 100% for Con A) suggests that, under the conditions of the coupling reaction, the lac-modification was sufficiently extensive so that most molecules of any cell-surface protein, represented by Con A receptors and Ig, were modified and redistributed. We, of course, cannot rule out that a particular atypical surface protein(s) may have escaped hapten labeling, if it presented no accessible azo-reactive residues.

Freeze-fracture analysis of membranes of capped, lac-modified cells provides evidence that a proportion of E-face IMP are associated with surface proteins. There was a 35-37% decrease in IMP density on E faces after lac-capping. About 5% of E-fracture faces showed areas of extreme IMP aggregation. The high particle density in these areas was in line with calculations based on the assumption that the decrease in IMP elsewhere could be accounted for by their movement into a cap. The low frequency of E-fracture faces showing extreme IMP aggregation could be a result of the high protein concentrations in the cap region. It is known in other systems (27) that fewer fractures occur through membranes having a high concentration of protein.

Our findings show no detectable correlation between P-face IMP and lac-modified surface proteins. Because the density of
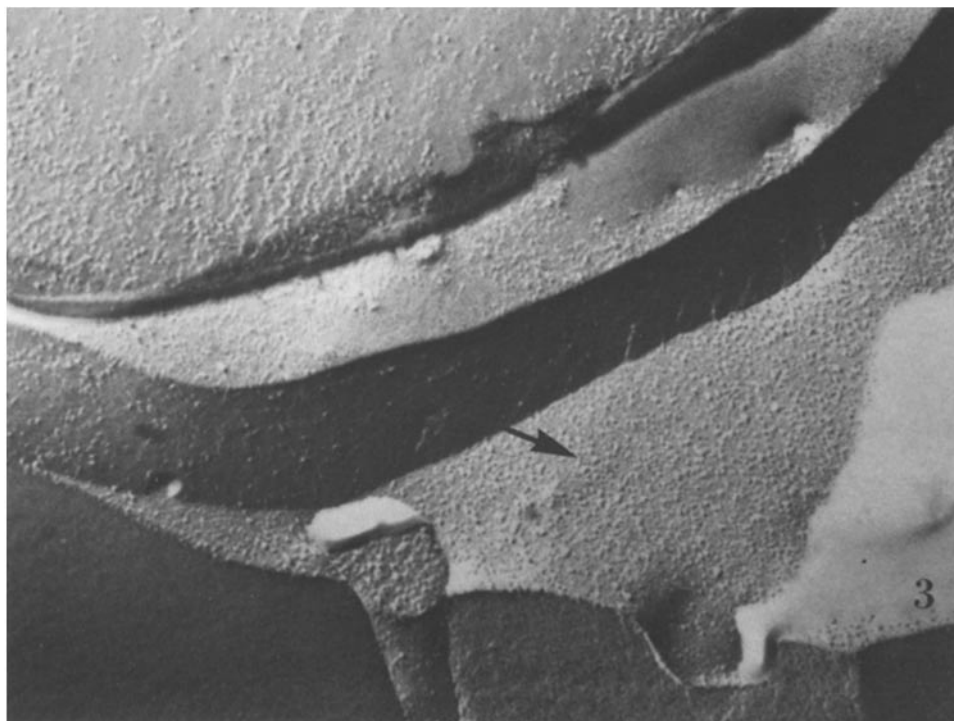


FIGURE 3   Freeze-fracture E-face replica from glutaraldehyde-fixed, lac-capped spleen cells. X 50,000. IMP on this E face are densely packed (see arrow).

lymphocyte IMP is almost two times higher on P than on E faces, a significant decrease in particle density resulting from the redistribution of a given small number of IMP would be more difficult to detect on P than on E faces. Apparently, however, virtually all P-face IMP and the majority of E-face IMP are not accessible as lymphocyte surface antigens, nor are they in stable association with the lac-modified cell surface proteins.

These results inevitably raise questions about whether the IMP, as visualized in freeze-fracture analysis, provide a representative and significant reflection of the organization of proteins in the lymphocyte membrane.

It has been estimated that the plasma membrane of a lymphocyte with a diameter of 8 $\mu$m and a smooth surface contains $\sim 10^7$ protein molecules, whereas the average number of IMP (both E and P face) observed per lymphocyte is $<10^5$ (4).[1] Although it has been assumed that the lymphocyte IMP are mainly proteins that span the membrane (3, 4), it may well be that the large majority of lymphocyte transmembrane proteins are not revealed as IMP by the freeze-fracture technique, possibly because they do not form molecular aggregates of large enough size (9).

The absence of any observed correlation between surface antigen mobility and that of P-face IMP is in striking contrast to other evidence on the organization of the lymphocyte membrane and transmembrane interactions involving surface mobility. It has been shown that antigens of the major histocompatibility complex examined thus far (14, 21), as well as B lymphocyte membrane IgM (22), are almost certainly transmembrane proteins. If IMP did indeed provide an accurate representation of lymphocyte transmembrane protein organization, the following additional evidence might well lead to the expection of coordinate mobility between some P-face IMP and surface proteins: (a) every antigen on the lymphocyte surface can be capped by appropriate application of antibodies or lectins (25); (b) capping of any surface antigen results in aggregation of contractile proteins on the cytoplasmic side of the membrane (2); and (c) both H-2 and cross-linked surface immunoglobulin have been isolated from lymphocyte membranes in complexes associated with actin (10, 15).

The puzzling contraindications resulting from freeze-fracture analysis of lymphocyte membranes cannot yet be clearly explained. If IMP reflect only a small fraction of the total transmembrane proteins, this fraction must be largely inaccessible to antibodies or lectins that bind to the lymphocyte surface. One difficulty in defining the situation further is that serious artifacts may be incorporated into freeze-fracture analysis of some cell membranes as a result of differential association of transmembrane polypeptides with the separate halves of the membrane bilayer, as well as by the breaking of some covalent bonds in the freeze-fracture procedure (6).

In studies on association of IMP and cell-surface proteins in the erythrocyte membrane (20, 24, 27), redistribution of IMP was induced first, after which a corresponding redistribution of cell-surface proteins was observed. Using the experimental system developed for this research, Shotten et al. (24) observed an association between both P- and E-face IMP and lac-

modified surface proteins in erythrocyte ghosts. When IMP were aggregated by manipulations of pH or ionic strength to remove spectrin, the lac-modified surface proteins were also aggregated. Similarly, all studies indicating an association of erythrocyte IMP with selected surface antigens, such as blood group A antigen, Con A, and PHA binding sites, have also required initial procedures to disrupt membrane organization resulting in the aggregation of IMP. In such studies, the proportions of P- and E-face IMP actually associated with the antigens could not be determined.

Although the present study does demonstrate a relationship between some E-face IMP and lymphocyte surface antigens, its main result is to establish that IMP do not provide a basis for distinguishing certain transmembrane protein molecules or revealing significant transmembrane associations in the lymphocyte and possibly other complex cell types.

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