LIGAND-INDUCED MOVEMENT OF LYMPHOCYTE SURFACE MACROMOLECULES

IV. Stimulation of Cell Motility by Anti-Ig and Lack of Relationship to Capping*

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Some of the early events transpiring in the lymphocyte upon combination of a ligand—usually an antiimmunoglobulin (Ig)—with its antigen receptor have been disclosed recently. One striking feature is the rapid change in the initial surface distribution of the ligand-receptor complex which, in most species, is readily agglutinated into one small area of the cell termed the cap (1–3). Other events which take place simultaneously with or following capping include endocytosis of the ligand-receptor complex (3) with subsequent catabolism (4, 5), the shedding of some of it into the extracellular milieu (4–6), and/or its persistence at the surface for finite periods of time (6). The mechanisms involved in the redistribution of complexes into caps, as well as in the other events described above, are not totally understood. On the basis of experiments using metabolic inhibitors or variations in temperature, it became evident that capping required a metabolically active cell (1, 2, 7). Capping of anti-Ig-Ig complexes did not proceed at 4°C, nor at 37°C in the presence of inhibitors of glycolysis or of oxidative phosphorylation. Similar results were obtained when studying histocompatibility molecules on lymphocytes (8) or fibroblasts (9). On the basis of these results, it was speculated that capping resulted from cell movement (1, 3, 9). Either the heavy lattice of complexes was dragged towards the posterior end as the cell moved, or alternatively, the cell simply crawled out of the lattice of complexes "somewhat like a snail emerging from its shell" (6). Indeed, we obtained morphological evidence of some lymphocytes in the process of movement while capping anti-Ig-Ig complexes (3). Along these lines, concanavalin A has been found to be localized to the end of motile lymphocytes (10.) The relationship between movement and capping became blurred when it was found that cytochalasin B, a drug that presumably stops cell motion, did not affect capping (7). [High doses of cytochalasin B did result in decreased capping (1)]. Nevertheless, it has never been tested directly whether exposure to anti-Ig stimulated the B lymphocyte to move, and, if so, whether cytochalasin B affected this process.

The present series of experiments were undertaken to investigate two points: (*a*) whether lymphocytes were stimulated to move by ligand-receptor com-

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plexing; and (*b*) the relationship between capping of the surface complexes and cell movement. We conclude from the series of experiments described herein that anti-Ig stimulates random motion of lymphocytes but that capping proceeds in the absence of overt cell motion.

MATERIALS AND METHODS

Preparation of Cells.—Cells were teased from the spleen or thymus of A/St mice (West Seneca Laboratories, Buffalo, N. Y.) into Hanks's balanced salt solution (BSS)[1] with 10 mM HEPES and 1% fetal calf serum (FCS) (Microbiological Associates, Bethesda, Md.). This was done either at 2–4°C, on ice, or at room temperature. In all experiments, the cells were centrifuged on a Ficoll-Hypaque gradient in order to eliminate dead cells, red blood cells, and debris. All cell preparations contained approximately 90% small lymphocytes, more than 98% of which were viable as evidenced by Trypan blue dye exclusion test.

Antiserum.—The antiserum was a rabbit anti-mouse Ig employed in previous experiments (3, 6, 7). Its IgG fraction, isolated by diethylaminoethyl (DEAE) column chromotography, contained antibodies to all mouse Fc classes and to Fab determinants. In some experiments we used a fluorescent conjugate with a fluorescein-to-protein molar ratio of 6:8. Absorption of the fluorescent conjugate with insolubilized Ig removed all the surface Ig staining of B cells (3). A monovalent fragment of the IgG was used in some experiments. Its preparation was described previously (3).

Testing for Movement in Lymphocytes.—The rationale is discussed in the Result section. Movement was studied by microscopical examination of cells mounted on a glass slide and sealed with a cover slip. The microscope stage was kept at 37°C by using an air-stream stage incubator (Nicholson Precision Instruments, Inc., Bethesda, Md.). Two procedures were followed in order to score the number of lymphocytes exhibiting motility at a given time: in procedure A, 5 to 10×10^6 lymphocytes were incubated with the appropriate antibodies in a volume of about 100 μl at 4°C for 30 min, washed, and then resuspended in ½ ml of media (BSS with HEPES and 1% FCS); the suspension was placed on a 2.0 cm diameter well of a plastic culture dish (Multi Dish Disposo-Tray, Linbro Chemical Company, Inc., New Haven, Conn.) sitting on an ice bucket and the cells allowed to settle for about 20 min. The tray was then taken to a 37°C water bath for varying lengths of time. An equal volume of either 5% glutaraldehyde or 2% paraformaldehyde was added to terminate the reaction. In procedure B, ½ ml of media containing 5 to 10×10^6 cells were harvested and processed at room temperature, placed on the plastic dish, and allowed to settle for 10 min; the reagents were added, and the tray then incubated at 37°C for various times as in procedure A.

After fixation the cells were washed with distilled water and then mounted between slide and cover slip and examined microscopically at × 400 either the same or the following day. The cells were scored for percentage exhibiting morphology typical of motile lymphocytes (see Results). Small lymphocytes that showed a significant deviation from a round shape were counted as positive. In the vast majority of cases such cells showed a definite projection from one side of the cell that resulted in the typical "hand-mirror" shape, in some cases the cells showed only a pronounced oval shape (Figs. 1–7). 300 to 500 cells were routinely counted. Most experiments report the means from duplicate samples. The deviation in counts over and under the mean did not exceed 15%. In many experiments the procedures were done using fluorescent-tagged anti-Ig. The cells were scored for motile forms as well as for the fluorescent surface reaction.

Immunofluorescence.—Lymphocytes, either fresh or after fixation in paraformaldehyde,

[1] *Abbreviations used in this paper:* BSS, Hanks's balanced salt solution; DFP, Diisopropyl fluorophosphate; DNP, dinitrophenol; FCS, fetal calf serum; and FITC, fluorescein isothiocyanate.

were incubated with 20 μg of fluorescent anti-Ig at 4°C for ½ h, washed, and examined under the ultraviolet microscope.

Microscopy.—We used a Zeiss microscope (Carl Zeiss, Inc., New York). For immunofluorescence a fluorescein isothiocyanate (FITC) interference filter (Optisk Lab, Dyngby, Denmark) was employed.

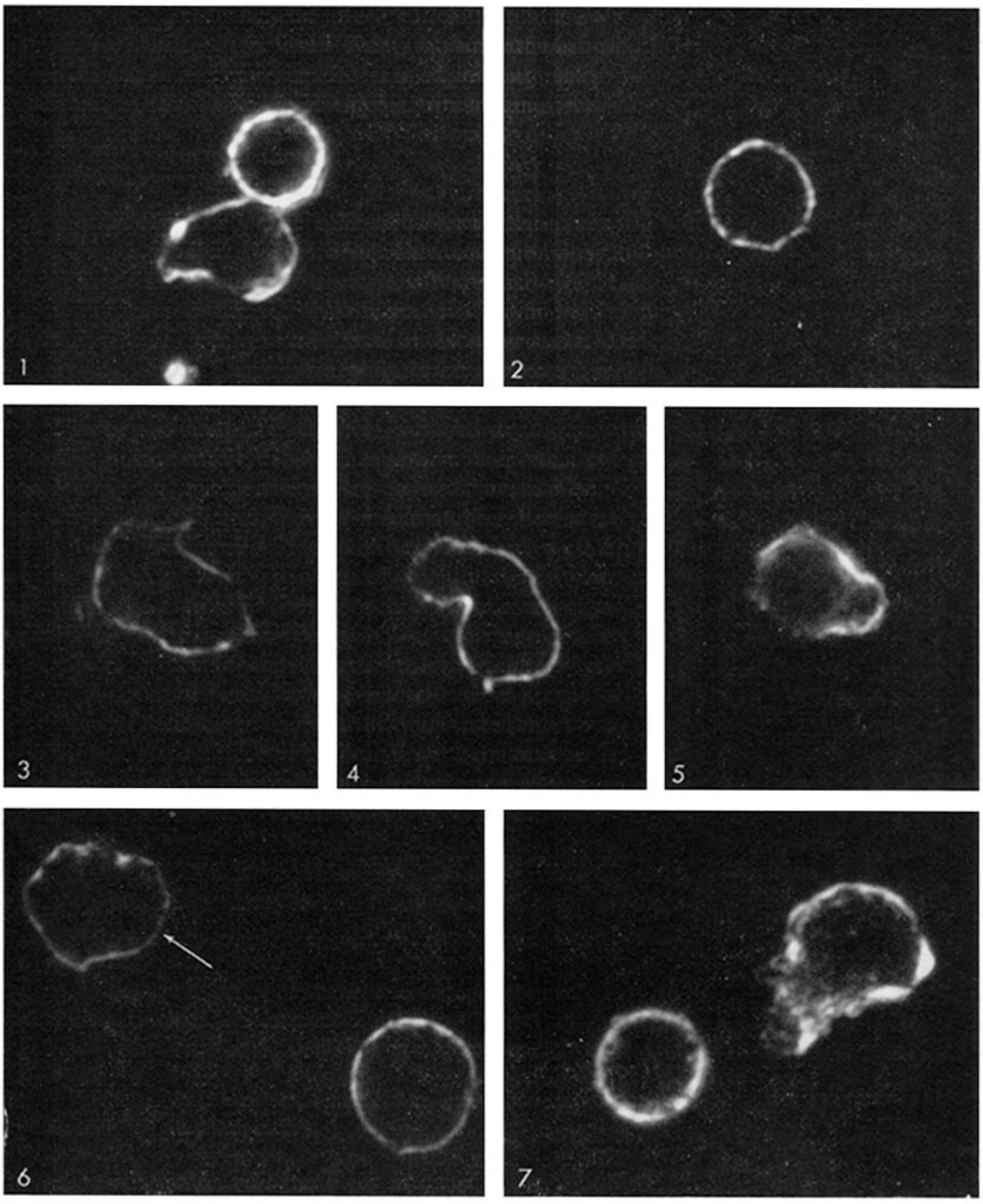
Miscellaneous Reagents.—Cytochalasin B was from ICI Research Laboratories, Cheshire, England; it was dissolved in dimethyl sulfoxide to a concentration of 1 mg/ml.

2-deoxyglucose and diisopropyl fluorophosphate (DFP) were from Aldrich Chemical Co., Inc. Milwaukee, Wis. DFP was diluted in BSS immediately before use.

Dinitrophenol (DNP), oligomycin, and iodoacetamide were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Methodology.—It was our purpose to relate movement of lymphocytes to the immunological reaction occurring at their membrane. Movement of lymphocytes was studied by continuous microscopic observations on preparations of cells mounted on slides and kept at 37°C. Since this type of examination was tedious and did not lend itself to exploring and quantitating a series of experimental variables, we also resorted to the different procedures outlined in the previous section. It is well-known since the early studies of Lewis in 1931 (11), and Rich, Wintrobe, and Lewis in 1936 (12), that lymphocytes in the process of movement exhibit a series of typical changes in morphology. These changes consist of simple elongation of the cell, or more typically, the hand-mirror or pear shape in which the lymphocyte has a round part containing the nucleus and an elongated strip of cytoplasm usually at the posterior end. This posterior end of the cell, on occasion, has a series of fingerlike projections termed uropod by McFarland (13). McFarland, as well as others (14, 15), noted the appearance of these pear-shaped cells in cultures. Since one could relate the dynamic state of the cell, that is, whether it was moving or not, to its morphology, we employed the following technique. Lymphocytes that had settled on the surface of a culture dish were incubated at 37°C for various periods of time and then fixed by addition of an equal volume of 5% glutaraldehyde or 2% paraformaldehyde; the cells were washed and examined microscopically. Lymphocytes in the process of local or translational movement exhibited a series of distinct shapes depicted in Figs. 1–7, easily distinguished from the round, stationary lymphocytes. Hence, we were separating the lymphocytes on the basis of their morphology at a given time, into those with forms corresponding to cells in movement and those corresponding to stationary cells. Our purpose, then, was to determine if our experimental variable changed the number of lymphocytes in a dynamic state; obviously, any variation in rate or extent of movement of a given cell would not be detected. Throughout this paper, we will use the term "moving" or "mobile" lymphocytes to indicate those cells exhibiting morphological change typical of those cells in motion and depicted in Figs. 1–7. We realize that such an indirect assay of movement may lead to an underassessment of the situation but for practical reasons this approach was adopted.



FIGS. 1–7. Dark-field photomicrographs of spleen lymphocytes after exposure to anti-Ig (procedure A). The motile lymphocytes can be easily distinguished (Figs. 1, 3, 4, 5, cell with arrow in Fig. 6, and 7). Fig. 2 shows the typical stationary lymphocytes × 360.

Lymphocytes with Spontaneous Movement.—Microscopic examination of murine spleen lymphocytes disclosed a few with random translational movement. These few lymphocytes moved usually by first sending out a small cytoplasmic projection after which the nucleus followed, leaving a posterior tail of cytoplasm. This whole process took about 3–5 min under our experimental conditions. On occasion, the lymphocytes sent out a long, cytoplasmic projection that later (i.e., from 1 to 5 min), returned to the main body of the cell. In these cases, the cell did not change to a great extent from its original position and evidently did not translate itself to a different area of the slide. In essence, the changes in morphology of the cells related to either true translation, in most instances, or less frequently to local cytoplasmic movement. Both procedures A and B would not discriminate between the two forms. Normal spleen populations were studied by these two procedures. Lymphocytes were examined after settling on dishes at 4°C, and following incubation at 37°C for various lengths of time (procedure A). Moving lymphocytes were not seen in cells examined after 4°C but up to 10% could be detected after warming to 37°C. The number of spontaneously moving lymphocytes was not significantly influenced by the length of time at 37°C as can be seen in one of the groups depicted in Fig. 8. The number of moving lymphocytes from cell suspensions harvested at room temperature, never exposed to cold temperatures, and cultured on dishes at 37°C (procedure B) was increased, occasionally to as high as 15%. In general, lymphocytes that were cooled first on ice seemed to be able to move less well on warming. We were interested in determining whether the spontaneous moving lymphocytes were of the B or T class. Cells incubated on a culture dish at 37°C were fixed in paraformaldehyde, washed, stained with fluores-

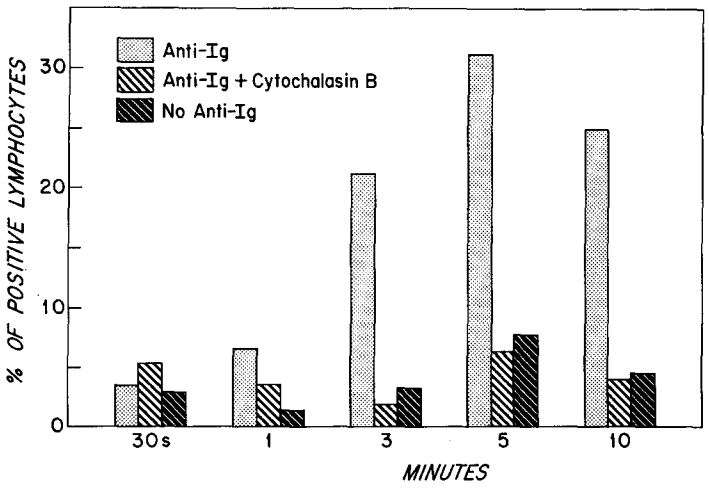


FIG. 8. In this experiment lymphocytes were incubated with anti-Ig at 4°C, washed, placed in a culture dish, and then incubated at 37°C for the designated periods of time. One group of lymphocytes was not exposed to anti-Ig.

cent anti-Ig, and examined under the fluorescent microscope, taking into consideration both the presence or absence of surface Ig as well as their shape. Of the total moving lymphocytes (4.5% in this experiment), 72 and 28% were of the T or B class, respectively (the classification into T or B cells is based on the absence or presence of surface Ig). Thus, the majority of cells exhibiting spontaneous moving forms belonged to the T class (Table I). (These results agree with the study of Rosenstreich et al. who found that most lymphocytes in the guinea pig exhibiting spontaneous "uropods" were T cells [14].) The spontaneously moving B cells exhibited surface Ig cells throughout its surface, an

TABLE I

Class of Lymphocytes with Spontaneous or Anti-Ig Induced Movement

	No anti-Ig	Anti-Ig	
		Exp. 1	Exp. 2
Motile Forms, %	4.5	16.4	13.0
B Cells: % moving	2.7	30.9	27.3
T Cells: % moving	5.6	8.5	5.8
Moving lymphocytes			
B Cells, %	27.7	67.0	69.7
T Cells, %	72.3	33.0	40.3
Ratio B:T	0.4	2.0	1.7

This experiment combines results of immunofluorescence and cell morphology. Untreated lymphocytes were incubated at 37°C for 5 min, fixed, and then stained with fluorescent anti-Ig. In experiments 1 and 2, lymphocytes were incubated with fluorescent-labeled anti-Ig (20 μg) at 4°C, washed, and then incubated at 37°C for 5 min.

indication that in the moving cells there was no concentration of uncomplexed surface Ig towards its posterior end.

Effect of Anti-Ig.—Murine splenic lymphocytes (10^7) were incubated with FITC anti-Ig (100 μg) at 4°C for 30 min, washed, and resuspended in culture media. Portions of the cell suspension were placed between slide and cover slip and examined microscopically, the microscope stage being maintained at 37°C with the aid of the air curtain apparatus. An alternative procedure was followed consisting of microscopic examination of the labeled cells following an initial brief incubation at 37°C on a culture tray. The cell suspension was placed on the culture dish, allowed to settle—on ice—and incubated 1–3 min at 37°C and then examined microscopically. There were marked changes in morphology in the cells that exhibited membrane fluorescence. The following patterns were observed. Some cells projected broad cytoplasmic extensions to which the nucleus appeared to follow leaving a long prolongation of the cytoplasm in its posterior end; this cytoplasmic tail eventually flowed back into the whole body of the cell. The whole process took about 1–5 min, and led to a definite translation (i.e., a gain of a few microns). At the time these changes were

developing, capping of the anti-Ig-Ig complexes was taking place. The cap was situated towards the posterior end of the cell. Such an organized pattern of motility resulting in translation was not observed in all cells. Many exhibited cytoplasmic extensions to one side or the other of the cap zone; these extensions eventually returned to the body of the cells. Other cells exhibited a pronounced oval shape at the time of capping, returning to a round shape in a few minutes but not changing definitely from their original position. Some cells exhibited a cycle of translation, then changed direction, exhibiting cytoplasmic extensions to one side. This whole series of changes took place within 15 min at the end of which most cells had capped the complexes and apparently stopped moving. The best way to observe these changes was by allowing the process to start in the culture dishes and then mounting and examining the cells. Since the whole process described above took place fairly rapidly, it was not possible to estimate the number of Ig-positive cells that exhibited the changes in motion. For this reason, we employed procedures A and B in which the reaction was stopped with strong fixatives and which permitted us to obtain an estimate of the percent of active cells at a given time. It was evident, however, from the microscopic examination of freshs cell that many cells did cap without having any overt change in their morphology. From here on we describe results using one of the two procedures.

There was a clear increase in the number of moving lymphocytes when incubated with anti-Ig by either procedure A or B. The parameters of this anti-Ig stimulated movement were investigated in several experiments.

Fig. 8 includes a time sequence study using procedure A (which consists of incubating the cells at 4°C with anti-Ig followed by washings and then incubation at 37°C). The increase in number of motile forms is best seen after 5 min. Table II shows the results of one experiment using procedure B (continuous exposure to anti-Ig) either at 21 or 37°C. At both temperatures the increase in motile forms is noted after 10 min and a decrease after 1 h. We set up experiments trying to explain the decrease in motile forms after 1 h. At this time the surface of the lymphocytes is clean of receptor Ig—all surface Ig that complexes with anti-Ig is interiorized within 10 min (3, 6, 7). Lymphocytes (2×10^7) were exposed to anti-Ig (200 μg) at 4°C for ½ h, washed, and incubated in suspension for 1 h at 37°C; the aliquots of cells (5×10^6) were then placed on the culture tray and incubated with 50 μg of anti-Ig for 10 min. A control set included lymphocytes not pulsed initially with anti-Ig. The net number of motile cells in cells not pulsed initially with anti-Ig was 18.6%; the number of motile forms in cells pulsed initially with anti-Ig was 2.3%. Hence, the experiment suggested that the presence of surface Ig was necessary for the phenomenon to take place.

Table II also shows the effects of various amounts of anti-Ig and of normal rabbit Ig on the number of moving lymphocytes. Note that 10 μg of anti-Ig

TABLE II

Effect of Anti-Ig on Splenic Cells

Stimulant	Dose	Time of incubation	Temperature	Procedure	Mean number of motile forms
	μg	(*min*)	−°C		%
None		10	21	B	4.9
None		20	21	B	7.2
None		60	21	B	6.1
Anti-Ig	50	10	21	B	15.1
Anti-Ig	50	20	21	B	12.4
Anti-Ig	50	60	21	B	8.7
None		10	37	B	10.5
None		20	37	B	10.3
None		60	37	B	4.0
Anti-Ig	50	10	37	B	22.5
Anti-Ig	50	20	37	B	22.7
Anti-Ig	50	60	37	B	9.7
None		5	37	A	4.2
Anti-Ig	1	5	37	A	15.9
Anti-Ig	10	5	37	A	22.7
Anti-Ig	50	5	37	A	22.6
Anti-Ig	100	5	37	A	22.5
None		10	37	B	12.3
Normal rabbit Ig	100	10	37	B	9.4
Anti-Ig	100	10	37	B	21.7

The figure for percent of motile forms represents mean of two determinations. Anti-Ig refers to the IgG of a rabbit anti-mouse Ig serum.

increased the number of moving cells about fivefold (from 4.2 to 22.7%). Normal rabbit Ig had no effect.

In order to determine the class of lymphocytes involved in the anti-Ig triggered movement, the cells were incubated with FITC anti-Ig and studied as in procedure A, assaying microscopically for cells bearing surface Ig or not and showing morphology of moving lymphocytes. In one of the experiments we counted 16% of moving lymphocytes of which 67% showed surface Ig; it was calculated that 31% and 9% of all Ig positive (B) and Ig negative (T) cells, respectively, were motile. The exact data of this and a repeat experiment as well as on the experiment with spontaneous moving is shown in Table II. It is apparent that B cells account for the vast majority of lymphocytes stimulated to move by anti-Ig. It should be noted that no more than ⅓ of B cells moved after stimulation, regardless of dose (Table II); perhaps the remainder were moving but were fixed at a time when the did not exhibit the typical morphology; or perhaps only a certain percentage of the B cell population was able to be stimulated for movement.

There was no increase in the number of motile cells after reaction of thymus cells with anti-Ig antibody (Table III).

An Fab fragment of the rabbit anti-Ig did not increase the number of moving spleen lymphocytes. Using procedure A, the number of spontaneously moving cells was 6.2%; after incubated with 10 μg of Fab anti-Ig the figure of moving cells was 7.1%; the same amount of the bivalent anti-Ig increased this figure to 22.7%. The same results were obtained by procedure B. Cells were incubated for 10 min at 37°C with 50 μg of anti-Ig and 30 or 120 μg of its Fab fragment. The number of spontaneous moving forms was 12.6%; the bivalent anti-Ig increased this figure to 27.1%. The Fab fragment did not result in any change from the controls (13.7% with 30 μg; 14.4% with 120 μg). We concluded that

TABLE III

Effect of Anti-Ig on Thymic Cells

Cells	Dose of anti-Ig	Time of incubation at 37°C	Procedure	Mean number of moving cells
	μg	min		%
Spleen	No anti-Ig	5	A	5.1
Spleen	100	5	A	15.9
Thymus	No anti-Ig	5	A	17.7
Thymus	100	5	A	10.1
Thymus	No anti-Ig	10	B	17.4
Thymus	5	10	B	14.2
Thymus	10	10	B	13.7
Thymus	50	10	B	15.0

The mean number of motile cells is taken from two determinations.

the increase in the number of moving cells by anti-Ig depended upon a bivalent, cross-linking, ligand.

Relationship between Capping and Cell Motility.—In several experiments it was noted that if the lymphocytes were not allowed to settle onto a surface the number of motile forms was markedly decreased. Presumably, cells require attachment to a substrate for the local and translational movement that we observed. The above observations made it possible to test for capping of the anti-Ig-Ig complexes in lymphocytes exposed to FITC anti-Ig and incubated at 37°C in suspension where motility was reduced or on the surface of a dish. It was observed that capping proceeded just as rapidly and involved as many cells regardless of whether they were allowed to settle onto the surface of a culture dish or were maintained in suspension. Table IV describes this experiment. Of the lymphocytes incubated in suspension, 72–75% capped the anti-Ig-Ig complexes but no more than 11% were moving; of the lymphocytes incubated on a dish, 68% and 71% capped the complexes and about 27% moved.

Fig. 9 shows the formation of caps as a function of time in cells settled on a

TABLE IV

Role of Substrate in Anti-Ig Induced Movement

	Moving cells		Caps	
	%		%	
Incubation with FITC-anti-Ig				
1. $2 \times 10^6 \times$ ml on dish	29.3	26.0	70.8	68.2
2. $2 \times 10^6 \times$ ml in suspension	10.1	10.7	72.8	71.4
3. 0.66×10^6/ml in suspension	10.3	8.7	74.6	71.9
No incubation with anti-Ig				
4. 2×10^6/ml on dish	11.8	10.6	—	—
5. 2×10^6/ml in suspension	9.2	9.6	—	—
6. 0.66×10^6/ml in suspension	9.6	8.7	—	—

Each figure represents one individual determination. Spleen cells were incubated with fluorescein-labeled anti-Ig at 4°C for 30 min, washed, portioned as in the different groups, incubated for 10 min at 37°C, and fixed in paraformaldehyde. A group was not incubated with anti-Ig. Note that the number of cells moving spontaneously is not changed regardless of whether cells are settled on a dish or are in suspension. However, the anti-Ig induced movement is seen only with cells settled on a dish.

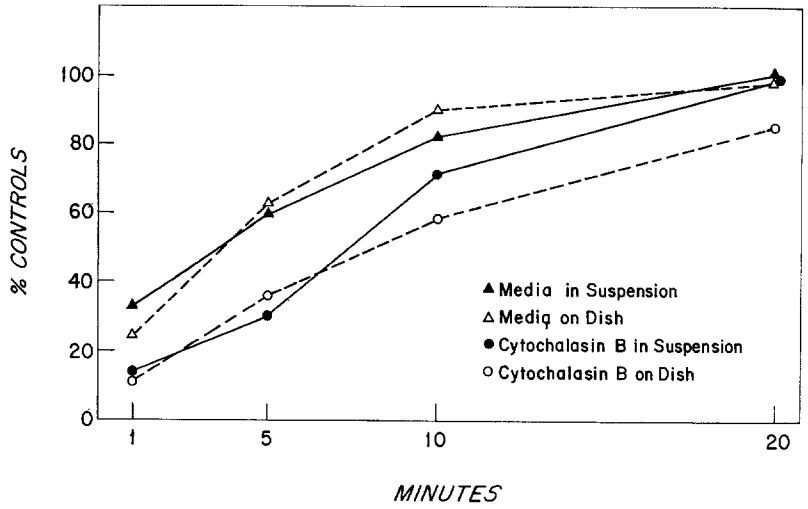


FIG. 9. Lymphocytes were incubated with fluorescent anti-Ig at 4°C, washed, and portioned as above; in regular media (5×10^6/ml) in test tubes with frequent mixing; in regular media allowing the cells to settle; and in media containing Cytochalasin B (10 μg/ml) either in suspension or in a dish. At the different times the cells were examined for percentage exhibiting caps. Mean number of cells with caps when incubated in regular media after 20 min was taken as the 100% value.

dish or in suspension. There was no difference in both sets of cells. Peak time of cap formation was the same as the peak time of increase in motile forms.

Although movement did not seem to be required for capping, it might still have an effect on the morphology or rapidity of cap formation, thus further

experiments were designed to assess the effect of cell motion on capping. Splenic lymphocytes were allowed to settle onto a dish surface, then fluorescent anti-Ig was added and the cells were brought to 37°C for 10 min. After fixation with paraformaldehyde, the cells were examined for motile forms as well as for cap morphology. Caps were scored according to the fraction of the circumference that they occupied. Thus, a "¼ cap" was a small, dense cap occupying 25% or less of the cell circumference, and a "¾ cap" was a large expanse of fluorescence covering at least 75% of the circumference. The results (Fig. 10) indicated that moving cells show a larger proportion of tight (¼) caps, whereas those not

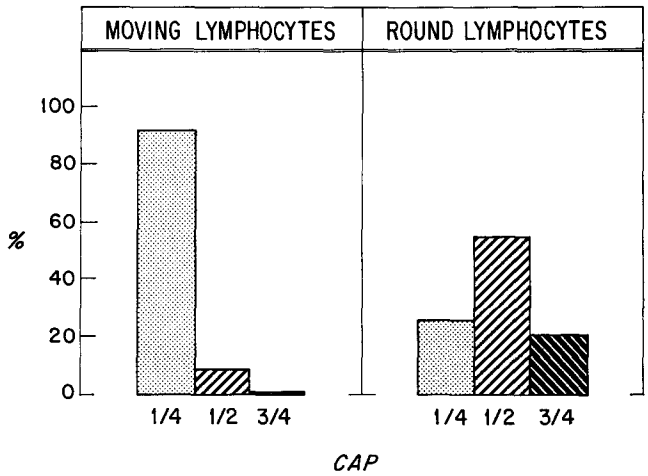


FIG. 10. Cell area occupied by the anti-Ig-Ig caps in lymphocytes that were round or in the process of movement. The explanation of the experiment is given in the text.

moving tended to have larger, more diffuse caps. This, taken together with the observation that many of the caps on moving cells involve the end region, makes it likely that when a cap forms on a moving cell it tends to be pulled to the posterior aspect of the cell and is perhaps concentrated into a smaller area by virtue of the cell motion. We conclude that, although capping takes place in the absence of overt cell motion, it can be modified by it.

Effects of Cytochalasin B on Capping and Movement.—Several drugs were used to pretreat the cells in a further attempt to dissociate motion from capping. The first of these was cytochalasin B. Fig. 8 shows the results of having 10 μg/ml of cytochalasin B in the medium at the time of addition of anti-Ig. There was nearly complete loss of the increase in moving forms produced by anti-Ig. However, the background of spontaneous motile formation was not totally eliminated. That the drug did not interfere with binding of anti-Ig to the lymphocyte or with capping is illustrated in Fig. 9. Cells in suspension in the presence of cytochalasin B, 10 μg/ml, were labeled with fluorescent anti-Ig and capping was observed to proceed to the same degree as in control cells

without the drug. Note that the tempo of cap formation was slower with cytochalasin. At 10 min there was about one-half the number of caps, but at 20 min the number was the same as controls. Also, most caps in cytochalasin B-treated cells tended to occupy one-half of the cell circumference, as opposed to the caps in untreated cells (Fig. 10). We concluded that the concentration of cytochalasin B that effectively inhibited anti-Ig induced movement did not interfere with capping.

Effects of DFP, Colchicine, and Metabolic Inhibitors on Capping and Movement.—DFP is known to irreversibly phosphorylate serine esterases and thus inhibit some cellular functions that depend on enzyme activation (16, 17). We first examined the effect of pretreating the cells with DFP and washing them free of the drug prior to interaction with anti-Ig. Presumably any serine esterases that were in the active state would be inactivated. Such pretreatment did not effect subsequent anti-Ig stimulated movement (Table V). However, exposure of the cells to 10^{-3} M DFP concurrently with exposure to anti-Ig reduced the level of motile forms close to background levels (Table V and Fig. 11). It can also be seen in Table V that either pretreatment with DFP or the presence of DFP during reaction with anti-Ig did not inhibit cap formation. Thus, we have again been able to inhibit motility in response to anti-Ig without altering cap formation. In addition, it seems that the interaction of anti-Ig on the cell surface activates a serine esterase, which is necessary to initiate subsequent cell movement. This result was obtained in several different experiments and using different batches of DFP (Table VI).

Colchicine, when placed in the medium in concentrations from 10^{-4} to 10^{-6} M failed to inhibit movement in response to anti-Ig. It also failed to inhibit capping. The results of an experiment using cytochalasin B, colchicine, and DFP either before or after addition of anti-Ig are seen in Fig. 11.

Other agents which were investigated included the metabolic inhibitors iodoacetamide, dinitrophenol, and oligomycin, all of which inhibited anti-Ig

TABLE V

Effect of DFP on Anti-Ig Induced Movement and Capping

Step 1	Step 2	Motile forms at 10 min	Caps
Pretreatment 30 min, 21°C	Add FITC-anti-Ig plus:		
		%	%
DFP 10^{-3} M	—	23.0	70.0
" $5\ 10^{-4}$ M	—	20.2	65.7
" 10^{-4} M	—	19.2	Not done
—	DFP 10^{-3} M	12.6	64.5
—	" 5×10^{-4} M	11.8	65.3
—	" 10^{-4} M	18.9	Not done
—	—	20.3	60.0
—	No anti-Ig	8.1	—

Between step 1 and 2 the cells were washed three times with media. Cells were examined for both motile forms and anti-Ig-Ig caps.

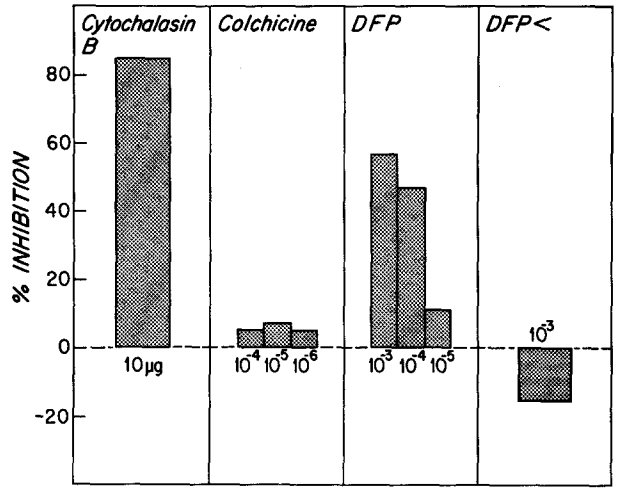


FIG. 11. Lymphocytes were incubated with anti-Ig (100 μg) at 4°C, washed well, placed in a dish, and incubated 5 min at 37°C. The net percentage of motile forms was 24.5%. Similarly treated lymphocytes were also incubated at 37°C in media containing Cytochalasin B, colchicine, or DFP. One portion was first incubated with DFP for 15 min at 37°C before incubation with anti-Ig at 4°C (DFP <).

TABLE VI

Four Experiments Using DFP

Anti-Ig	DFP	Motile forms			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
μg	*M*	%	%	%	%
50	—	28.8	22.5	13.9	28.6
—	—	4.5	—	3.6	17.1
50	10^{-3}	10.3	10.2	3.3	19.5
50	5×10^{-4}	12.8	16.2	7.9	22.4
50	10^{-4}	21.7	12.7	10.0	—

DFP was placed in the medium together with anti-Ig. Experiments 1 and 2 used procedure A, and 3 and 4 used procedure B. In procedure A, the cells were resuspended in media containing DFP.

stimulated movement (Table VII). These agents are also inhibitors of capping (7). In another experiment 2-deoxyglucose was used and found to be modestly inhibitory at 10^{-2} M.

DISCUSSION

The series of experiments described herein leads us to two conclusions: (*a*) that the complexing of anti-Ig with the surface Ig of a B lymphocyte stimulates it to move randomly; and (*b*) that capping is not directly associated with or caused by cell translation.

TABLE VII

Effect of Various Drugs on Movement by Anti-Ig

Drug	Concentration	Anti-Ig	Motile forms	
			Exp. 1	Exp. 2
		μg	%	%
Iodoacetamide	10^{-3} M	50	12.0	—
DNP	5×10^{-3} M	50	7.7	—
DNP	10^{-3} M	50	2.1	—
Oliogomycin	1 μg × ml	50	4.3	—
2-deoxyglucose	10^{-2} M	50	—	16.5
2-deoxyglucose	10^{-3} M	50	—	20.5
—	—	50	21.7	20.8
—	—	—	12.3	8.6

Experiments were done using procedure B.

Microscopic observations on lymphocytes treated with anti-Ig antibodies disclosed many with random movement at a time when capping of the complexes was taking place. The movement of the lymphocyte depended upon the reaction of its surface Ig with a bivalent antibody and required a metabolically active cell. Indeed, normal rabbit IgG, or monovalent anti-Ig did not stimulate movement; nor did anti-Ig stimulate movement on B cells that had lost most of their surface Ig (by a previous exposure to anti-Ig). The anti-Ig stimulated movement was associated with a DFP-sensitive step that requires better characterization but which implies that the activation of a proesterase is essential in the phenomenon. Activation of serine esterases is needed for the degranulation of mast cells (16) or the chemoattraction of neutrophils (17).

Capping of the anti-Ig-Ig complexes was not the result of movement of the lymphocytes. The translational movement of the lymphocyte was greatly minimized under three experimental conditions and yet under each of them capping proceeded. For example, treatment with 10 μg/ml of cytochalasin B abolished cell motility and only resulted in a slowing of the capping. Similar results were obtained by not allowing the cell to adhere to the surface of a dish or by the use of DFP. Two other experiments brought further evidence for the lack of association between capping and cell movement. First, cell movement was initiated by doses of anti-Ig that did not result in capping (1 μg, Table II); and second, human B cells which cap the anti-Ig-Ig complexes poorly (5) nevertheless are stimulated to move by anti-Ig.[2] Our working hypothesis at present is that capping results from intrinsic flow of the membrane and/or from active movements of the surface of the lymphocytes. In previous work, electron microscopic analysis of the surface of lymphocytes in the process of capping suggested increased membrane activity in the form of waves or undulations (6). Although capping and cell movement could be dissociated, we have shown

[2] K. A. Ault and E. R. Unanue, unpublished observations.

also that one process influenced the other. Indeed, lymphocytes in the process of moving exhibited tighter and denser caps occupying a small area at the posterior end of the cell; in contrast, the cap in stationary lymphocytes tended to occupy a larger segment of the cell circumference. It would appear that the mobile cell orients the cap towards its posterior end, and at the same time, both processes, the putative surface activity and the motion of the cell, result in the tighter aggregation of the lattice of immune complexes. Like the lymphocyte, it has been shown that the neutrophil caps surface complexes (of concanavalin A) independently of motility.[3]

Although in the lymphocyte and using anti-Ig, one can dissociate the cell movement from capping, one must consider studies using other cells which do indicate an association between movement of aggregates on a membrane and cell translation. Studies of Abercrombie et al. (18), have shown that fibroblasts in the process of movement transport surface-bound particles to the posterior end of the cell; a mechanism similar to this was postulated by Edidin and Weiss in order to explain capping of *H-2* complexes in fibroblasts (9). If by the term capping we simply denote the accumulation of aggregates towards one area of the cell surface, then one is forced, on the basis of the various experiments done with several cells, to postulate two forms of it: one independent of cell motion studied herein in the lymphocyte which takes place quite fast and is conditioned to the variables discussed in our previous studies (a cross-linking ligand, surface sites in close approximation and throughout the surface, 37°C, etc.); a second that is merely represented by the trailing of large aggregates to the posterior end of an actively moving cell as suggested by the experiments with fibroblasts. Both processes are operationally different altogether, although morphologically both are represented by the accumulation of complexes towards one zone of the cell surface. At this point, one word of caution on the interpretation of capping observed on live cells with fluorescent markers seems pertinent. At times it becomes difficult to differentiate whether one is observing surface capping of complexes or simple accumulation of endocytic vesicles containing the complexes in the perinuclear region. In the human B lymphocyte, for example, the anti-Ig-Ig complexes are rapidly interiorized without much aggregation or capping, and they rapidly flow into the perinuclear area, giving the impression of a cap (5). Similar results occur with rabbit B cells (19).[2] The term "cap" should be used as defined by Taylor et al. (1), as truly a surface accumulation of aggregates and care must be exercised to test whether the phenomena one is observing is truly on the surface.

The different events that take place in a lymphocyte upon reaction with ligands are summarized in Table VIII. It is our belief that the careful dissection of these early steps will aid in unraveling and understanding the response of

[3] G. B. Ryan, J. Z. Borysenko, and M. J. Karnovsky: Capping of concanavalin A on neutrophil polymorphonuclear leukocytes is independent of cellular movement. Abstract submitted to the American Society for Cell Biology.

TABLE VIII

Effect and Fate of Anti-Ig-Ig Complexes on Lymphocytes

Effects	Experimental manipulations				
	Cross-linking ligand	Metabolic inhibitors	Cytochalasin B	Concanavalin A*	DFP
Capping	Required (1, 3)	Inhibit (1–3)	No effect (Reference 7 and this paper)	Inhibits (20)	No effect‡
Endocytosis	Favors (1, 2, 21)§	Inhibit (7)	No effect (7)	Not tested	Not tested
Shedding‖	Required (4)	No effect (7)	No effect (7)	Not tested	Not tested
Cell movement	Required‡	Inhibit‡	Inhibits‡	No effect¶	Inhibits‡

* Yahara and Edelman have shown that the exposure of lymphocytes to concanavalin A at 21°C inhibits the capping of anti-Ig-Ig complexes.

‡ This paper.

§ There can be slow endocytosis of complexes formed by monovalent antibodies (21, 7).

‖ Refers to the release of anti-Ig-Ig complexes into the culture supernatant.

¶ Our unpublished results.

this cell to antigen. The event described in this paper, movement, could be an important step in immune induction principally as it relates to the encounter of clonally selected cells with antigen.

SUMMARY

Microscopic examination of spleen lymphocytes discloses a small number moving at random at a given time. The majority of lymphocytes with this spontaneous movement are thymic derived. Addition of anti-Ig antibodies stimulates random movement of B lymphocytes. This movement depends upon a bivalent antibody and a metabolically active cell. The movement is inhibited by DFP, suggesting the involvement of a serine esterase. Also the anti-Ig stimulated movement of the lymphocyte is inhibited by cytochalasin B or by not allowing the cells to settle onto a surface. Lymphocytes treated with DFP or cytochalasin B, or untreated lymphocytes in suspension, capped the anti-Ig-Ig complexes. Hence, one can dissociate the surface capping of anti-Ig-Ig complexes from cell movement. We postulate that capping may result from superficial movements of the surface and/or from membrane flow, both of which are not related to actual translation of the cell on a surface. Four effects have now been observed following combination of a ligand with the antigen receptor on the B lymphocytes: redistribution on the surface of the complexes; pinocytosis and catabolism; shedding into the extracellular environment; and stimulation of translational movement.

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