

LIGAND-INDUCED MOVEMENT OF LYMPHOCYTE MEMBRANE MACROMOLECULES

II. MAPPING OF SURFACE MOIETIES*

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Analysis of the cell surface movements of membrane moieties of lymphocytes induced by ligands is of interest both in regard to yielding information on the structural organization of the membrane, and also in terms of biological and immunological functional significance. The purpose of this paper is to present data on the fine structural aspects of these events and the topography of cell surface markers before, during, and after such movements. The observations made are consistent with and support several theses concerning the nature of such intramembranous movements, and the prerequisites necessary for such events to occur, and have marked bearing on current theories concerning the structure and behavior of cell membranes.

Materials and Methods

Reagents.—Antisera were prepared as in the previous paper. These antisera, together with concanavalin A (Con A)¹ and the labels used for their ultrastructural detection, are shown in Table I. Immunoglobulin (Ig) was isolated from the three antisera by the preparative methods outlined previously (1). Rabbit anti-mouse Ig (RAMG) was chemically coupled to ferritin using difluorodinitrodiphenyl sulfone (FNPS) as conjugating reagent (2). 40 μ g of globulin and 116 μ g of ferritin (Pentex 6 times crystallized, lot No. 7-1, Pentex Biochemical, Kankakee, Ill.) were dissolved in a total of about 5 ml of phosphate-buffered saline (PBS), pH 7.0. About 4.5 ml 2% Na₂CO₃ was added and then, with stirring at 4°C, 0.5 ml of FNPS (0.5% in acetone) was added dropwise. Stirring was continued for 24 hr at 4°C. The solution was then dialyzed for 5 days against PBS in the cold, and spun for 30 min at 7500 g. Free Ig and ferritin were separated from the conjugated Ig by electrophoresis. The solution was electrophoresed for 20 hr on a Pevikon block (500 v, 56 amp) in barbital buffer, pH 8.6, 0.05 M, with sodium phosphate buffer, pH 7.45, 0.2 M, in the outer troughs. The fraction containing the ferritin-Ig

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¹ *Abbreviations used in this paper:* ALG, anti-lymphocyte globulin; anti-H-2, antibodies made in DBA/2 mouse to histocompatibility antigens of C57BL/6; B lymphocytes, lymphocytes derived from bone marrow; Con A, concanavalin A; FNPS, difluorodinitrodiphenyl sulfone; Ig, immunoglobulin; PBS, phosphate-buffered saline; RAMG, rabbit anti-mouse Ig; T lymphocytes, thymic-derived lymphocytes.

conjugate was eluted and concentrated in an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.).

The concentration of globulin and ferritin in the conjugate was measured by immunoprecipitation using the Mancini method (3). The labeling frequency of ferritin to globulin had an average of 0.5 molecules of ferritin per molecule of globulin.

The immunological specificity of the conjugate was determined by immunoelectrophoresis. Positive immunoprecipitin lines were found against all mouse Ig classes. In addition, mouse Ig cross-linked with cyanogen bromide to a Sepharose 4B column (4) completely absorbed the antibody activity of the conjugate. The absorbed ferritin conjugate did not react thereafter with Ig of mouse lymphocytes derived from bone marrow (B lymphocytes).

Anti-lymphocyte globulin (ALG) was conjugated to ferritin in a similar manner, except that the quantities involved were scaled down to about half. The average ratio of ferritin to ALG was about 0.3.

TABLE I
Ligands and Their Specificities

Ligand	Specificity	Label
IgG of rabbit anti-mouse Ig sera	All Fc classes and Fab determinants	Hemocyanin (<i>B. canaliculatum</i>) Ferritin
Concanavalin A	Carbohydrate moieties	Hemocyanin (<i>B. canaliculatum</i>) Ferritin
IgG of rabbit anti-rat lymphocyte	Uncharacterized surface antigens	Ferritin
Serum of DBA/2 immunized to C57BL cells	Histocompatibility antigens of C57BL	—

RAMG was also conjugated with hemocyanin obtained from the marine whelk *Busycon canaliculatum*. The use of this marker was suggested by the work of Smith and Revel (5), who showed that as a visual marker on replicas it has an unambiguous shape, and a size suitable for adequate resolution in the electron microscope.

Marine whelks (Woods Hole Biological Laboratories, Woods Hole, Mass.) were bled by shattering the shells over the cardiac region and also by incising the mantles. The hemolymph (20–50 ml from each whelk) was filtered through glass wool and spun at 4°C, 2000 g, to get rid of debris. The supernatant was then centrifuged for 2 hr at 100,000 g, and the sediment suspended in 1% NaCl. This procedure was repeated three times, the final pellet being suspended in 3% NaCl. The solution was sterilized by filtration through a Millipore filter (Falcon Plastics, Oxnard, Calif.), pore size 0.45 μ , and the protein content measured by the Lowry method (6) against a standard curve constructed from micro-Kjeldahl estimations.

For conjugation of RAMG to hemocyanin, glutaraldehyde was used as a coupling reagent, based on the method of Avrameas (7). The hemocyanin in 3% NaCl was dialyzed at 4°C overnight against 0.1 M phosphate buffer, pH 6.8, and the protein concentration corrected for changes in volume. 264 mg of hemocyanin and 28 mg of RAMG were dissolved in 50 ml 0.1 M phosphate buffer, pH 6.8, and at 20°C, with stirring, 1 ml of 5.0% glutaraldehyde was added to give a final concentration of 0.1%. Stirring was continued for 2 hr, and then the solution was dialyzed against PBS, pH 7.0, overnight in the cold. The sediment formed was centrifuged off at 2000 g, and the supernatant was sterilized by passage through a Millipore filter (Falcon Plastics), pore size 0.45 μ . The conjugate was not purified further. Con A binding to cells was

revealed by the method of Smith and Revel (5) in which membrane-bound Con A is labeled directly by exposure to hemocyanin: carbohydrate moieties in the hemocyanin directly link to the Con A.

Handling of Cells.—Lymphoid cells were harvested from spleen and thymus of mice or rats by the techniques described in the previous paper (1). The requisite antibody conjugate or Con A was added to $1-3 \times 10^7$ cells in about 200 μ l of Hanks' balanced salt solution, at doses specified below. The final volume per tube was 300 μ l. The cells were then held at 4°C for 30 min. In the case of Con A, after washing, the cells were incubated with 500 μ g of hemocyanin for 30 min.

After washing three times at 4°C with Hanks' solution, by centrifuging at 400 g and re-suspending, the cells in some tubes were fixed immediately. Other tubes, now containing washed cells in 300 μ l Hanks' solution, were rapidly brought to 37°C in a water bath for periods of time ranging from 1 min to 2 hr. In one experiment the cells were cultured in minimal essential medium containing 5% fetal calf serum for 24 hr at 37°C. After the requisite period of incubation at 37°C the cells were immediately fixed by pouring 3 ml of fixative into the tubes.

Ultrastructural Methods.—

Conventional thin sections: Cells were fixed in Karnovsky's formaldehyde-glutaraldehyde fixative (8), diluted 1:2 (i.e., 1.7% formaldehyde-1.3% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.2, 8 mg/100 mg CaCl₂). Fixation was for 1 hr at 4°C. The cells were washed twice with 0.1 M cacodylate buffer, and postfixed in 1% O₃O₄-water at 4°C for 1 hr. The cells were then dehydrated and embedded in Epon 812 by conventional methods. Thin sections were cut and stained variously with lead citrate and uranyl acetate. Where ferritin conjugate was used, alkaline bismuth subnitrate (9) was used to enhance the size and electron opacity of the ferritin particles, and the sections were counterstained with alkaline lead and uranyl acetate.

Freeze-etching: Fixation was for 15 min in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, at 4°C. The cells were washed twice in the buffer and then washed once in 0.05 M tris(hydroxymethyl)aminomethane-HCl, pH 7.4. After centrifugation at 300 g, 3 ml distilled water was added to the pellet, and the cells were resuspended and respun at 500 g. The supernatant was decanted and the pellet was transferred to Balzers specimen holders (Balzers High Vacuum Corp., Santa Ana, Calif.) and rapidly frozen in Freon 22 cooled with liquid nitrogen. The specimens were then cleaved, etched for 5–30 min at -104°C in a Balzers high vacuum freeze-etch unit BA 360 M, and shadowed with platinum at an angle of 45°. The replicas were strengthened by evaporation of carbon at an angle of 90°, thawed, and the cells digested away from the replica with hypochlorite (Chlorox). The replicas were mounted on grids and examined by conventional transmission electron microscopy in an AEI-801 electron microscope (AEI Scientific Apparatus, Ltd., Essex, England), at 60/80 kv and 30–40 μ objective apertures.

Freeze-cleavage: Fixation was the same as above, but after washing in buffer, the cells were soaked in 25% glycerol in 0.1 M cacodylate buffer, pH 7.3, for 3–5 hr at 4°C. Thereafter the cells were frozen and handled as above, except that they were routinely etched for 5 min. In some experiments, however, the specimens were not etched at all.

Replicas of nonfrozen cells: 500,000 cells in 0.1 ml of Hanks' balanced salt solution were spun onto clean cover slips in a Shandon-Elliott cytocentrifuge (Shandon Scientific Co., Inc., Sewickley, Pa.) for 10 min at 1500 rpm. Without allowing the cover slips to dry, the cells were fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 5 min, washed in buffer, and then postfixed in 1% O₃O₄-water for 5 min. They were then processed by the method of Smith and Revel (5), i.e. rapidly dehydrated in ethyl alcohol, and dried from amyl acetate (or M-180 Freon TF) in a stream of hot air. Replication was performed with platinum-carbon at an angle of 45°, and carbon coating was done at 90°. Thereafter the replicas were separated from the cover slips by partially dissolving the latter in hydrofluoric acid. The replicas were then treated with hypochlorite (Chlorox) and handled as described above.

RESULTS

Treatment of Mouse Spleen Cells with Ferritin- or Hemocyanin-Labeled RAMG.—

Distribution of label at 4°C: In thin sections from preparations of cells incubated at 4°C for 30 min, the cell membrane was randomly labeled with RAMG-

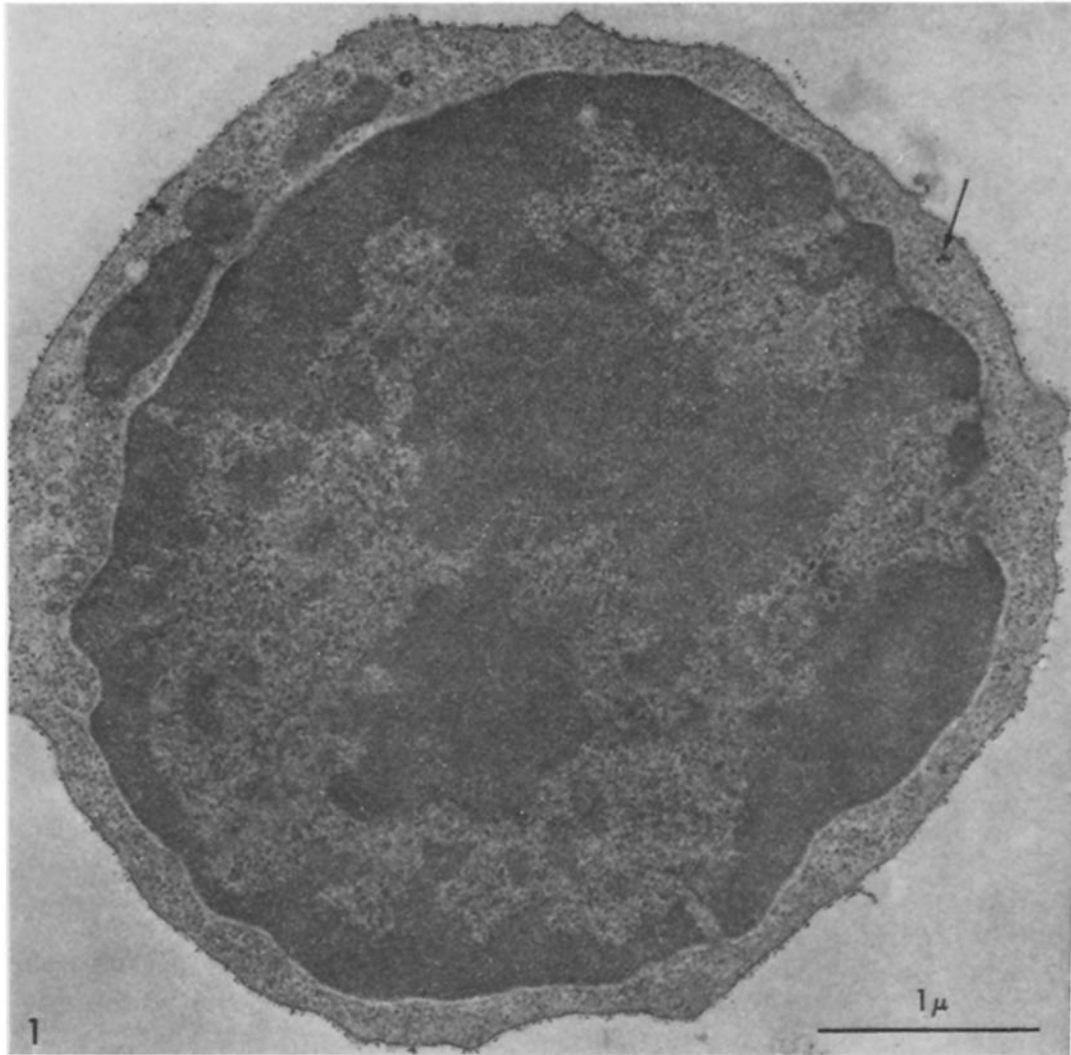


FIG. 1. B lymphocyte of mouse spleen labeled with RAMG-ferritin at 4°C for 30 min. Note the random and extensive labeling of the surface membrane. A few pinocytotic vesicles containing ferritin are visible (*arrow*). Section stained with alkaline bismuth. $\times 28,000$.

ferritin or RAMG-hemocyanin, in the form of labeled segments of membrane interspaced with short unlabeled segments (Fig. 1). The labeled segments varied in length and were randomly and irregularly distributed. Within each labeled segment, the ferritin particles were spaced at a distance (center-to-center) of about 200 Å from each other, and were situated about 200 Å from the cell membrane. When the plane of sectioning was more or less perpendicular to the cell membrane, the ferritin marker was usually arranged in a single layer, although sometimes two layers were noted. This latter is probably due to the presence of ferritin molecules attached at different levels to the cell membrane cut somewhat tangentially within the thickness of the section (~ 700 – 900 Å thick). Variation of the dose of RAMG-ferritin per 10^7 cells from 20 to 80 μg did not make any marked difference in the degree of labeling. A slight degree of pinocytosis was seen at 4°C; the small pinocytotic vesicles were close to the cell surface (Fig. 1).

Labeling of spleen cells with RAMG-hemocyanin gave essentially similar results, except that the labeled segments of the cell membrane showed less concentration of label and more irregular spacing (Fig. 2). Furthermore, the label tended to be stacked in two to three layers rather than in a single layer. This may also be a reflection of the label's large size (~ 300 Å diameter) and the thickness of sections; thus label at different levels on the membrane surface cut somewhat tangentially in the thickness of the section would be seen, in a two-dimensional view, as stacked layers.

The hemocyanin molecules were also seen situated at about 200 Å from the cell membrane. In suitably oriented cuts, a fine, slightly electron-opaque strand about 50 Å wide was seen to connect the hemocyanin molecule to the cell membrane (Fig. 3). This possibly represented the immunoglobulin antibody.

With both RAMG-ferritin and RAMG-hemocyanin, about 50% of splenic cells were labeled. These have previously been shown to be B cells. (Thymic cells [T cells] treated with conjugates showed no labeling as would be expected from previous results with fluorescein- and ^{125}I -labeled RAMG.) Cells treated with unconjugated ferritin or hemocyanin showed no labeling, as did cells treated with conjugates absorbed with specific Ig.

Freeze-etch preparations showed interconnected patches covering the whole surface of the cell forming a lacy, continuous, irregular network (Figs. 4 and 5). The molecules of label were closely contiguous throughout the network and there were small, irregular, bare patches of membrane seen between the interstices of the network.

Prefixation of cells with buffered 2% formaldehyde (freshly prepared from paraformaldehyde [8]), followed by RAMG-ferritin, showed no marked difference in distribution of label. Nonfrozen replicas gave essentially similar results in the distribution of label, but the cells were subject to some degree of distortion and deformation (Fig. 6). It was readily apparent that the patterns of distribution seen in thin sections and in replicas were entirely reconcilable with each other.

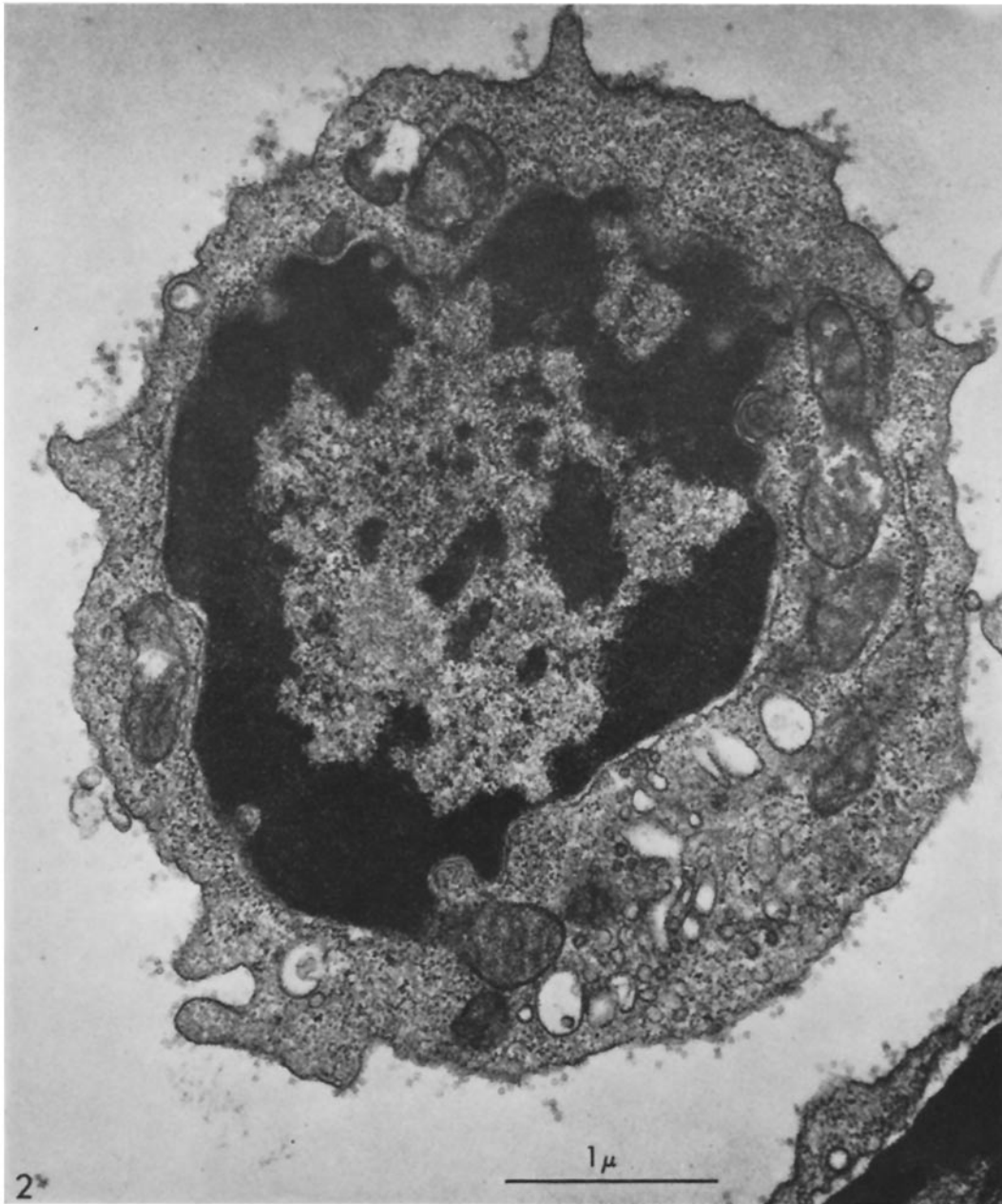


FIG. 2. Same as Fig. 1 except labeling is with RAMG-hemocyanin. There is extensive random labeling of the surface membrane. $\times 28,000$.

Redistribution of label on warming to 37°C: Within 10 min of warming the cells to 37°C, capping or polarization of the markers to one pole of the cell was readily apparent in thin sections. This pole of the cell was frequently the Golgi pole of the cell, as could be established when the centrosphere region was present within the plane of section (Figs. 7-9). At the capped pole, the surface membrane was sometimes thrown into folds and pseudopodia (Fig. 8). The markers

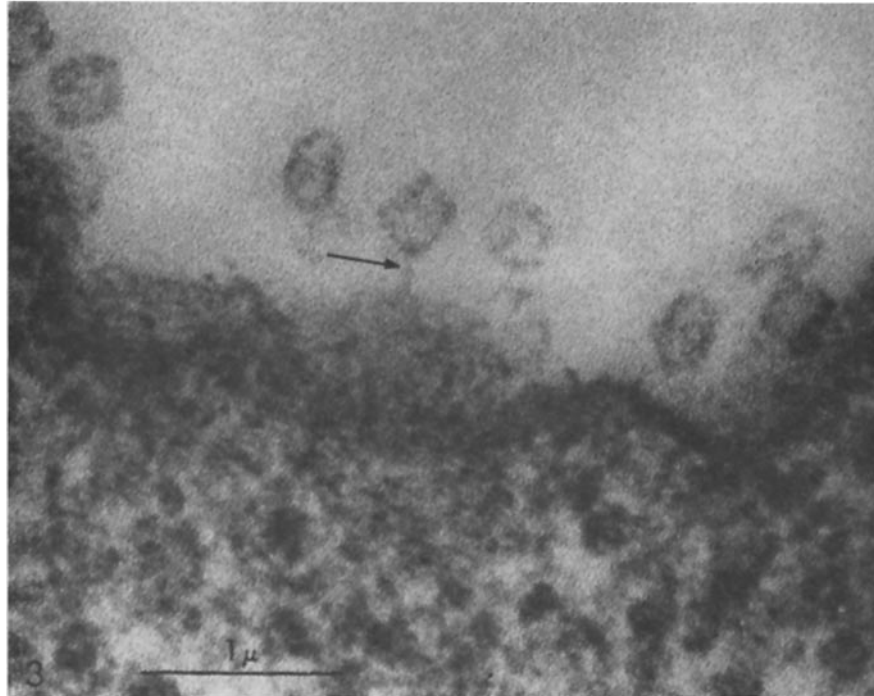


FIG. 3. Same as Fig. 2; higher magnification. At arrow, a thin filament connects the hemocyanin molecules to the cell membrane. (This can also be frequently seen if Fig. 2 is examined with a hand lens.) $\times 250,000$.

now covered the surface membrane of the capped pole in an almost continuous fashion, and appeared more tightly packed together than at 4°C. (This, however, may be an optical effect due to increased concentration of marker per unit area at the cap in the thickness of the section.) The marker was now stacked in many layers, particularly evident in the hemocyanin preparations (Fig. 8). Tilting of the specimen through 60° showed that this stacking of the marker in layers was genuine, and was not due to tangential cuts.

Endocytosis was readily apparent in the form of small vesicles and tubules containing label (Figs. 8 and 9). Some of the vesicles lay deep within the cell, close to the nucleus (Fig. 9). The cells appeared larger than unlabeled or un-



FIG. 4. B lymphocyte, mouse spleen, labeled with RAMG-ferritin at 4°C for 30 min (freeze-etched preparation). There is dense labeling of the cell surface in the form of a random network. $\times 26,000$.

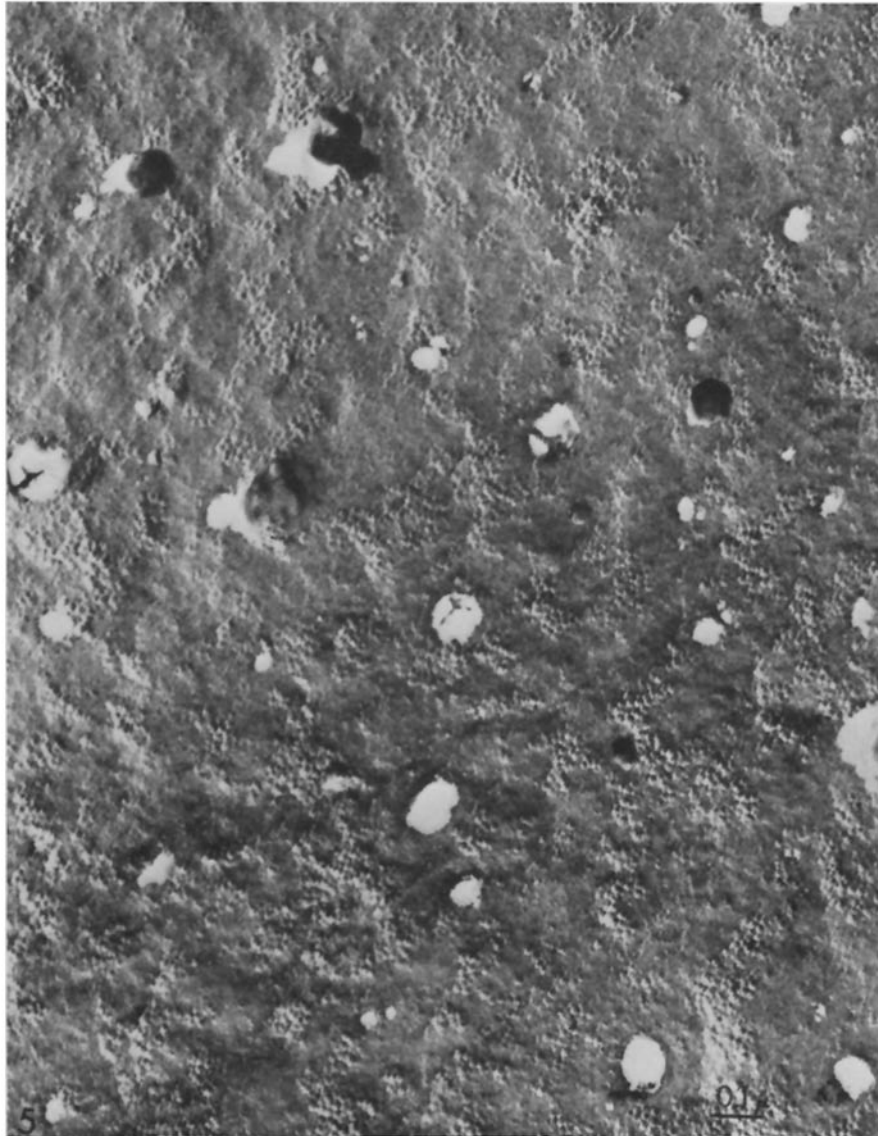


FIG. 5. Higher magnification of Fig. 4. Bare patches of membrane between the interstices of the network of label are seen. $\times 63,000$.

capped cells, and the Golgi apparatus was more prominent. Numerous small, unlabeled vesicles were associated with the lamellae of the Golgi apparatus (Figs. 8, 9). Occasionally apparent fusion of these small vesicles with the larger endocytotic vesicles containing label was observed (Fig. 9). The appearance

was highly suggestive of increased formation of primary lysosomes arising from the Golgi area.



FIG. 6. Same as Figs. 4 and 5, except RAMG-hemocyanin used, and the preparation is of a nonfrozen replica. The distribution of label is essentially similar to Figs. 4 and 5. $\times 63,000$.

At time intervals later than 10 min similar changes were observed, but by 30 min the cap had virtually disappeared from the cell membrane. Numerous endocytic vesicles of various sizes were observed in the cells as late as 24 hr after capping was induced, although at this stage the proportion of labeled cells ap-

peared to have decreased markedly, and there was much cellular debris and label extracellularly. One was struck by the apparent increase in activity of the cells at all stages after capping began as evidenced by enlargement of the cells, marked membrane activity at the capped pole, endocytosis, and enlargement of the Golgi apparatus, which contained numerous small vesicles.

Freeze-etched specimens showed the capping process clearly and dramatically (Fig. 10). The markers appeared to be streaming into the cap, and the underlying membrane appeared to be thrown up into wavy undulations toward the capped pole. The marker particles were packed solidly together in the capped area and little naked membrane could be seen. Occasionally, where cleavage had occurred through the cap into the underlying membrane, it could be seen that the marker molecules in the cap were stacked several layers deep. Intramembranous particles revealed on the cleaved faces showed no clumping or aggregation corresponding to the aggregation (capping) seen on the surface of the membrane. These latter observations will be presented in detail at a later date.

H-2 Loci on Mouse Thymocytes.—

C57BL thymocytes were incubated with DBA/2 anti-C57BL/6 antibody at 4°C for 30 min. The dose of antibody was 50 $\mu\text{g}/10^7$ cells. (This is a dose that saturates all histocompatibility antigens [*H-2*] on these cells. In control experiments using anti-*H-2* labeled with ^{125}I , we found saturation when using over 10 μg of antibody per 10^7 cells.) After washing, 20 μg of RAMG-ferritin was applied to the cells for a further 30 min at 4°C. After washing, some samples were immediately fixed, others were incubated at 37°C for 10 min, and then fixed.

Thin sections showed, at 4°C, relatively sparse segments of the cell membrane which were labeled. These segments varied in length. The ferritin molecules were stacked two or three layers deep, about 200 Å apart. There was some endocytosis, the vesicles lying under the cell membrane.

Freeze-etching revealed large patches of labeled membrane scattered irregularly and randomly on the membrane (Figs. 11, 12). These patches were usually several thousand angstroms in width. The patches were not interconnected and were separated by considerable expanses of bare membrane of the order of several thousands of angstroms in width.

After warming to 37°C for 10 min, thin sections showed some apparent aggregation of labeled segments. Endocytosis was quite marked.

Freeze-etching of cells incubated at 37°C revealed no definitive caps, although there did appear to be some aggregation of label to form larger patches. These observations are in accord with those made with fluorescein and ^{125}I label, in which little capping but some degree of aggregation was detected (1).

Binding of ALG to Rat Thymocytes and B Cells.—

In these experiments only freeze-etching of cells held at 4°C was done. Rat thymocytes and spleen cells were treated with ALG-ferritin at concentrations of 11 and 42 $\mu\text{g}/10^7$ cells at 4°C for 30 min, washed, and fixed as usual for freeze-etching. The doses used were calculated to be saturating doses.

The etched surfaces of thymocytes and spleen cells appeared similar. The ferritin was distributed in small, irregularly scattered, noninterconnected clusters (Fig. 13). Each cluster contained 2-12 or so ferritin particles closely approximated. Many of the clusters were within a few hundred angstroms of each other. Within the spleen cell population, no subpopulations of cells could be distinguished. The sampling problems inherent in the technique would not allow for subtle differences to be detected with ease.

Mapping of Concanavalin A Binding Sites.—

2×10^7 thymocytes in 200 μ l of Hanks' solution were treated at 4°C for 30 min with 0, 40, 200, and 400 μ g of Con A. After washing, the cells were treated with 100 μ l of PBS containing 500 μ g of hemocyanin for 30 min at 4°C. The cells were then washed, fixed in the cold, and processed for freeze-etching.

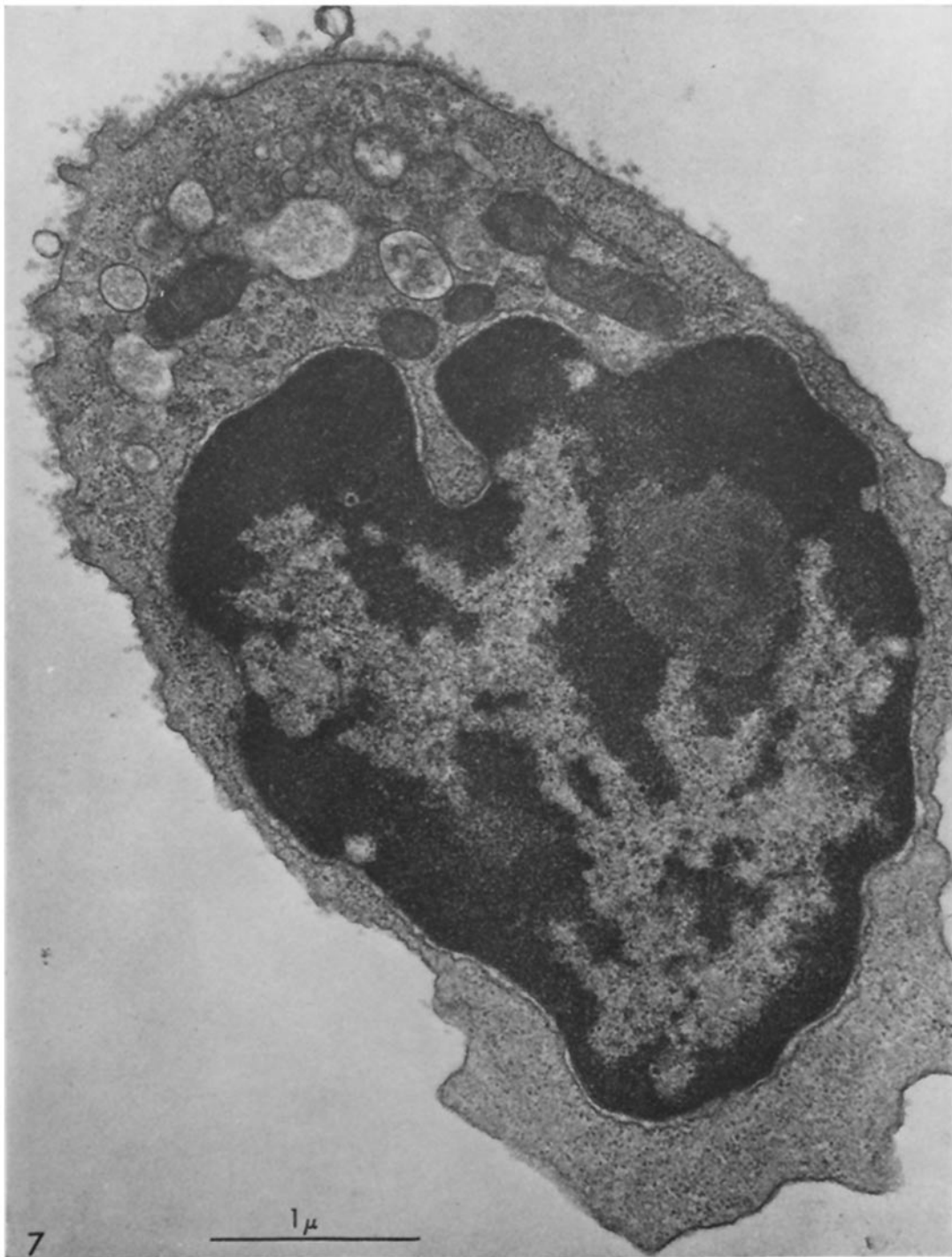
In another experiment, B cells, derived from spleen suspensions treated with anti- θ anti-serum were similarly treated. Details of the anti- θ treatment are given in the previous paper (1). 2×10^7 cells for 200 μ l of medium were treated with 500 μ g of Con A for 30 min at 4°C, washed, and then treated with 100 μ l of PBS containing 500 μ g of hemocyanin for 30 min at 4°C. After washing, the cells were fixed in the cold and processed for freeze-etching.

In both T and B cells the distribution of the hemocyanin-labeled Con A was essentially similar. It presented as an extensive, lacy, irregular network, with bare patches of membrane between the interstices of the network (Figs. 14, 15). Essentially the same pattern was seen at all concentrations of Con A used, although the labeling was heavier at the higher doses. Cells treated with Con A alone or with hemocyanin alone were negative. Light prefixation of the cells with 2% buffered formaldehyde (freshly prepared from paraformaldehyde [8]) for 2 min, followed by treatment with Con A and hemocyanin, made no differences in the distribution of label.

DISCUSSION

It is apparent from our work reported in the previous paper (1) and that of others (10, 11) that different moieties of the lymphocyte membrane have varying reactivity in regard to movement and endocytosis after they have been treated with a ligand. Movement and endocytosis of four moieties were analyzed in our previous paper (1). The movement was determined by observing whether there was formation of one or several aggregates on the membrane. We observed that Ig and Con A receptors moved and capped rapidly, while the ALG antigens moved less rapidly unless an additional ligand was brought into play; *H-2* antigens moved less, despite direct or sandwich reactions. Endocytosis was also variable, and was not correlated with efficiency of movement.

FIG. 7. B lymphocyte of mouse spleen treated with RAMG-hemocyanin at 4°C, then warmed to 37°C for 10 min. The label is now concentrated at the Golgi pole of the cell, forming a so-called cap. The membrane elsewhere is now virtually clear of label. The density of label at the cap is increased, and the label is stacked in several layers. Compare with Fig. 2. $\times 30,000$.



In our previous study (1), we analyzed three factors in the movement of these surface molecules: (*a*) temperature, which is important in cap formation, (*b*) amount of ligand, and (*c*) presence of one or more ligands in the reaction. The present study, using ultrastructural labels such as ferritin and hemocyanin, per-

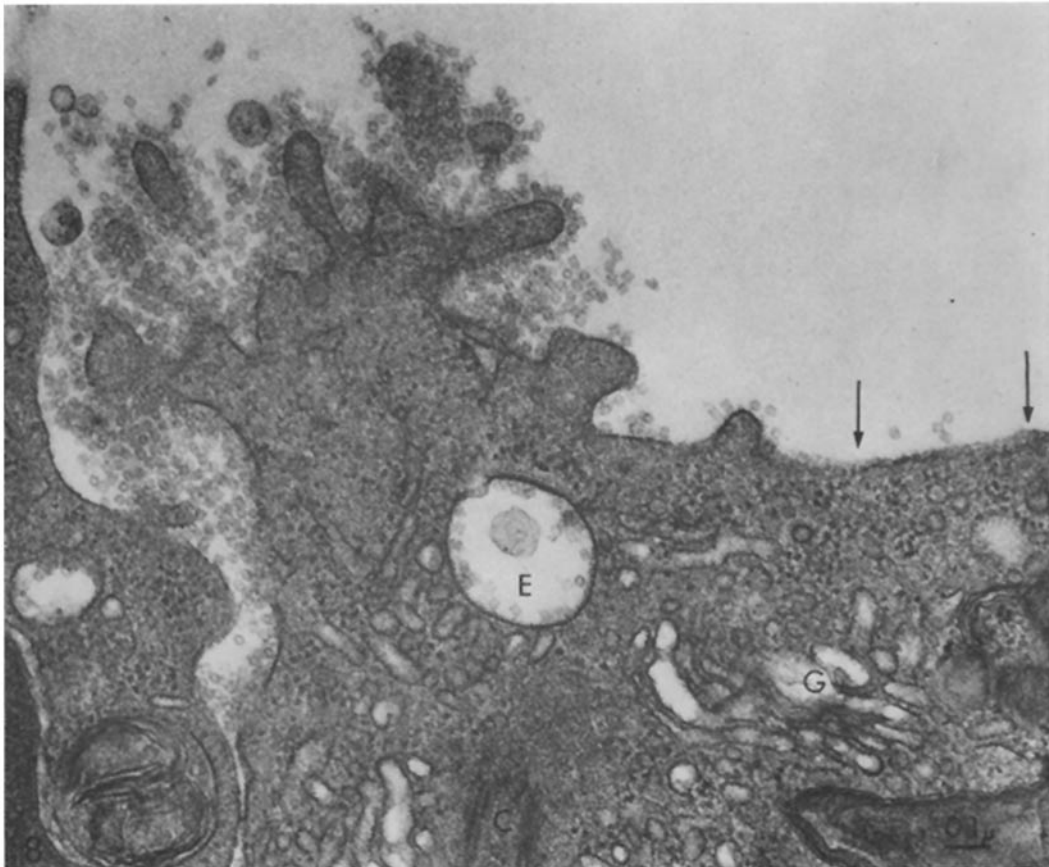


FIG. 8. Similar to Fig. 7; higher magnification of a cap. The capped pole of the cell shows surface membrane activity with formation of pseudopodia. The label in the cap is densely packed and stacked in several layers. The adjacent surface membrane is virtually clear of label (*arrows*). Endocytosis is occurring (*E*). The Golgi apparatus (*G*) is prominent, displaying dilated lamellae and numerous small vesicles. *C* = centriole. $\times 53,000$.

mits one to analyze the phenomenon of movement of surface molecules in finer detail. It is apparent that the movement and capping of the moieties reacted with ligands is occurring at the level of the cell membrane, and that endocytosis is indeed occurring in the form of small, membrane-bound vesicles and tubules budding off the cell membrane. The present experiments using freeze-etching methods have permitted us to establish the relationship between movement

upon reaction with one or two ligands and the distribution of molecules on the membrane.

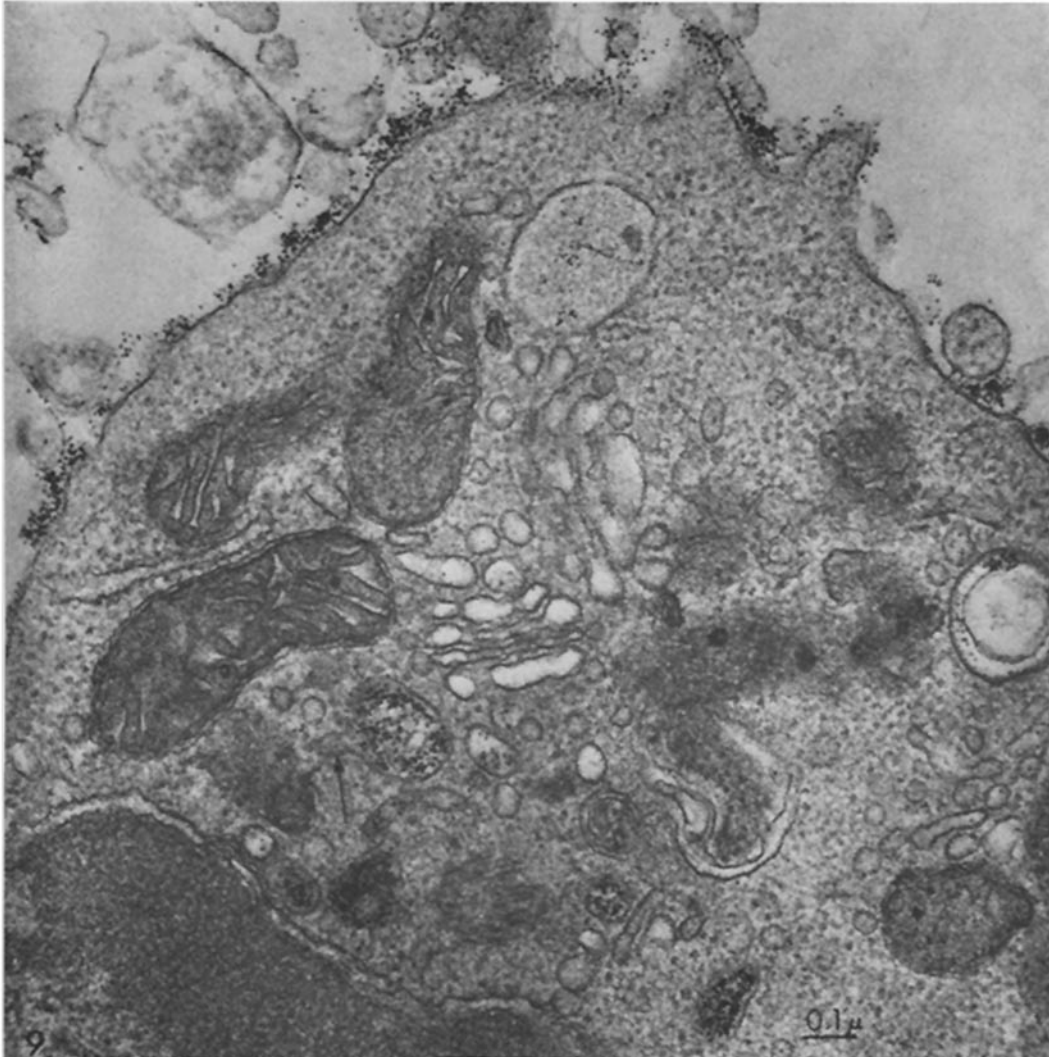


FIG. 9. Similar to Figs. 7 and 8, except RAMG-ferritin was used as label. The cap is still partially present on the cell surface. Endocytosis is occurring, and the Golgi apparatus is active. Apparent fusion of a small Golgi vesicle with an endocytotic vesicle is seen at the arrow. Section stained with alkaline bismuth. $\times 63,000$.

The entities which moved and capped efficiently upon treatment with a ligand (e.g. Ig and Con A-binding sites) were distributed in a continuous network, with close packing of the marker molecules, ferritin and hemocyanin. Al-

though we do not know at present the stoichiometry of the ratio of visualized particle(s) to binding sites, it is reasonable to assume that at the most two to three particles represent one receptor molecule in the case of Ig, and that each marker molecule (hemocyanin, ~ 300 A diameter) represents a binding site in

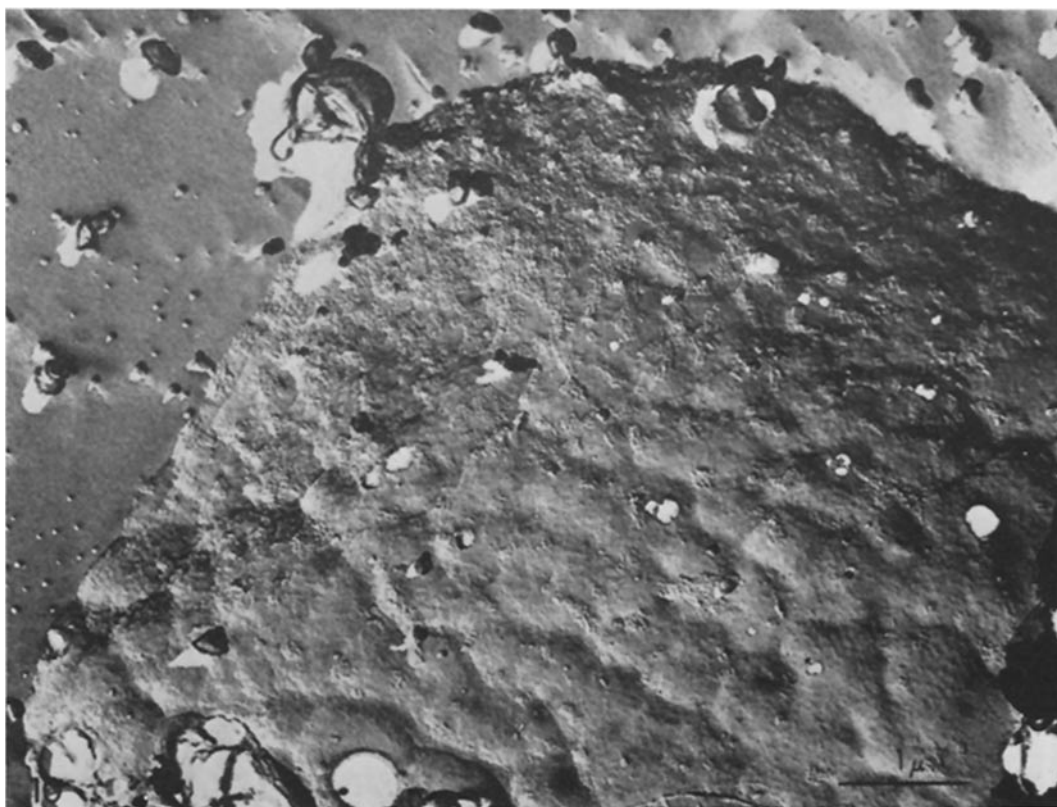


FIG. 10. B cell, mouse spleen, labeled at 4°C for 30 min with RAMG-hemocyanin, and then warmed to 37°C for 10 min; freeze-etched preparation. The streaming of the label into the capped area is seen. The label is densely packed in the cap area. Towards the bottom of the micrograph, the membrane is virtually clear of label. Note the undulations of the membrane surface. $\times 16,000$.

the case of Con A. Therefore, in the case of Ig the detected moieties are relatively closely packed and separated by distances easy to bridge by a single bivalent antibody molecule. The span of a single antibody is about 140 A (12). With these receptors an amount of ligand that cross-links all molecules leads to rapid movement, aggregation, and capping. In the case of Con A, which is apparently multivalent (13), the cross-linked sites are presumably close together, as the greatest dimension of the molecule is ~ 90 A (14, 15); alternatively, aggregates of Con A could cross-link sites somewhat further apart.

In the case of ALG receptor sites, we have no stoichiometric information which relates the number and packing of marker molecules to the number of underlying receptor sites. Nevertheless, there was a random distribution of anti-

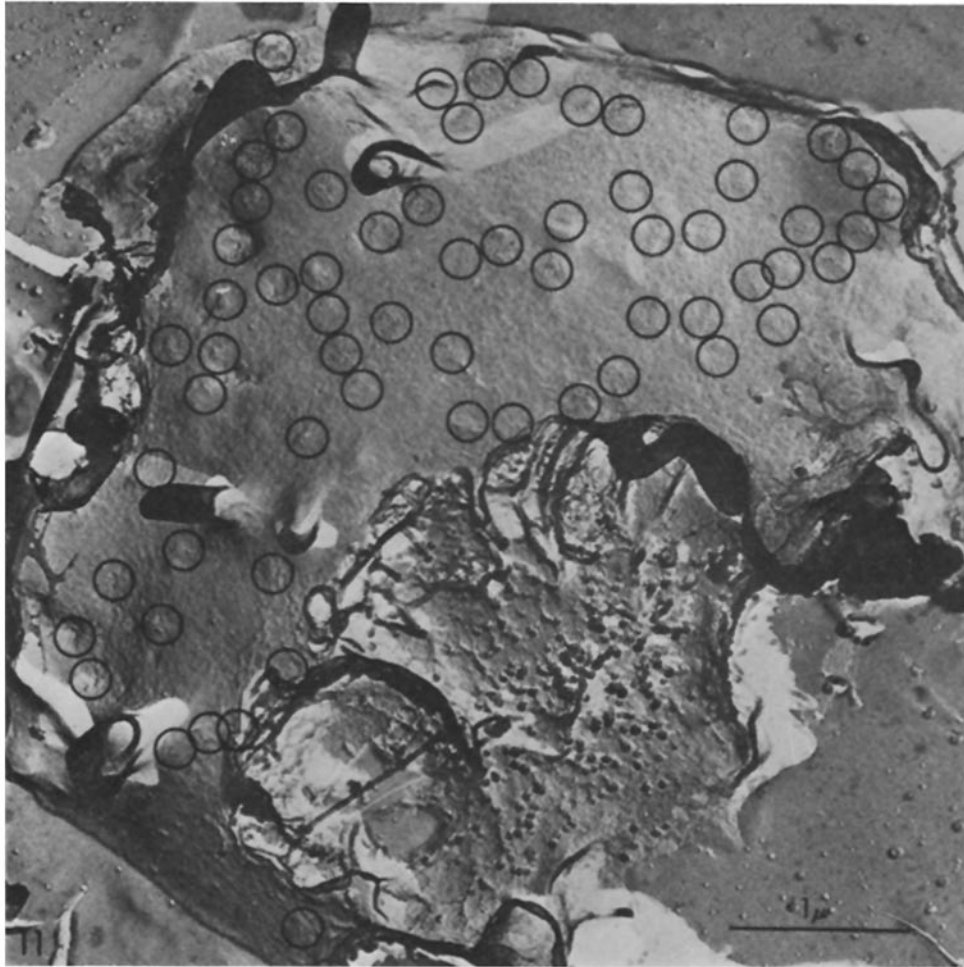


FIG. 11. Mouse thymocyte labeled at 4°C with anti-*H-2* antibody, followed by RAMG-ferritin. The label is randomly distributed in patches over the cell membrane. Each patch is roughly 1000–3000 Å in width, and most patches are separated from each other by bare membrane, at distances apart of several thousand angstroms. $\times 24,000$.

gen in small clusters, variable in size, over the whole surface of the membrane. The distance between these clusters was of the order of hundreds of angstroms. These distances are, in general, too large to be spanned by a single antibody molecule but yet can be reached by a sandwiched antibody reaction (which can

be calculated to span about 420 Å). Indeed, rapid cap formation occurred with ALG sandwiched to a second antigen.

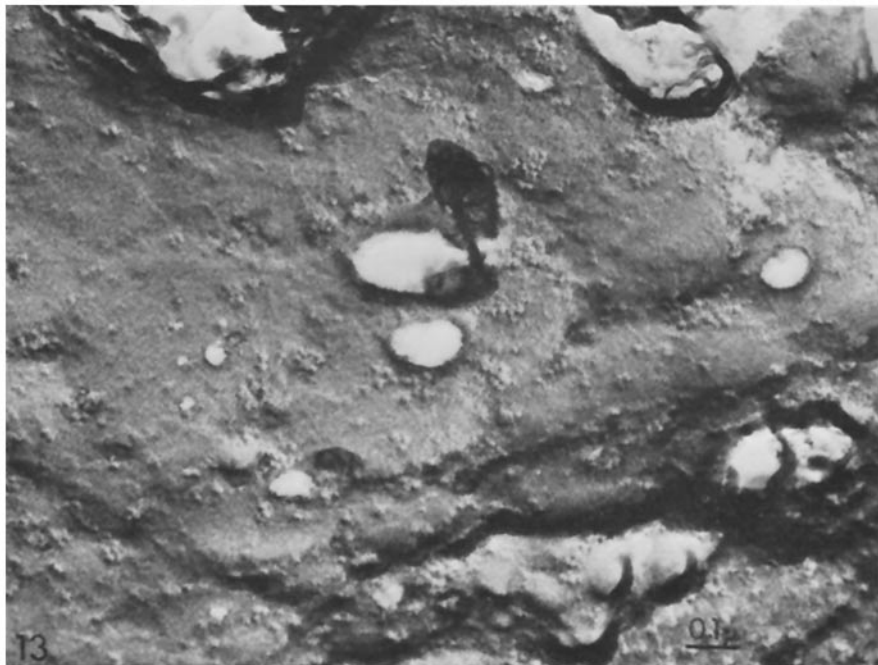
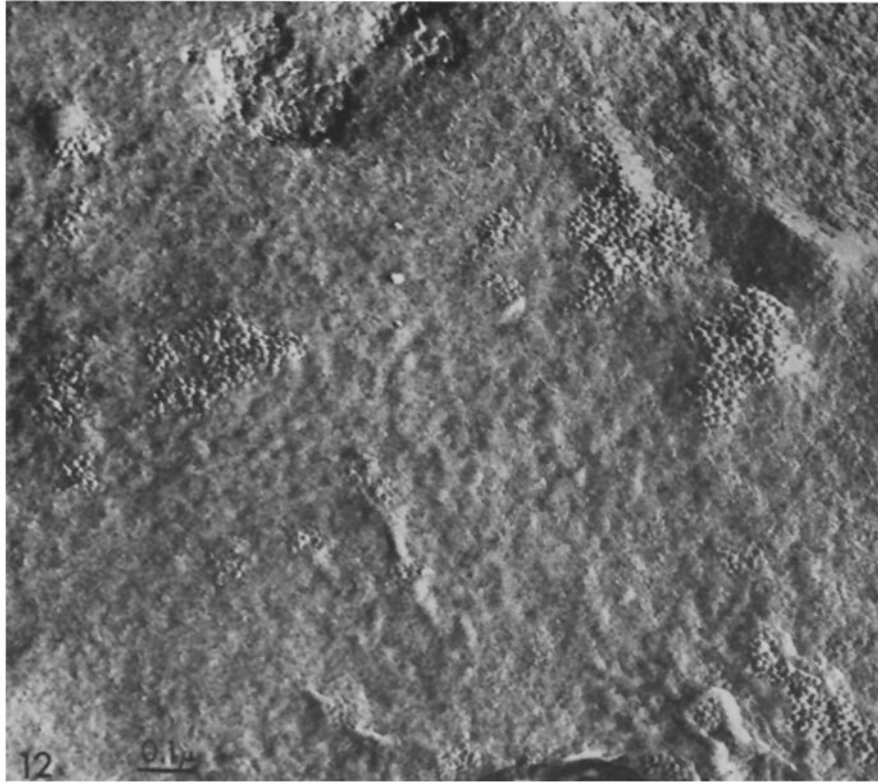
In regard to *H-2*, the mapping showed large patches of closely packed label, separated by bare membrane at distances of the order of several thousand angstroms. We are aware that we are revealing the location of *H-2* by an indirect sandwich technique, and that this may be hazardous, in that cross-linking and aggregation could occur even at 4°C. Thus, the patches we observe could represent some local degree of aggregation, but even if this were so, it is not unreasonable to assume that *H-2* is distributed in the form of widely dispersed patches. This would conform with the concepts of others (16–18). Nevertheless, with *H-2* we have found that capping occurs extremely slowly and then only to a small extent, although aggregation does occur. This would indicate that most of the clusters of *H-2* sites are more than 420 Å apart, i.e., the calculated span of a double antibody indirect sandwich. The results with *H-2* complexes suggest that even sites widely dispersed, either singly or in groups, move randomly in the membrane, albeit slowly; with time there is the chance that sites may come sufficiently close to each other to link and form an aggregate. This process is in part limited, no doubt, by the progressive removal by endocytosis of the complexes from the membrane, although, as pointed out previously, endocytosis and aggregation are not necessarily concomitant events.

In general then, we propose that sites, or clusters of sites, closer than 140 Å can readily be linked by a single antibody to form one or more aggregates; that sites farther apart than 140 Å but less than 420 Å form an immediate lattice only upon sandwiching with two ligands. Similarly, sites, or clusters of sites, farther apart than 420 Å also would not aggregate markedly and rapidly even when treated with antibody and anti-antibody. In the latter two instances, aggregation may occur late as a result of random movement of the sites. To obtain more precise information on the spacing of sites, or clusters of sites (patches), techniques yielding higher spatial resolution than those we have used would have to be applied, and the stoichiometry of the labeling of sites would have to be known. The Nicolson-Singer technique (19) of visualizing sites at high resolution should prove useful in the first regard, and studies are in progress in our laboratories along these lines.

It is of interest to consider what these experiments may tell us in relation to current concepts of membrane structure. The favored current concept is the fluid mosaic model, in which heterogeneous globular protein molecules are partially embedded in a fluid matrix of phospholipid, organized as a discontinuous fluid bilayer (20). The experiments of Frye and Edidin (21), in which intermixing of membrane antigens of heterokaryons was elegantly demonstrated, are

FIG. 12. Same as Fig. 11; higher magnification. $\times 63,000$.

FIG. 13. Mouse thymocyte labeled at 4°C with ALG-ferritin. The label is randomly distributed in small clusters, situated within a few hundred angstroms of each other. Compare Fig. 12. $\times 63,000$.



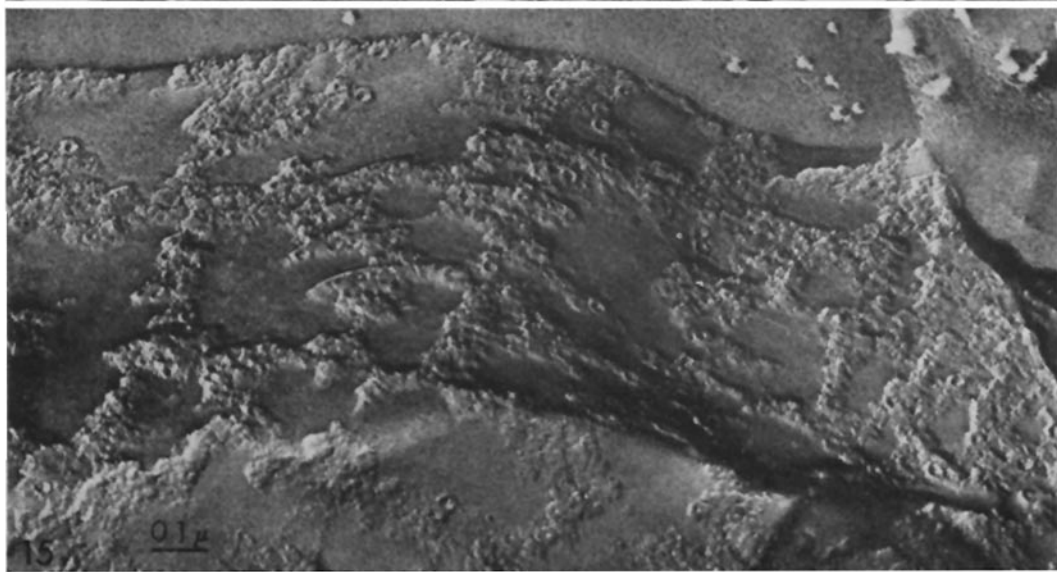
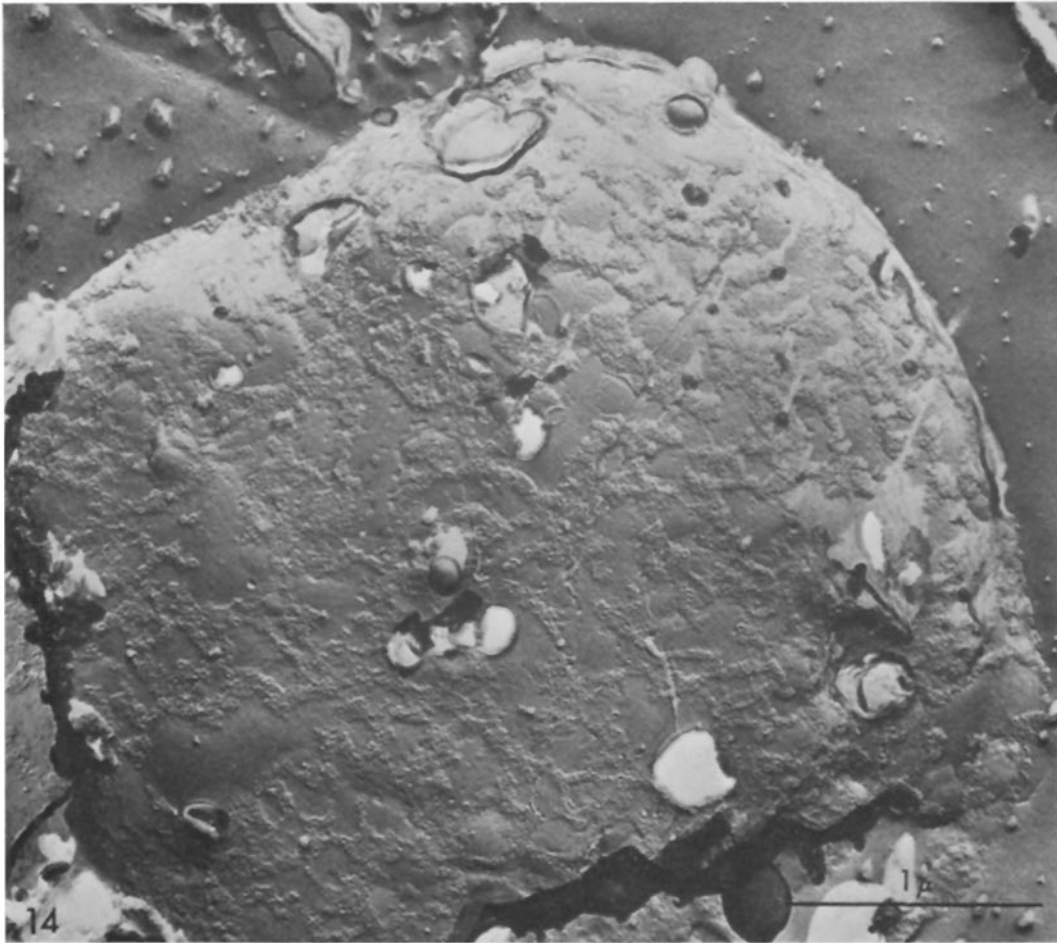
frequently adduced as evidence for the diffusional mobility of membrane protein components. It is of interest that in their experiments *H-2*-antigen-antibody complexes (revealed by a sandwich method), moved more slowly than antigens recognized by heterologous antibodies (equivalent to heterogeneous membranous antigens reacting with ALG). Presumably, what these authors were observing was intermixing of aggregates: no phenomenon equivalent to capping was observed. Also, the rapid capping of lymphocytes when treated with anti-Ig is cited as evidence for movement of protein moieties within fluid lipid layers of the membrane (20).

Distinction should perhaps be made between random movement of membrane moieties in their natural state and movement eventuating in aggregation and/or capping. The latter obviously occurs and requires cross-linking but there has not been direct proof of the former. In the experiments of Frye and Edidin, bivalent antibodies were utilized and the effects of cross-linking cannot be entirely excluded. In our experiments (1) using monovalent anti-Ig, a ring reaction was observed covering the whole cell surface but movement of individual moieties could not be ascertained because of the uniform fluorescence. Observation of localized areas of bound fluorescein-labeled Fab' in experiments similar to those of Frye and Edidin (21) would be very helpful in this regard. On the other hand, we believe that cell movement itself is required for the formation of a cap (although not necessarily for formation of several large aggregates) since certain metabolic inhibitors (e.g. inhibitors of glycolysis) suppress capping completely (manuscript in preparation). Capping possibly occurs when the cell crawls out of its lattice of antigen-antibody complex, somewhat like a snail emerging from its shell. Taylor et al. have also related cell motility to capping (10).

The topography of the membrane moieties reveals, in two dimensions, a long-range random, but short-range organized, pattern of distribution, which is consistent with the fluid mosaic model. The slow movement of *H-2* loci in the heterokaryon experiments (20) could be explained on the basis of the organization of the loci in large patches, which would then diffuse slowly. A similar explanation could be applied to our relative failure to move *H-2* sites, even with an indirect sandwich technique. However, consideration should be given to the view that the very organization of *H-2* loci into patches or domains implies an imposed restriction of diffusional movement on the components of such domains, perhaps a less fluid lipid environment or some degree of bonding of *H-2* antigens to lipid or other protein components. Thus, these domains would be able to move, but slowly and in a restricted fashion, and the restriction of movement of the domains may be a reflection of the possibility that *H-2* antigens, unlike

FIG. 14. Mouse thymocyte labeled with Con A-hemocyanin at 4°C. The label is densely distributed in a random network. $\times 33,000$.

FIG. 15. Same as Fig. 14; higher magnification. $\times 63,000$.



IgG (besides being farther apart from each other), may be deeply embedded in the lipid matrix of the membrane. There is evidence that to isolate the complete *H-2* molecule ionic, proteolytic, and detergent treatments of membranes are required (22).

We feel, however, that caution should be exercised insofar as the significance of the movement of Ig. The possibility should be kept in mind that the movements and capping of Ig may be events occurring largely external to the lipid layers of the membrane. Our observation that the cap frequently demonstrates stacked layers of the marker is of some interest. If, as is probable, the cap represents an agglutinated, cross-linked mass of antigen-antibody complex, then it is suggested that at least part of the receptor moiety in the membrane is avulsed or exfoliated from the membrane during movement and/or cap formation. Otherwise it is difficult to explain the presence of components of the cap situated several thousand angstroms away from the cell membrane unless membrane moieties are also present at that distance to provide cross-linking with the antibody, so that the cap "holds together." In other experiments to be published, we have made the aggregates of Ig flake off to some degree by simple agitation of the cell.

As a working hypothesis we therefore propose that capping is dependent on cross-linking of sites which are in close and sufficient proximity, or which can approximate sufficiently through random movement in the membrane. Superficial membrane moieties could be thus easily moved, perhaps by avulsion of the membrane moiety into an extracellular liquid phase. Membrane moieties fixed and embedded in the membrane would have little chance of capping, especially if these sites or clusters of sites are far apart and move randomly in a limited and slow fashion.

SUMMARY

Anti-immunoglobulin (Ig) coupled to ferritin or hemocyanin was used to map the distribution of Ig molecules on lymphocytes derived from bone marrow (B lymphocytes) by freeze-etching. The labeled anti-Ig was distributed all over the membrane in the form of random interconnected patches forming a lacy, continuous network. This was the pattern of lymphocytes labeled at 4°C with the anti-Ig. After warming at 37°C, the labeled molecules concentrated into a single area of the cell (forming the cap) and were rapidly internalized in small vesicles. Freeze-etching showed close packing of the labeled molecules in the cap area. There was evidence that in the cap area the Ig molecules were exfoliated from the plane of the membrane, suggesting that the Ig may be superficial to the bilipid layer, or weakly anchored to the membrane.

Similar studies were made using antibodies to histocompatibility antigens. Thymocytes were labeled with anti-*H-2* and ferritin anti-Ig at 4°C. Freeze-etching showed large patches scattered over the membrane and separated from each other by several thousand angstroms. This distribution may, in part, ex-

plain why *H-2* antigens do not readily form a cap; the large patches are beyond the reach of even a double ligand (sandwich) reaction.

The antigens that reacted with heterologous anti-lymphocyte globulin (ALG) were found in small noninterconnected clusters a few hundred angstroms apart. Such clusters presumably cannot be linked by a single antibody but can by a sandwich (ligand to ligand-antigen) reaction. In previous studies it was found that ALG antigens form a cap only after a sandwich reaction. Finally, the receptors for concanavalin A (Con A) were found in a lacy, irregular interconnected, random network. The spatial distribution of these moieties on the membrane may, in great part, determine their movement after reaction with one or two ligands.

Note Added in Proof.—We have recently studied the distribution of *H-2* sites on melanoma cells of C57BL/6 (melanoma B16 from Jackson Laboratory, Bar Harbor, Maine) using the same antisera described in this and the accompanying paper. In many of these tumor cells there is a strong positive fluorescent reaction in the form of a ring at 4°C which upon warming forms a single aggregate, i.e., the cap. The intensity and pattern of fluorescence suggests that the amount of *H-2* antigens is high in these cells and this could account for the differences observed in the present studies reported with thymocytes. Distribution and quantitation of *H-2* sites are currently being studied on these tumor cells. Also the distribution of *H-2* antigens on thymocytes has now been studied with the same anti-*H-2* antibody used here but labeled directly with ferritin. The pattern of distribution of *H-2* sites was identical to that reported here.

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