

## LIGAND-INDUCED MOVEMENT OF LYMPHOCYTE MEMBRANE MACROMOLECULES

### I. ANALYSIS BY IMMUNOFLUORESCENCE AND ULTRASTRUCTURAL RADIOAUTOGRAPHY\*

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Lymphoid cells possess a variety of macromolecules on their membranes, some of which seem to be exclusive for a specific population of lymphocytes (1) or particular to a certain stage of their differentiation (2). Several studies, in particular those of Boyse, Old, and their collaborators (2, 3), have been instrumental in identifying many of these different moieties and also have given some indication of their distribution on the surface of the cells (4, 5). These surface molecules have been identified to a great extent by immunological methods or through reactions involving plant agglutinins. The functions of many of these membrane moieties, which include histocompatibility antigens and other alloantigens such as the thymus leukemia antigen (6), theta isoantigen (1), and the Ly A-B antigens (7), as well as the receptors for plant lectins, are not known at present. Immunoglobulins (Ig)<sup>1</sup> are included among the molecules on lymphocyte membranes. The Ig molecules have been demonstrated directly by the use of immunofluorescence (8-10), radioautography (8, 11), or immunocytocidal tests (12), and have also been labeled while on the membrane with radioactive <sup>125</sup>I (13). Ig molecules are easily detectable only on bone marrow- or bursal-derived lymphocytes that comprise the progenitors of antibody-forming cells (B lymphocytes) and appear to be the receptors for antigen (14, 15). The number of such molecules of Ig has been estimated to range from 50,000 to 150,000 per B lymphocyte (9). Recent studies have shown that the Ig molecules on the surface, upon interaction with specific antibody, can be displaced readily and become agglutinated at one pole of the cell, forming what Taylor et al. termed a "cap" (16), and are subsequently internalized in vesicles. This process leaves the B lymphocyte denuded of its antigen receptors for several hours (17). This phenomenon of

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<sup>1</sup> *Abbreviations used in this paper:* ALG, anti-lymphocyte IgG; B lymphocytes, the progenitors of antibody-forming cells which are derived from the bone marrow in mammals; Con A, concanavalin A; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; T lymphocytes, thymic-derived lymphocytes.

movement of Ig molecules on or in the membrane is of interest not only because of its possible importance in the immune expression of the B lymphocytes but also because of its relevance to our understanding of the molecular composition of the plasma membrane. Indeed, the studies with Ig, as well as those of Frye and Edidin (18) with heterokaryons (using antibodies to histocompatibility antigens and heterologous antibodies), imply that some membrane moieties may be loosely bound to a fluid lipid layer and are capable of being displaced with relative ease.

In this report we have evaluated the fate of several moieties on lymphocyte membranes after reaction with a suitable ligand. We have chosen to study Ig on B lymphocytes and major histocompatibility antigens, antigens that react with a heterologous anti-lymphocyte antibody and the carbohydrate moieties that bind to the plant lectin concanavalin A (Con A) in both B and thymic-derived (T) lymphocytes. Our studies clearly show different patterns of reactivities of these four moieties. Some cap rapidly upon direct reaction with a ligand, others cap only after a second ligand is brought into the reaction, and others do not cap at all; some are rapidly internalized while others remain on the membrane.

#### *Materials and Methods*

*Cells.*—Lymphoid cells were harvested from spleen and thymus of mice or rats by conventional procedures using sterile techniques. The mice, about 20–25 g of either sex, were of the A/St (West Seneca Laboratories, Buffalo, N. Y.) or C57BL/6 and DBA/2 strains (Jackson Laboratory, Bar Harbor, Maine). Rats were from the Lewis strain, of about 150 g, males (Microbiological Associates, Bethesda, Md.). In most experiments the cells were washed twice with Hanks' balanced salt solution usually containing 5% v/v fetal calf serum, centrifuged at 400 g for 7–10 min, and then placed on a gradient of Ficoll-Hypaque in order to separate live from dead cells (19). In this procedure, the dead cells together with red cells and some live cells, pelleted to the bottom of the centrifuge tube while the live undamaged cells remained in the upper layers. The upper layer had virtually no dead cells as judged by the trypan blue exclusion test. The upper layer of live cells was further washed once and incubated with the appropriate reagents. In most experiments  $5\text{--}10 \times 10^6$  cells were incubated with the appropriate antiserum in a total volume of 100–200  $\mu\text{l}$  at 2–4°C for 30 min and then washed twice. Great care was taken to maintain the temperatures during the whole procedure of incubation and washing at 2–4°C. In most experiments cells were resuspended in 100–200  $\mu\text{l}$  of medium and incubated in a 37°C water bath for variable periods of time. Further details of the procedures are stated when describing the appropriate experimental protocols.

#### *Antisera.*—

(a) Rabbit anti-mouse Ig was the same antiserum described in our previous studies (11, 17). This was produced in rabbits hyperimmunized with a gamma globulin-rich fraction of mouse serum. This antibody precipitated all mouse Ig classes and Fab fragments.

(b) Rabbit anti-rat Ig was produced as above.

(c) Rabbit anti-rat lymphocyte serum (ALG). New Zealand rabbits were twice injected (with a 2-wk interval between) with  $10^9$  Lewis rat thymus and lymph node cells. 7 days after the second injection the rabbits were exsanguinated and the serum was decanted for  $\frac{1}{2}$  hr at 60°C. The serum was absorbed once with Lewis rat red blood cells ( $\frac{1}{3}$  volume of red cells to  $\frac{2}{3}$  volume of rabbit serum) at 37°C for 1 hr and then at 4°C for about 18 hr.

(d) Rabbit anti-mouse lymphocyte serum was prepared as above but using mouse lymph node cells.

(e) Sheep anti-rabbit Ig was prepared in sheep hyperimmunized several times for 9–12 months with purified rabbit IgG. It precipitated rabbit IgG as well as some beta globulins.

(f) DBA/2 anti-C57BL/6 antibody. Antibodies to some of the histocompatibility antigens of C57BL were obtained by immunizing DBA/2 on eight weekly occasions with progressively increasing amounts of C57BL spleen cells ( $10^5$ – $5 \times 10^7$  cells). The recipient mice were exsanguinated 1 wk after the last immunization. We thank Dr. Howard Engers from our laboratories for preparing this antibody.

(g) Anti- $\theta$  serum (1) was prepared in AKR mice by repeated injection with CBA thymocytes.

*Fractionation of Antisera.*—IgG was isolated from most of the rabbit antisera by diethylaminoethyl cellulose (DEAE-cellulose) chromatography. First the globulins from the sera were precipitated in 50% ammonium sulfate, dialyzed against 0.01 M phosphate buffer, pH 8.0, and then passed through a DEAE-cellulose column; the material eluted at 0.01 M contained pure IgG. Mouse IgG from DBA/2 anti-C57BL serum was obtained by preparative electrophoresis using Pevikon as the supportive medium. Fab' rabbit Ig was prepared by digestion with papain for 18 hr at 37°C (20); the digested material was passed through a DEAE-cellulose column; the product eluted at 0.01 M was then passed through a Sephadex G-100 column in order to eliminate any possible amount of undigested Ig. No undigested Ig could be detected in the preparation of monovalent antibody by Ouchterlony analysis.

*Iodination.*—A chloramine-T method in microvolumes was used for most Ig (21). Specific activity of the iodinated reagents varied from 10 to 35  $\mu\text{Ci}/\mu\text{g}$ . Anti-*H-2* antibodies were iodinated using lactoperoxidase as described by Marchalonis (22). Except for Con A, all labeled reagents were passed through a G-25 column to eliminate free nonconjugated I. The labeled Con A was dialyzed against buffered saline for 24 hr.

*Conjugation to Fluorescein Isothiocyanate (FITC) and Fluorescence Microscopy.*—Conventional methods were used. The molar fluorescein:protein ratio varied from 4 to 7. For fluorescence microscopy we used a Zeiss microscope (Carl Zeiss, Inc., New York) with an FITC primary filter manufactured by Optisk Laboratorium in Lyngby, Denmark. Suspensions of cells were placed between slides and cover slips and examined at variable times. The cells were examined immediately after reaction in the cold since the movement of some reactants on the membrane can take place, with time, on cells mounted on slides which are warming up. Usually about 300 cells were scored for patterns of fluorescence.

*Ultrastructural Radioautography.*—The procedure employed was detailed previously (11). We used Ilford L4 liquid emulsion (Ilford Ltd., Ilford, England). Sections were exposed to the emulsion for variable periods of 5 days to 3 wk. About 50–100 cells were examined.

*Miscellaneous Reagents.*—Con A was obtained from Miles Laboratories, Inc. (Kankakee, Ill.) as a twice-crystallized product. It was conjugated to FITC or to  $^{125}\text{I}$  by the same procedures described above.

## EXPERIMENTAL PROTOCOLS AND RESULTS

### *Anti-Ig*

In our previous studies employing FITC- or  $^{125}\text{I}$ -labeled anti-Ig at 4°C, the B lymphocytes among spleen cells were shown to have Ig molecules distributed all over the plasma membrane. It was subsequently shown that the anti-Ig-Ig complex moved on the membrane and was internalized after warming the cell suspension. In these first experiments we studied the fate of the Ig-anti-Ig complex after the increase in temperature of the cells.

*Quantitation of the Phenomena.*—The movement of the anti-Ig-Ig complex on the cell surface depended upon the amount of anti-Ig that reacted with the

surface Ig. This was tested in experiments using immunofluorescence or ultrastructural radioautography.

*Experiment 1 (Table I):*  $5 \times 10^6$  murine spleen cells were incubated with 0.1, 1.0, 10, and 100  $\mu\text{g}$  of FITC-labeled anti-Ig in a total volume of 100  $\mu\text{l}$  for 30 min, washed, and resuspended in 200  $\mu\text{l}$  of medium. A sample was examined immediately or after warming at 37°C for 10–20 min.

Cells that were exposed to 10  $\mu\text{g}$  or more of anti-Ig exhibited irregular fluorescence all around the cell membrane if examined immediately after incubation at 4°C. When warmed, the fluorescence reaction became concentrated in one pole of the cell, i.e., the cap. Later on small dots were seen in the area under the cap, an indication that the conjugate was being taken into cytoplasmic vesicles. The fluorescence pattern clearly changed from a circumferential ring type, at 4°C, to a cap immediately after warming, to discrete round vesicles clearly separate from the edge of the cell several minutes after warming. Figs. 1–5 are fluorescence micrographs of the different patterns using anti-Ig or other antibodies.

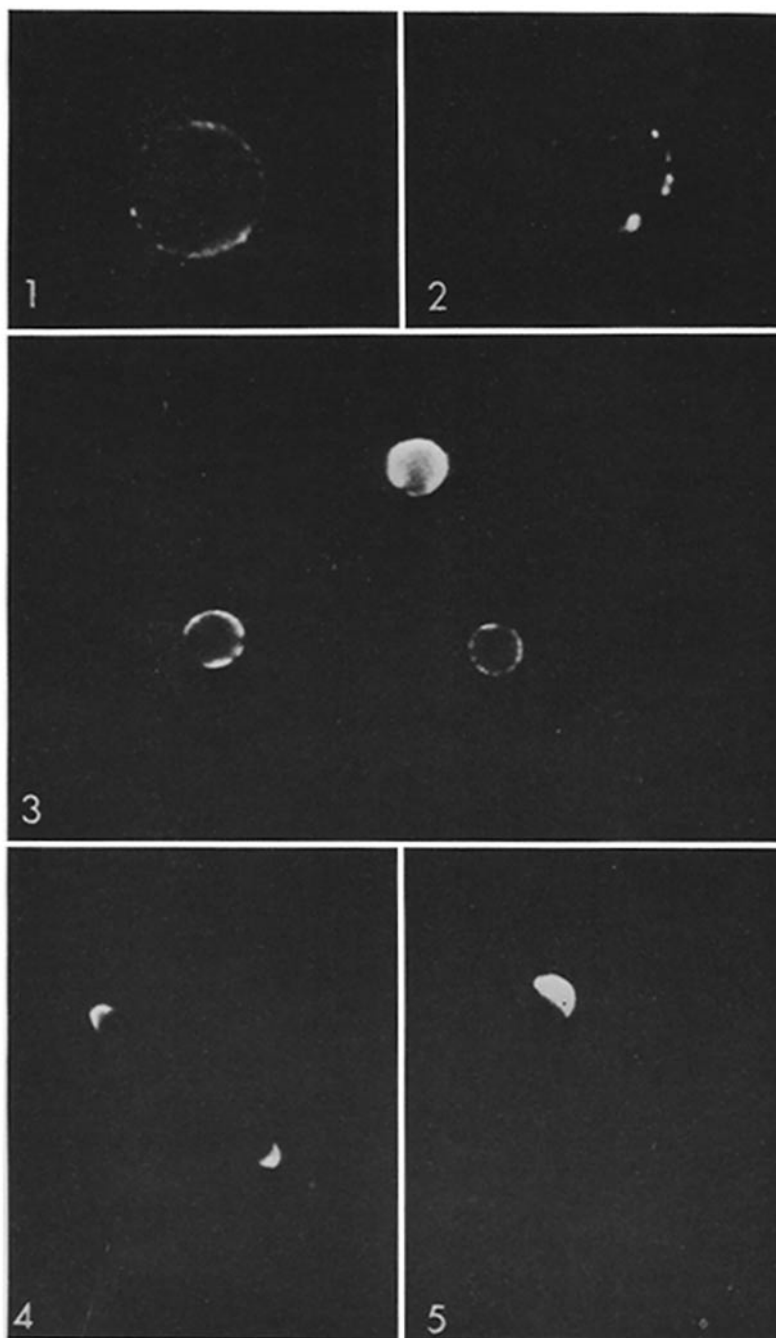
Cells exposed to 0.1 to 1.0  $\mu\text{g}$  of anti-Ig exhibited weak fluorescence all around the cell membrane; this fluorescent reaction did not form typical caps when the cells were warmed at 37°C. At the 1.0  $\mu\text{g}$  dose occasional discrete heavy aggregates were noted on the surface of the cells after 10 min of incubation. The cells examined after 20 min showed discrete dots separated from the edge.

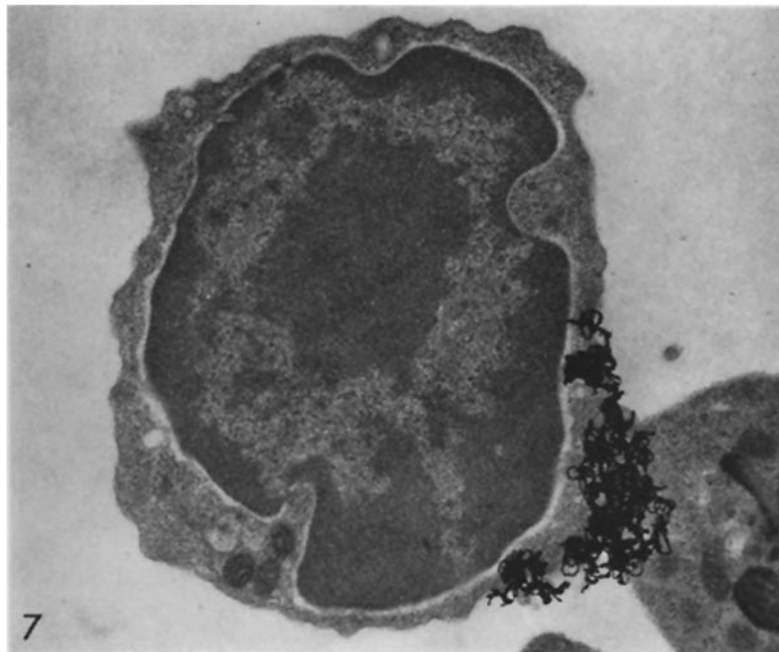
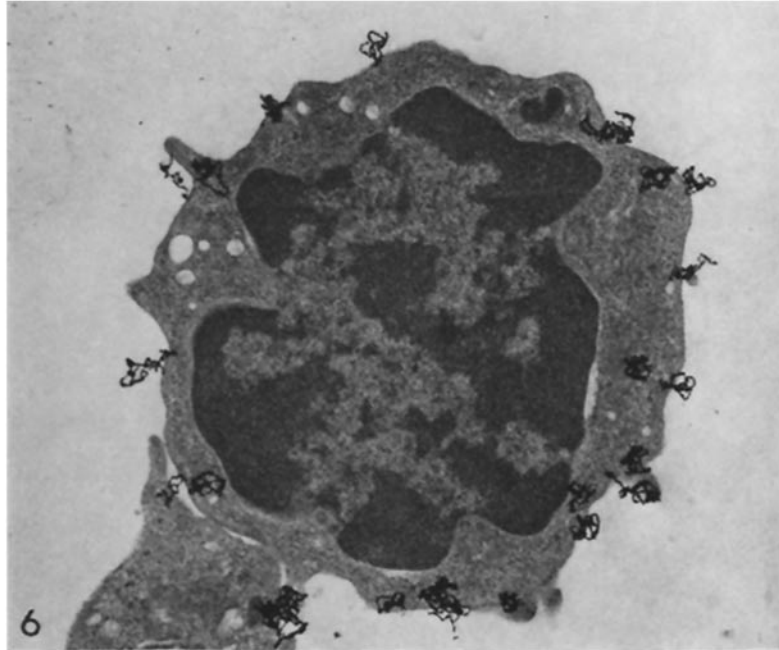
*Experiment 2:* Murine spleen cells ( $10^7$ ) were incubated with either 2 or 20  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled anti-Ig (specific activity 20  $\mu\text{Ci}/\mu\text{g}$ ) for 30 min at 4°C, washed three times, resuspended in 200  $\mu\text{l}$  of medium, and placed at 37°C for periods of 1 min to 2 hr. The cells were fixed for ultrastructural studies after incubation in the cold and after warming for the different time periods. The specificity of the reaction between  $^{125}\text{I}$ -labeled anti-Ig and Ig on B lymphocytes was reported in the previous study. Since the anti-Ig used previously is the same employed now, we have not carried out any further specificity controls in the present series. In the previous study specificity was checked by testing the  $^{125}\text{I}$ -labeled anti-Ig after absorption of its antibody activity.

As in the previous study, cells examined immediately after reaction with  $^{125}\text{I}$ -labeled anti-Ig exhibited grains all over the circumference of the cell

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Figs. 1–5. Figs. 1–5 are fluorescence micrographs of lymphocytes demonstrating different patterns of positivity. Fig. 1 is a lymphocyte after reaction with FITC-labeled anti-Ig at 4°C and examined immediately; it shows typical ring reaction. Fig. 2 represents a lymphocyte after incubation with FITC-labeled anti-Ig at 4°C and then incubated for 30 min at 37°C; note round spots of fluorescence. Figs. 3 and 4 show lymphocytes after reaction with ALG and FITC-labeled anti-rabbit Ig. In Fig. 3 the cells were examined immediately after incubation with the antibodies at 4°C (note ring reaction); Fig. 4 cells were examined after 1 hr at 37°C (note two lymphocytes with caps and no evidence of interiorization). After 1 hr at 37°C all lymphocytes incubated with FITC-labeled anti-Ig had the pattern of Fig. 2. Fig. 5 is a typical cap in a spleen lymphocyte after incubation with FITC-labeled anti-Ig at 4°C and subsequent warming for 1 min. Original magnification  $\times 250$ .





(Fig. 6). However, the incubation of spleen cells for as little as 1 min at 37°C resulted in the rapid movement of labeled anti-Ig towards one pole of the lymphocyte plasma membrane (Fig. 7). Within 2 min at 37°C the anti-Ig was totally concentrated in one zone of the surface membrane with little or no label remaining on other portions of the plasma membrane. Anti-Ig was always found to be concentrated on the portion of the cell surface associated with the centrosphere area and where the majority of cytoplasm was located. At 5 min, endocytosis was apparent with label observed in vacuoles of various sizes (Fig. 8). By 10 min, the majority of labeled cells had labeled Ig within the cytoplasm and some of the cells had endocytosed most of the capped Ig (Fig. 9). After 1 hr the endocytosed Ig became aggregated in the centrosphere area generally associated with the Golgi apparatus. It was therefore clear that a temperature change from 4 to 37°C brought about the immediate agglutination of all the anti-Ig-Ig membrane complex to one zone of the membrane followed by the immediate endocytosis of the complex. Lymphocytes incubated with 1  $\mu$ g of  $^{125}$ I-labeled anti-Ig showed no concentration of grains in one pole after incubation at 37°C: the grains were present as aggregates on the membrane immediately after warming (1 min) and later (10 min) were associated with intracellular vesicles.

As would be expected, the phenomenon of movement and endocytosis of Ig on B lymphocytes was not exclusive to murine cells. Rat spleen cells treated as above with 20 and 100  $\mu$ g of FITC-labeled anti-rat Ig experienced the same phenomenon of movement, capping, and endocytosis of Ig.

*Experiment 3 (Table I):* In this experiment we further studied the relationship between amounts of Ig and cap formation. The murine spleen cells ( $5 \times 10^6$ ) were exposed to non-labeled anti-Ig at concentrations of 1–100  $\mu$ g, as detailed above, washed, and then warmed for 20 min at which time they were returned to 4°C and then incubated with FITC-labeled anti-Ig. It was expected that if the anti-Ig were bound to all available mouse Ig then the complex would be interiorized and the subsequent reaction with FITC-labeled anti-Ig would be negative.

Cells incubated with 10  $\mu$ g or more of anti-Ig exhibited weak (or no) fluorescence. When present, fluorescence was localized at one pole of the cell. We concluded that at these doses the initial anti-Ig-Ig complex was all interiorized or was present in a small amount in one pole of the cell, and that no free Ig molecules remained on the rest of the surface. In contrast, cells exposed to the lower doses, which did not form a single agglutinate (Experiment 1), still exhibited Ig molecules over the entire surface.

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FIG. 6. Electron microscope radioautograph of a mouse spleen lymphocyte treated with  $^{125}$ I-labeled anti-Ig at 4°C. Note random distribution of grains on the cell surface.  $\times 13,500$ .

FIG. 7. Electron microscope radioautograph of a mouse spleen lymphocyte incubated with  $^{125}$ I-labeled anti-Ig first at 4°C for 30 min, then washed and incubated at 37°C (for 1 min). The label becomes located at one pole of the cell forming a cap.  $\times 17,500$ .

We have seen no inhibition of cap formation by large doses of anti-Ig. In another experiment the murine spleen cells were incubated with 10–400  $\mu\text{g}$  of anti-Ig in the cold, warmed, incubated in the cold with FITC-labeled anti-rabbit Ig, and examined immediately after washing. All cells showed concentrations of fluorescence in caps and not in rings. Cells not warmed before reacting with the fluorescent label showed fluorescence in rings.

*Experiment 4 (Table I):* The amount of anti-Ig molecules bound to the surface Ig of B lymphocytes was determined with the use of  $^{125}\text{I}$ -labeled antibody. Spleen cells were incubated with a solution consisting of 0.075  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled anti-Ig (4.0  $\mu\text{Ci}/\mu\text{g}$ ) plus increasing amounts of unlabeled anti-Ig for 30 min at 4°C, washed three times, and counted in a gamma ray spectrometer.

Part of the results are shown in Table I which summarizes this experiment plus the fluorescent antibody studies. It can be seen that at the dose of 1  $\mu\text{g}$  of anti-Ig about 8000 molecules were bound per B lymphocyte while at 10  $\mu\text{g}$  about 56,000 molecules were bound. Thus with 1  $\mu\text{g}$  the 8000 molecules of anti-Ig would obviously be incapable of reacting with all the  $10^5$  molecules of Ig thought to be present on each B cell.

*Effect of Cross-Linking.—*

*Experiment 5:* The effect of cross-linking of Ig by anti-Ig in the movement and interiorization phenomenon was investigated by using papain-treated anti-Ig by immunofluorescence. Spleen cells ( $5 \times 10^6$ ) were incubated with 5–10  $\mu\text{g}$  Fab' anti-Ig conjugated to FITC for 30 min at room temperature; a sample of cells was washed and examined and a second sample was treated with 50  $\mu\text{l}$  of sheep anti-rabbit Ig serum for  $\frac{1}{2}$  hr, then washed and examined.

Cells treated with Fab' anti-Ig exhibited fluorescence around the membrane and did not show evidence of capping or interiorization in vesicles. However, after reaction with anti-rabbit Ig most cells showed fluorescence concentrated in one pole, i.e., in typical caps.

*Endocytosis of Fab' Anti-Ig-Ig Complexes.—*

*Experiment 6 (Table II):* The fate of the complex of Fab' anti-rabbit Ig-anti-mouse Ig-mouse Ig after cap formation was studied. Spleen cells were first incubated at 4°C with Fab' anti-Ig, then with anti-rabbit Ig, washed, and finally placed in culture for  $1\frac{1}{2}$  hr. Cells were examined at various time intervals.

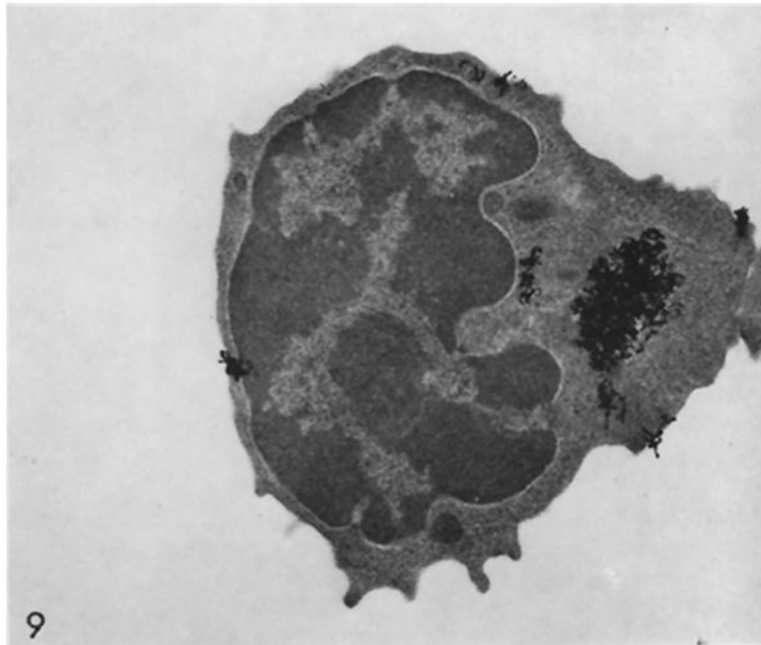
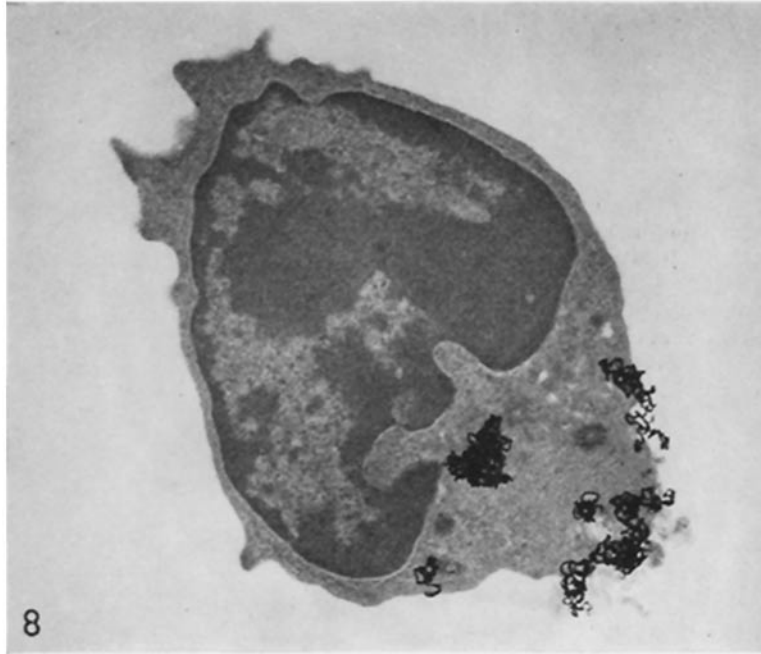
The results showed that endocytosis as evidenced by a beaded pattern of fluorescence developed at a much slower rate in caps formed by this trilayered

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FIG. 8. Electron microscope radioautograph of a mouse spleen lymphocyte incubated 10 min at 37°C with  $^{125}\text{I}$ -labeled anti-Ig. Label is exhibited both at the centrosphere pole, on the cell surface, and intracellularly.  $\times 10,500$ .

FIG. 9. Electron microscope radioautograph of a mouse spleen lymphocyte incubated with  $^{125}\text{I}$ -labeled anti-Ig for 10 min at 37°C as in Fig. 8. Note that most of the label is located intracellularly in the centrosphere area.  $\times 17,500$ .





complex. Table II shows the detailed results of this experiment as well as of a similar experiment (number 10) using ALG.

### ALG

#### *Quantitation of the Phenomena.—*

*Experiment 7:* We first investigated the fate of ALG on the lymphocyte membranes using fluorescent antibody. In these experiments the ALG was not labeled with FITC and it was detected only after its reaction with a labeled anti-rabbit Ig.

Rat spleen cells ( $10^7$ ) were incubated with 5, 10, 50, and 100  $\mu\text{g}$  of ALG at  $4^\circ\text{C}$ , washed, and divided into two samples. One was treated with FITC-labeled anti-rabbit Ig for  $\frac{1}{2}$  hr at  $4^\circ\text{C}$  and examined immediately; the second was warmed for 10 min–1 hr, then brought back to  $4^\circ\text{C}$ , exposed to the FITC-labeled anti-rabbit Ig, and examined. Thymus cells were also examined after incubation with 10 and 100  $\mu\text{g}$  of ALG, as above.

TABLE I  
*Effects of Anti-Ig on Membrane Ig of B Lymphocytes\**

Ig	Fluorescent anti-Ig		$^{125}\text{I}$ -labeled anti-Ig (Experiment 4)	
	No previous reaction with unlabeled anti-Ig (Experiment 1)	Previous reaction with unlabeled anti-Ig (Experiment 3)	% Binding	Molecules Ig per B cell
$\mu\text{g}$ to $5 \times 10^6$ cells				
0.1	Very weak; ring-type fluorescence	Positive in rings‡	Not done	Not done
0.24	Not done	Not done	0.54	1,600
1.0	Weak; few large clumps on surface	Less intense; rings	0.48	8,200
10.0	Strong positive; caps	39% reduction in number of positive cells; reaction in positive cells is weak and in cap	0.37	55,600
100.0	As above	67% reduction in number of positive cells; reaction in positive cells is circumscribed to weak spot in cap	0.11	192,700

\* This table summarizes experiments 1, 3, and 4.

‡ Spleen cells were used in all the three experiments; about 50% are positive with anti-Ig.

All lymphoid cells treated in the cold with ALG showed an interrupted ring-type pattern of fluorescence. Warming the cells at  $37^\circ\text{C}$  for up to 2 hr did not result in a significant change of pattern; clearly there was no capping or polarization of the fluorescent reaction.

*Experiment 8:* An ultrastructural study was carried out using ALG- $^{125}\text{I}$ . Rat spleen cells ( $10^7$ ) were incubated with 1–50  $\mu\text{g}$  of antibody and fixed after 30 min at  $4^\circ\text{C}$  or after incubation

at 37°C for 1, 2.5, 10, and 20 min, and 1 and 2 hr. (ALG was labeled at a specific activity of 13.1  $\mu\text{Ci}/\mu\text{g}$ .)

At 4°C the labeling of cells by ALG was similar to anti-Ig, i.e., a random distribution pattern of label over the total cell surface. When the temperature was raised to 37°C, in contrast to anti-Ig, the capping phenomenon, i.e. the agglutination of label on one zone of the surface, was not apparent at time periods of up to 2 hr (Fig. 10). In about 20% of the cells the label coalesced into larger aggregates on several areas of the membrane. Evidence of endocytosed ALG was not seen until at least 1 hr of incubation at 37°C. This endocytosis was always incomplete and in all cells some (i.e. 20–70% of grains)

TABLE II  
*Endocytosis of ALG or Anti-Ig after Cap Formation\**

Complex	Time of examination(% of cell showing fluorescence in vesicles)				
	10 min	30 min	40 min	1½hr	3 hr
Experiment 6					
Fab' anti-rabbit Ig anti-Ig-membrane	2–3	34–46	51–66	57–81	
Ig					
Experiment 10					
Anti-rabbit Ig-rabbit anti-Ig-membrane Ig	75–82	83–88	—	96–100	—
Anti-rabbit Ig-rabbit ALG-membrane antigen	4–11	8–9	—	17–23	15–16

\* Experiment is detailed in text. About 100 cells were counted in each slide. Figures indicate results obtained with doses of 5–10  $\mu\text{g}$  of rabbit anti-Ig, bivalent or monovalent, and 10–60  $\mu\text{g}$  of the ALG. No differences were noted with the different amounts used.

of the labeled ALG always remained on the cell membrane. The cytoplasmic vacuoles containing ALG were generally dispersed randomly in the cytoplasm and were not necessarily concentrated in the centrosphere area. The concentration of ALG, from 1 to 50  $\mu\text{g}/10^7$  cells, did not appreciably change this labeling pattern. Also, all spleen cells were labeled to the same extent and appeared to behave similarly.

*Effect of Cross-Linking with Anti-Rabbit Ig.*—We next investigated the effect of interlinking the ALG-membrane antigen complexes on their movement and endocytosis. These experiments were done both by immunofluorescence and ultrastructural radioautography.

*Experiment 9:* Both rat spleen and thymus cells ( $10^7$ ) were exposed to 10–50  $\mu\text{g}$  of ALG at 4°C for 30 min, washed, and then incubated also at 4°C with 5  $\mu\text{g}$  of FITC-labeled anti-rabbit Ig for another 30 min. After washing, the cells were examined immediately or after incubation at 37°C for up to 1 hr. Note that in the previous fluorescence experiment (number 7) the cells were warmed *before* the reaction with labeled anti-Ig; in this experiment we reacted the cells with ALG and the anti-rabbit Ig sequentially and then warmed the suspension.

All spleen and thymus cells examined immediately after reaction at 4°C showed typical ring reaction as described above. After incubation at 37°C the fluorescent reaction became rapidly concentrated in a typical cap in about 60–75% of all cells. Of great importance was the fact that the change in pattern of fluorescence from a cap to discrete small dots that was seen in the case of cells exposed to anti-Ig (see experiment 1) was not present with ALG. This suggested that the membrane complex was not being endocytosed.

*Endocytosis of the ALG-Antigen Complexes.—*

*Experiment 10:* This apparent lack of endocytosis of the ALG-anti-rabbit Ig complex was further tested in the same experiment but this time comparing cells treated with either anti-Ig or ALG and using the indirect method of detection, i.e., using FITC-labeled anti-rabbit Ig. Murine cells were used in these experiments. 10 million cells were exposed to from 10 to 60  $\mu$ g of either anti-Ig or ALG for  $\frac{1}{2}$  hr at 4°C, then washed and incubated with FITC-labeled anti-rabbit IgG for another  $\frac{1}{2}$  hr. After washing, the cells were placed in culture and examined at intervals of up to 3 hr.

This experiment is summarized in Table II. Note that 10 min after incubation at 37°C about 80% of cells exposed to anti-Ig exhibited fluorescence in small dots, indicating internalization of the immune complexes. Such cells had clearly shown the shift from the pattern of fluorescence of a cap to that representative of endocytosis. The ALG-treated cells had a different reaction in that cap formation was maintained without much evidence of endocytosis. After  $1\frac{1}{2}$  and 3 hr at 37°C the intensity of fluorescence clearly was decaying; in about 15–20% of the cells a few small fluorescent dots were discerned.

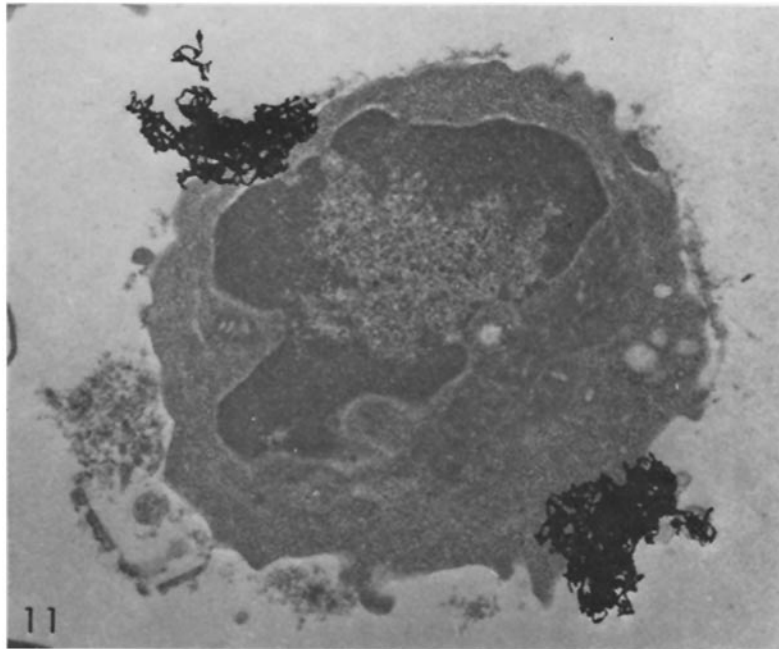
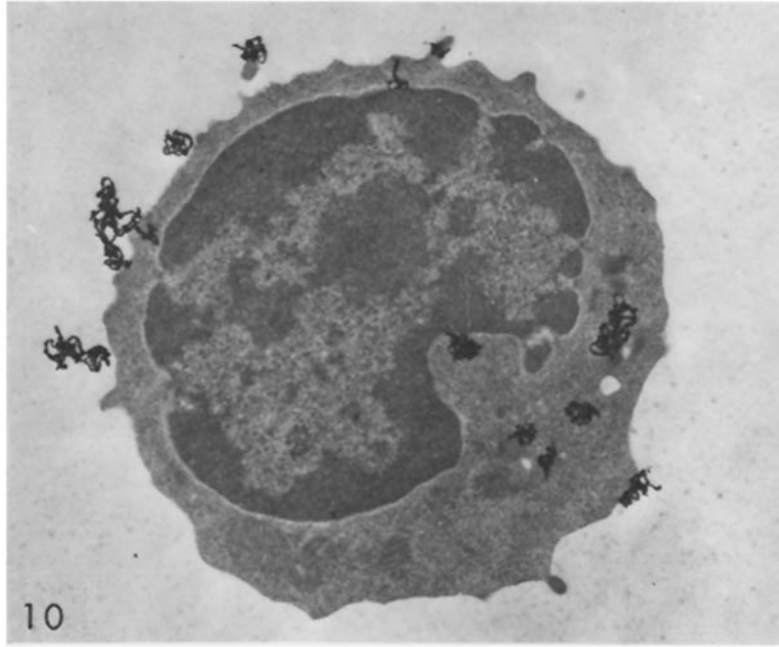
*Experiment 11:* Ultrastructural radioautography confirmed these observations.  $10^7$  rat spleen cells were incubated with 10  $\mu$ g of  $^{125}$ I-labeled ALG (specific activity of 15.5  $\mu$ Ci/ $\mu$ g) for  $\frac{1}{2}$  hr at 4°C, washed, and then incubated with 50  $\mu$ l of sheep anti-rabbit serum (not fractionated). The cells were washed after 30 min and examined after incubation at 37°C for 10 min and 1 hr.

The cells examined immediately after reaction in the cold showed grains randomly distributed over the membrane surface. After 10 min 26% of the labeled cells showed large aggregates of grains in two or three areas of the surface (Fig. 11) and 47% showed concentrations of grains in just one area, in the typical cap similar to that observed with anti-Ig. After 1 hr, 48 and 52% of cells had grains either in large patches or on a cap but there was no

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FIG. 10. Electron microscope radioautograph of a mouse spleen lymphocyte incubated with ALG- $^{125}$ I for 1 hr at 37°C. In contrast to  $^{125}$ I-labeled anti-Ig-treated cells (Fig. 7) the label does not become polar. Note also that the ALG- $^{125}$ I is not completely endocytosed.  $\times 17,500$ .

FIG. 11. Electron microscope radioautograph of a mouse spleen lymphocyte treated with ALG- $^{125}$ I at 4°C followed by sheep anti-rabbit IgG for 10 min at 37°C. Two large aggregates are noted on the cell surface.  $\times 13,500$ .



evidence of pronounced endocytosis. At 1 hr only 6% of the cells contained an occasional grain inside vesicles.

*Use of Fab' ALG.—*

*Experiment 12:* The fate of monovalent Fab' ALG was compared with that of the bivalent molecule. The studies with the monovalent Fab' were made only by ultrastructural radioautography using 60  $\mu\text{g}$  of Fab' ALG labeled with  $^{125}\text{I}$  at a specific activity of 21  $\mu\text{Ci}/\mu\text{g}$  and following similar protocols to those used in experiment 11. Cells were examined after reaction with Fab' ALG or Fab' ALG plus anti-rabbit Ig.

The Fab' ALG was located randomly over the cell membrane after the reaction at 4°C. When the cells were warmed to 37°C some endocytosis took place but as with the bivalent molecule the process of internalization was slow and not extensive. Thus, at 1 hr about 35% of lymphocytes showed some grains in vesicles. (In the previous experiment the number of cells exposed to the bivalent molecules and showing grains in vesicles varied from 34 to 42%.) Treatment of cells with anti-rabbit Ig after exposure to Fab' ALG changed the pattern of distribution. Now the label was present in a cap in  $\frac{1}{3}$  of cells and in several large aggregates in the remaining  $\frac{2}{3}$  both after 10 min and 1 hr at 37°C. Only 3% of the cells showed evidence of endocytosis after 1 hr at 37°C.

*Con A*

*Movement of Con A.—*The plant lectin Con A can bind to carbohydrates on cell surfaces. We now studied the pattern of distribution and the movement of Con A labeled with FITC or  $^{125}\text{I}$  on surfaces of lymphoid cells.

*Experiment 13:* Murine thymus, spleen, or spleen cells ( $10^7$ ) after anti- $\theta$  treatment were incubated with 2, 20, or 200  $\mu\text{g}$  FITC-labeled Con A at 4°C for 30 min, washed, and examined immediately or after warming at 37°C for up to 1 hr. ( $10^8$  spleen cells were incubated with 1 ml of anti- $\theta$  serum for 1 hr at 4°C, spun once, and then incubated in 2 ml of Hanks' medium containing 20% fresh guinea pig serum for 1 hr; the treated cells were then placed in a Ficoll-Hypaque gradient to separate off the dead cells.)

FITC-labeled Con A was present all over the membrane in the form of small discrete linear deposits at 4°C. All cells in thymus, spleen, or anti- $\theta$ -treated spleen treated with 20 and 200  $\mu\text{g}$  were strongly positive. About 5% of these cells treated with 2  $\mu\text{g}$  were negative, the remainder exhibiting weak positive reactions. After incubation of the cells at 37°C there was movement of the FITC-labeled Con A to one pole of the cell forming a typical cap. The cap formation was best seen in those cells incubated with 20 and 200  $\mu\text{g}$  of FITC-labeled Con A where more than 90% of cells regardless of their source exhibited the phenomenon; however, cells incubated with 2  $\mu\text{g}$  showed large clumps of fluorescence on the membrane and, in about  $\frac{1}{4}$ , typical caps.

*Experiment 14:* Con A was labeled with  $^{125}\text{I}$  to a specific activity of 33  $\mu\text{Ci}/\mu\text{g}$ . Spleen cells were incubated with 3  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled Con A for 30 min at 4°C, washed, resuspended

in medium, and incubated at 37°C for 1, 5, 10, and 60 min at which times the cells were fixed and examined by ultrastructural radioautography.

Before incubation at 37°C, the radioactive Con A was distributed equally over the entire cell surface. 1 min after warming the label appeared in several large patches in about 33% of the cells (Fig. 11); 5 and 10 min after warming the number of cells exhibiting large patchy aggregates on the membrane increased (42 and 43% at 5 and 10 min, respectively); about 12 and 6% of the cells examined at these times exhibited grains concentrated in a cap. Endocytosis of Con A was observed; at 5 min and 1 hr, 18 and 55% of the cells exhibited grains inside vesicles.

*Specificity of the Con A Binding.—*

*Experiment 15:* The specificity of the FITC-labeled Con A cell reaction was studied by determining whether this reaction could be inhibited by addition of appropriate carbohydrates in the medium. 5 million thymus cells were incubated with 20  $\mu$ g of FITC-labeled Con A in the presence of 0.1–0.5 M methyl  $\alpha$ -D-mannopyranoside for  $\frac{1}{2}$  hr at 4°C and then examined under the fluorescence microscope.

As would be expected, there was complete inhibition of the fluorescent reaction when FITC-labeled Con A was incubated in the presence of mannopyranoside.

*H-2 Antigens*

*Movement of Anti-H-2-H-2 Complexes.—*

*Experiment 16:* In the first experiments we tested the distribution and movement of these antibody-antigen complexes on thymus cells using both immunofluorescence and ultrastructural radioautography. This reaction was carried out only on thymus cells; in the spleen the B lymphocytes would react with the anti-mouse Ig. 10 million thymus cells were first incubated with 20–100  $\mu$ g of antibody in the cold, washed, and either maintained at 4°C or warmed at 37°C for 30 min. Whether or not the anti-H-2-H-2 antigen had moved or capped was determined by then examining the cells with FITC-labeled anti-Ig; i.e., the cells were incubated at 4°C with the FITC-labeled anti-Ig and examined immediately. For ultrastructural radioautography 10  $\mu$ g of the anti-H-2 antibody labeled at a specific activity of 4.03  $\mu$ Ci/ $\mu$ g was incubated with  $10^7$  spleen cells first in the cold and then warmed for 1 and 10 min and 2 hr.

By fluorescence microscopy immediately after their reaction with anti-H-2 antibodies in the cold, the thymus cells exhibited well-isolated discrete small patches all over the surface. The concentration of patches varied: in 92% of the cells they were few, while the remaining 8% exhibited a large number, more than 20–25. This pattern did not change in cells examined  $\frac{1}{2}$  hr after warming. (Note that cells were incubated in the warm after binding with anti-H-2, then cooled and tested with FITC-labeled anti-Ig.)

Ultrastructural radioautography clarified some of the aspects of the anti-H-2-H-2 antigen reaction. Most of the cells exhibited few isolated grains over the surface before incubation at 37°C. The degree of labeling was much lighter

than with the other three antigens used previously. This distribution changed after warming in that many of the grains coalesced into small aggregates over the entire surface. No formation of a large single aggregate, i.e. a cap, was observed. With time some endocytosis of the anti-*H-2-H-2* complex was noted although this was not extensive. For example, after 2 hr of incubation 53% of the cells had grains exclusively on the membranes; 20% contained, besides grains on the membrane, an occasional grain in vesicles; 27% of cells showed grains only in vesicles.

*Experiment 17:* Will anti-*H-2* antibodies-*H-2* antigens cap if sandwiched with a second antibody and then warmed? We tried to answer this question using immunofluorescence and ultrastructural radioautography. Thus  $10^7$  thymus cells were reacted with 50  $\mu\text{g}$  of anti-*H-2* antibody and then with 10  $\mu\text{g}$  of FITC-labeled anti-Ig, both reactions being done sequentially and at 4°C; the cells were then resuspended in medium and cultured for up to 3 hr with frequent samples being examined under the fluorescent microscope. For electron microscopy  $10^7$  cells were incubated at 4°C first with 50  $\mu\text{g}$  of anti-*H-2*, followed by 25  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled anti-Ig (13  $\mu\text{Ci}/\mu\text{g}$ ), washed, and incubated at 37°C for up to 2 hr.

10 min after warming only 1.2% of cells showed a typical cap; the remaining cells showed large isolated patches over the membrane. (It should be remembered that by this time all B cells that were treated with anti-Ig or T and B cells that were treated with ALG plus anti-rabbit Ig had formed typical caps.) The number of cells with caps increased slowly with time of incubation: 7.3% after  $\frac{1}{2}$  hr, 7.6% after  $1\frac{1}{2}$  hr, and 12.7% after 3 hr. By  $1\frac{1}{2}$  and 3 hr the intensity of the reaction was clearly decaying, suggesting that some of the complex had dissociated from the cell surface. In a further experiment, thymic cells treated with 50  $\mu\text{g}$  of anti-*H-2* were incubated with monovalent anti-Ig and examined after incubation at 37°C for up to 1 hr. After reaction at 4°C before warming, the cells exhibited a discrete linear interrupted pattern: only 20% of the cells were positive after sandwiching with our monovalent antibody. No capping or large aggregation occurred after incubation at 37°C. Comparable results were obtained by ultrastructural radioautography; i.e., few large aggregates became apparent on the membrane and in none of the cells was a single large cap observed. Endocytosis of the complex was evident although this process was not extensive: in most cells, grains were observed both inside and on the membrane.

#### DISCUSSION

Our experiments confirm that Ig molecules on the membrane of B lymphocytes upon reaction with antibody and at temperatures greater than 4°C can first move on the surface and then rapidly become interiorized in vesicles. The present experiments have quantitated this reaction with Ig-anti-Ig and have examined the phenomenon of surface movement using three other molecules. The main results of our studies are summarized in Table III.

The movement of molecules on lymphocyte membranes was ascertained by



determining whether cap formation or several heavy aggregates could form on the membrane after reaction with one or more ligands. There may be several factors involved in this membrane movement and only some will be analyzed now. First, besides temperature, the movement depends upon the interaction with one or more ligands. Clearly, the movement of molecules to form a cap

TABLE III  
*Summary of Experimental Results*

	Direct reaction	Indirect (or sandwich) reaction
Ig	Rabbit anti-mouse Ig-membrane Ig (a) Rapid capping followed by endocytosis (b) Capping depends upon linking of all Ig molecules: critical amounts of antibody and bivalency of antibody molecules are necessary (experiments 1-5)	Anti-rabbit Ig-rabbit anti-Ig-membrane Ig (a) Rapid capping followed by endocytosis (b) Slow endocytosis if monovalent anti-Ig used (experiment 6)
ALG	Rabbit ALG-membrane antigen (a) No capping (experiments 7 and 8) (b) Some endocytosis takes place with bivalent and monovalent ALG (experiment 8)	Anti-rabbit Ig-ALG-membrane antigen (a) Capping takes place but there is no endocytosis (experiments 9-12)
Con A	Con A-surface carbohydrate (a) Capping, endocytosis (experiments 13 and 14)	
Anti- <i>H-2</i>	Anti- <i>H-2</i> -membrane <i>H-2</i> (a) No capping; limited endocytosis (experiment 16)	Anti-mouse Ig-anti- <i>H-2</i> -membrane <i>H-2</i> (a) Capping in less than 15% of cells and only after long periods of time; endocytosis is more intense (experiment 17)

may take place directly after reaction with a single ligand, or after a second reaction, ligand to ligand-antigen reaction, or may not take place in an obvious manner. Both the receptors that bind to anti-Ig and Con A rapidly moved and formed caps, while in the case of the antigens recognized by ALG the movement took place only after sandwiching the reaction with a second antibody. (The reasons why Yahara and Edelman did not find movement of Con A receptors, in contrast to our studies, are not readily apparent [23].) The movement of immune complexes involving *H-2* antigens was not as evident as with the other three molecules. The fact that after a sandwich reaction large aggre-

gates were formed in most cells, and caps in a small number, indicates that movement of the *H-2* complexes did in fact take place. Perhaps the spacing of *H-2* determinants on the surface and/or their degree of anchoring in the lipid bilayer limit the rapid movement and formation of caps. Frye and Edidin were the first to show displacement of *H-2* determinants in experiments involving heterokaryons (18). In their studies cap formation was not seen and the displacement of *H-2* antigens was much slower relative to the displacement of antigens recognized by a heterologous antibody. Kourilsky et al. found aggregation of immune complexes formed with *HL-A* antigens and limited formation of caps (24). Our findings agree in general terms with these published reports. The reasons for these differences in surface movement among the four molecules studied may now become more apparent when we analyze their distribution on the membrane in our next paper; in essence these differences appear to depend upon the amount and proximity of each membrane moiety and perhaps upon their degree of anchoring in the cell membrane. Ig and Con A receptors are closely associated and easily linked by a single ligand, but ALG antigens are farther apart from each other and need two ligands to interlink; *H-2* determinants are separated from each other by large empty areas.

A second factor which certainly determines the fate of the membrane complex is the amount of ligand. A dose of anti-Ig capable of binding all available surface Ig molecules did agglutinate all Ig molecules in a single mass and left the membrane devoid of free Ig molecules; however, an amount of antibody that binds to only a few molecules resulted in several large agglutinates on the membrane and in many free molecules left *in situ*. No cap formation occurred with these limiting doses of anti-Ig. These results imply that in the case of Ig moieties these molecules are not all interconnected but are free from each other and that only after all are interlinked by antibody do they form a lattice which can be pulled into a single mass at one zone of the cell membrane. The results with monovalent anti-Ig support this statement.

The results of endocytosis of the different membrane complexes, although puzzling, may give some indication of some of the mechanisms underlying the internalization process. First it was apparent that not all complexes agglutinated in a cap or in several large aggregates suffered interiorization: Ig and Con A receptors were rapidly interiorized but the complex of antibody on ALG-membrane antigen was not, and moreover the complex of antibody on Fab anti-Ig-membrane Ig was interiorized in a limited way. Secondly, it was evident that with some complexes like those formed with ALG endocytosis took place slowly and to a limited extent and independent of cross-linking of antigenic determinants.

We explain our results by postulating that two forms of endocytosis take place in lymphocytes: a rapid and a slow one. In the first one, the process is immediate and requires that a ligand bind (*a*) to two molecules in close approximation and (*b*) at a site in the receptor molecule relatively close to its

point of attachment to the membrane. These two conditions perhaps lead to a conformational change on the surface that then initiates the internalization process. This would explain why anti-Ig-Ig is immediately interiorized but not anti-Ig sandwiched on ALG-membrane antigen; the latter membrane antigens are in fact held farther apart from each other. It would also explain the limited and slower endocytosis of the anti-rabbit Ig sandwiched on monovalent anti-Ig. The anti-rabbit Ig which serves as the ligand attaches to sites on the Fab molecule which are quite separate from the membrane itself. Finally, the second form of endocytosis which does not require cross-linking and which takes place in a slower fashion may be explained simply as a form of slow membrane turnover where certain zones, perhaps at random, are taken into the cells. This would imply that lymphocytes are slowly interiorizing portions of their membranes continuously.

There is little information available on the fate of different complexes on lymphocytes because, among several reasons, it is difficult to establish endocytosis unless ultrastructural techniques such as the ones used here are applied. Indeed, the fluorescent antibody method may not be the method of choice to study endocytosis of all complexes. For example, in the case of Ig or ALG we could determine with a reasonable degree of confidence when endocytosis was taking place: the distribution of the capped complexes on the surface was in a curved linear pattern, and when the complexes were inside cells, discrete dots were discernible, clearly distinguishable from the cap. These observations were in fact corroborated by the ultrastructural analysis. However, in the case of *H-2* complexes it was difficult, if not impossible, to determine by fluorescence whether they were in or on the cell: the *H-2* antigens on the cell membranes are distributed in small roundish well-separated areas. The main experiments in the literature that suggest endocytosis of complexes by lymphocytes are those of antigenic modulation (25). In this phenomenon an antigenic determinant is apparently lost from the membrane of a cell as a result of reaction with antibody at 37°C. (In this reaction cells are first exposed to antibody without complement for a certain period of time, and then subsequently tested for modulation by determining whether they lyse after reaction with antibody and complement.) Modulation took place upon reaction of thymus leukemia (TL) antigens with both bivalent and monovalent antibodies (26) and did not take place with *H-2* antigens. Anti-Ig antibodies readily modulated those cells carrying Ig on their surface (27). All these results of modulation, if indeed explained by endocytosis, would in general terms agree with our present series of studies.

We do not believe that the differences in movement and endocytosis among the different molecules depend upon the kind of antibody that was used or on the type of lymphocyte. Except for *H-2* (or Con A) the antibodies that were used were all rabbit Ig obtained late after immunization. Insofar as the type of lymphocytes is concerned, clearly both T and B demonstrated the

phenomenon; for example, the behavior to ALG and Con A was the same in cells obtained from thymus or spleen. Finally, it should be noted that movement and cap formation was not preconditioned to a rapid change in temperature (i.e., 4°C → 37°C). The experiment with monovalent anti-Ig (number 5) was done at 37°C in order to avoid any rapid shift in environmental temperature; here movement and capping readily took place after reaction with a second antibody.

The significance of the movement of molecules with regard to the triggering of lymphocytes is not altogether clear. Certainly this process per se is not sufficient to trigger B lymphocytes to detectable levels of activity in the absence of T cell function nor is it a process that inactivates the cell. It has been shown that B lymphocytes primed to an antigen (among spleen cells) do not form antibody after movement of their Ig receptors by anti-Ig unless there is a subsequent challenge with antigen; in this situation antigen at least could be stimulating T cell activity.<sup>2</sup> In the present series of results it was clear that Con A receptors of B cells could move and be endocytosed, yet such B cells alone do not proliferate upon Con A stimulation (28). T lymphocytes have been shown to proliferate upon interaction with phytohemagglutinin conjugated to large beads, a situation that perhaps may not lead to movement and endocytosis of all the phytohemagglutinin receptors (29). A situation akin to this last one apparently takes place when lymphocytes are triggered by antigen molecules fixed to the membrane of macrophages (30).

#### SUMMARY

The fate of different complexes on the membrane of thymocytes and spleen lymphocytes was studied with the use of both immunofluorescence and ultrastructural radioautography. The complexes of anti-immunoglobulin (Ig) with the surface Ig of B lymphocytes were present all around the membrane at 4°C; an increase in temperature produced a rapid aggregation of the complex into a cap which was readily interiorized in vesicles. Ultrastructural details of this process were given. The movement of the complexes depended upon the amount of anti-Ig and the temperature.

The complexes of anti-lymphocyte antibody with surface antigen(s) did not result in formation of a single large aggregate (or cap) unless an anti-antibody was brought into the reaction. The caps formed by this trilayered complex were not interiorized. Concanavalin A (Con A) bound to cell surface carbohydrate moieties and the complexes of Con A readily formed a cap and were interiorized. Finally, antibodies to *H-2* determinants did not form in most instances a single cap aggregate even when anti-antibodies were used. With time the *H-2* complexes tended to form several large aggregates with some endocytosis.

<sup>2</sup> Katz, David H., and Emil R. Unanue. 1972. The immune capacity of lymphocytes after cross-linking of surface immunoglobulin receptors by antibody. *J. Immunol.* In press.

*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.

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