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Restriction of the Mobility of Lymphocyte Immunoglobulin Receptors by Concanavalin A

(lymphocyte receptors/lectin/NaN_3/cell membranes/fluorescence microscopy)

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ABSTRACT **The induction by anti-immunoglobulin of patch and cap formation on mouse lymphocytes was inhibited by the addition of concanavalin A. This effect was reversed by α-methyl-D-mannoside, a competitive inhibitor of the binding of concanavalin A. Concanavalin A prevented both patch and cap formation, in contrast to the metabolic inhibitor NaN_3, which prevented only cap formation. This suggests that binding of concanavalin A induces changes on or in the lymphocyte membrane that inhibit free diffusion of immunoglobulin receptors.**

Cell surface receptors of different specificities may differ in their number, distribution, mobility, and effect on the cell membrane. Lymphocytes provide excellent opportunities to study these differences for they bind various lectins, and in the case of B lymphocytes, they are known to have immunoglobulin (Ig) receptors on their surfaces (1–4). Lymphocytes also possess about 1.4×10^6 Con A receptors *per* cell (5, 6). The redistribution of the Ig receptors into patches after treatment with fluorescent antibodies directed against Ig (2) and the observation that the patches may then form caps (7) suggest that the Ig receptors are freely movable in or on the cell surface, and raise the question of whether the Con A receptors show similar properties.

The present studies were designed to analyze the distribution and mobility of Con A receptors on splenic lymphocytes and to assess the effect of the binding of Con A on the mobility of Ig receptors, as well as on other membrane properties. It was found that the binding of Con A does not lead to patch formation and inhibits both patch and cap formation by antibodies to Ig. The site of action of Con A differs from that of NaN_3 and cytochalasin B, which inhibit cap formation only (7). The results suggest that Con A alters the cell surface or membrane in such a way as to inhibit free motion of Ig receptors.

MATERIALS AND METHODS

Preparation of Mouse Spleen Cells. Mouse spleen cells prepared from NCS mice (Rockefeller University) were used throughout the present experiments. Spleens were cut in small pieces and cell suspensions were made by gently pressing them through a stainless-steel wire mesh into Hank's balanced salt solution (GIBCO, Grand Island, N.Y.). Cell clumps were removed by low-speed centrifugation and cells were washed twice with phosphate-buffered saline, pH 7.4 containing 0.2% bovine serum albumin (PBS–BSA) at 4°C. Cell suspensions were made up to 2×10^7 cells/ml in PBS–BSA. For experiments in which binding of Con A was determined, cells were exposed to distilled water for 30 sec to eliminate contaminating erythrocytes (8).

Preparation of Fluorescein-labeled Anti-Mouse Ig (fl-anti-Ig). Antiserum was obtained by injection of mouse IgG in complete Freund's adjuvant intramuscularly into rabbits. Ig was purified from the antiserum by precipitation with $(NH_4)_2SO_4$ at 37% saturation and chromatography on DEAE-cellulose. Fractions eluted with the starting buffer (0.175 M sodium phosphate, pH 7.0), were dialyzed against water and lyophilized. The resulting preparations were conjugated with fluorescein isothiocyanate according to the method of Cebra and Goldstein (9). The preparations used had a fluorochrome–protein ratio (A_{500}/A_{280}) of 0.5.

Determination of the Percentage of Cap-Forming Cells with fl-anti-Ig. Cells were stained with fl-anti-Ig under various conditions as described in the figure legends, and were observed by fluorescence microscopy (5). About 45% of all

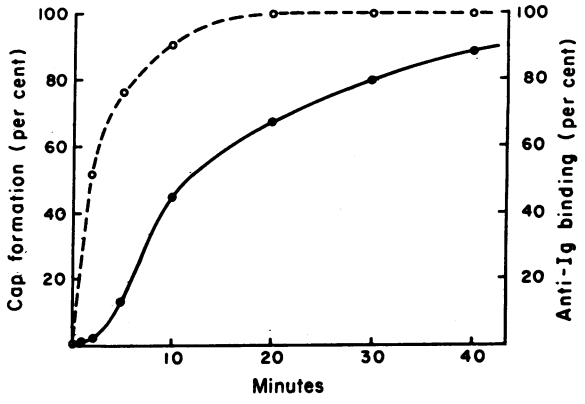

FIG. 1. Rate of cap formation after cells are mixed with fl-anti-Ig (●——●). 2×10^7 cells/ml were mixed with fl-anti-Ig (80 μg/ml) in PBS–BSA at 21°C. At various times after mixing, aliquots of cells were washed twice with PBS–BSA containing 10 mM NaN_3, and cap formation was determined. Rate of ^{125}I-labeled anti-Ig binding to cells (O– – –O): 2×10^7 cells/ml were mixed with 25 μg/ml of ^{125}I-labeled anti-Ig (7.6×10^4 cpm/μg) in 5.0 ml of PBS–BSA at 21°C. 0.5-ml Aliquots were sampled at various times after mixing and radioactivity was determined as described in the text.

Abbreviations: Con A, concanavalin A; Ig, immunoglobulin; fl-anti-Ig, fluorescein-labeled rabbit Ig directed against mouse Ig; PBS–BSA, phosphate-buffered saline, pH 7.4 containing 0.2% bovine serum albumin.

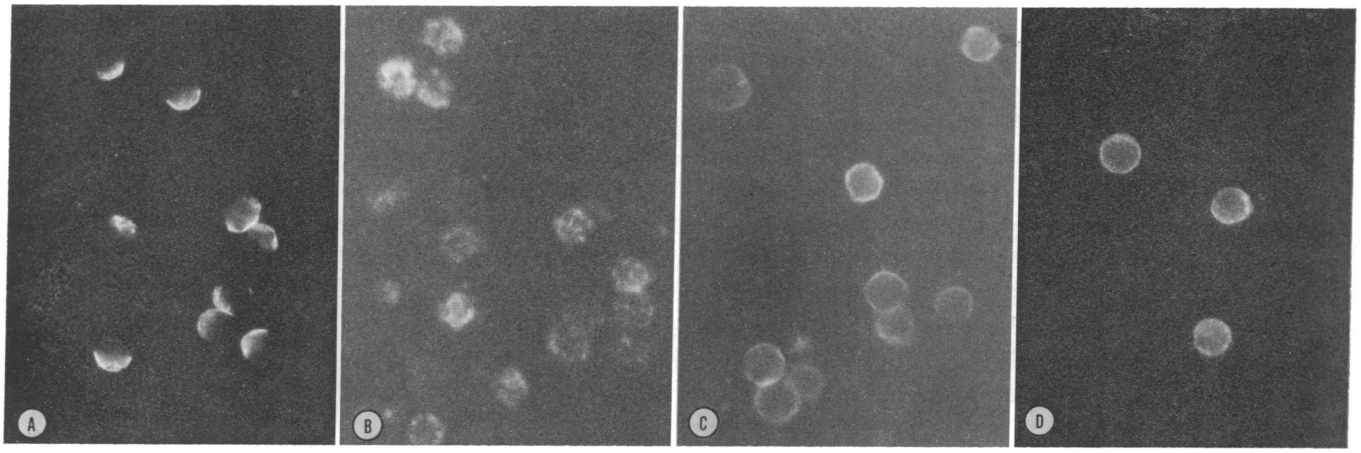

FIG. 2. Labeling patterns of cells with fl-anti-Ig and with fl-Con A. (*A*) Cells incubated with fl-anti-Ig (80 μg/ml) at 21°C for 30 min showing caps. (*B*) Prior addition of NaN_3 (10 mM) showing patches. (*C*) Prior addition of Con A (100 μg/ml) showing diffuse patterns. (*D*) Patterns after incubation with fl-Con A alone (100 μg/ml) at 21°C for 30 min.

cells were stained. Photographs were taken with Kodak Tri-X film, with exposure times of 5 min or longer. Between 100 and 200 stained cells were observed to determine the percentage of stained cells showing caps in any one experiment. The several patterns of staining, called here "caps", "patches", and "diffuse" are shown in Fig. 2. Occasionally, a very small percentage of stained cells that could not be classified as any of these three types was observed. Less than 20 μg/ml of Con A did not cause cell agglutination under the conditions used.

Binding Experiments with [^{125}I]anti-Ig and [^{125}I]Con A. Rabbit anti-mouse Ig and Con A were labeled with ^{125}I according to the method described by McConahey and Dixon (10). The specific activity of [^{125}I]anti-Ig was 7.6×10^4 cpm/μg and that of [^{125}I]Con A was 2.4×10^4 cpm/μg. A filtration method was devised to determine the amount of labeled protein bound to the cells. Whatman GF/C fiber glass filters used for filtration were soaked in PBS–BSA overnight at 4°C to prevent nonspecific binding. Labeled protein solutions used for the binding experiment were passed through a Millipore filter (HA 0.45 μm) before the reaction, and the concentration of protein and the radioactivity were determined after the filtration. Specific binding conditions are described in the figure legends. Just after the binding reaction, 0.5 ml of each cell suspension was diluted in 10 ml of PBS–BSA. Cells were collected by aspiration on a filter, at a flow rate of 20 ml/min, and were washed with 100 ml of PBS–BSA. The filters were rolled and put in small glass tubes, and their radioactivity was determined with an automatic gamma counter (Nuclear Chicago Co., model 4320).

RESULTS

Effects of NaN_3 and temperature on cap formation

Spleen cells prepared from unimmunized mice were treated with fl-anti-Ig in PBS–BSA, and cap formation was observed after incubation at 21°C for 30 min. Typical results are shown in Figs. 1 and 2*A* and are in good agreement with reported observations of others (7). After mixture of the cells with fl-anti-Ig, cap formation showed a time lag, whereas binding of anti-Ig occurred without any lag, and clearly the rate of binding was greater than that of cap formation (Fig. 1). This suggests that the initiation of cap formation in different individual cells is not synchronous and that cap formation depends on cooperative processes of binding within each cell. As can be seen in Fig. 3, cap formation was inhibited by low temperatures (<10°C) and high concentrations of NaN_3 (>5 mM), in agreement with the findings of Taylor *et al.* (7). In a separate experiment, we found that the extent of binding of anti-Ig was not affected by these conditions. Elevation of the temperature shortened the initial lag of cap formation and decreased the effect of a given concentration of NaN_3 (Fig. 3). Brief preincubation of cells in PBS–BSA containing the indicated concentrations of NaN_3 was necessary to determine the effect of NaN_3 on cap formation in a quantitative fashion. If this procedure was omitted, the inhibition curve was not reproducible.

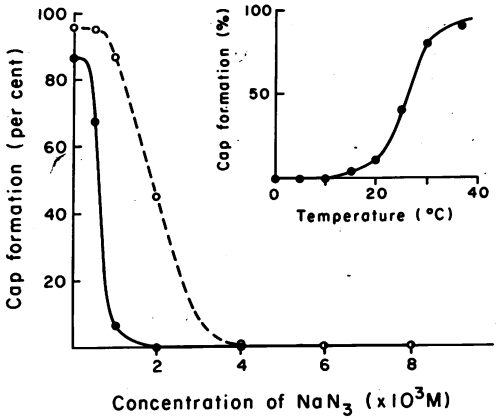

FIG. 3. Effects of NaN_3 and temperature on cap formation: (●——●, 21°C; O- - -O, 37°C): Cells were suspended in PBS–BSA containing various concentrations on NaN_3 and incubated for 5 min at 21 and 37°C, respectively. Fl-anti-Ig was added to these mixtures to a final concentration of 80 μg/ml, and the mixtures were incubated for 30 min. Cap formation was determined after washing the cells with PBS–BSA containing 10 mM NaN_3. *Inset:* 2×10^7 cells/ml were incubated with fl-anti-Ig (80 μg/ml) in PBS–BSA at various temperatures for 5 min. Further reaction was stopped by the addition of NaN_3, to a final concentration of 10 mM. Cap formation was determined as described in the text.

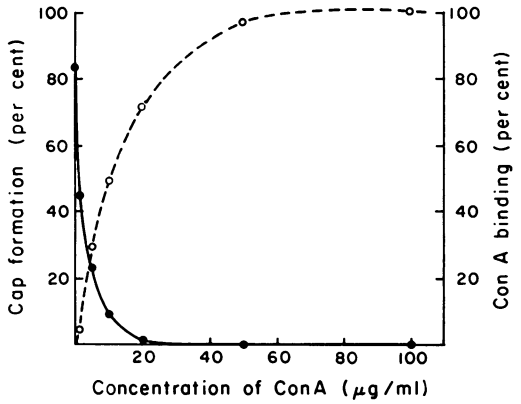

FIG. 4. Effect of Con A on cap formation (●——●): 2×10^7 cells/ml were incubated with various concentrations of Con A in PBS–BSA at 21°C for 10 min. Fl-anti-Ig was added to the mixture to give a concentration of 80 μg/ml, and the mixture was incubated for 30 min at 21°C. Cap formation was determined after washing cells. Binding of [^{125}I]Con A to cells (O- - -O): 2×10^7 cells/ml were incubated with various concentrations of [^{125}I]Con A (2.4×10^4 cpm/μg) at 21°C for 30 min in PBS–BSA. Cells were washed with PBS–BSA and the radioactivity was determined.

Effect of Con A on cap formation

An average of 1.4×10^6 Con A molecules can bind to mouse spleen cells (5). Cap formation was markedly inhibited by binding of Con A to these cells (Figs. 2*C* and 4). The inhibition showed the following properties: (*a*) The extent of inhibition of cap formation depended on the Con A concentration, as shown in Fig. 4. Also shown in Fig. 4 is the relationship between the amount of Con A added and the amount of Con A bound to cells. (*b*) Cap formation was inhibited when Con A was added to cell suspensions in PBS–BSA before or simultaneously with the addition of fl-anti-Ig. (*c*) If cells were incubated with Con A (100 μg/ml) at 21°C for 10 min, washed three times with PBS–BSA to remove free Con A molecules, and treated with fl-anti-Ig, cap formation was still inhibited. (*d*) Cap formation was also inhibited if cells were incubated with fl-anti-Ig at 0°C for 30 min, Con A (100 μg/ml) was added, and the mixture was brought to 21°C. (*e*) In contrast, incubation of cells with fl-anti-Ig at 21°C for 30 min, followed by the addition of Con A, did not show any inhibitory effect. This result indicates that once formed, patches and caps are not affected by Con A.

Effect of Con A on binding of antibodies to Ig receptors

As shown in Fig. 5, the Ig sites on 5×10^6 cells were saturated by about 0.025 μg of [^{125}I]-labeled anti-Ig. Assuming that at least one antibody molecule binds to each surface Ig molecule, the average minimum number of antibody receptors on mouse spleen cells is estimated to be 2×10^4 *per* cell. Inasmuch as only one-half of the population of cells was observed to be stained with fl-anti-Ig, this number may be as great as 4×10^4 receptors *per* cell. When Con A was added in saturating amounts to cell suspensions before the addition of fl-anti-Ig, binding was decreased by 20–30%. However, since cap formation was observed at anti-Ig concentrations as low as 15 μg/ml, these decreases in the binding of anti-Ig were not the cause of inhibition of cap formation.

Labeling patterns with fl-anti-Ig in the presence of NaN_3 and Con A

Cells were incubated at 21°C for 30 min with fl-anti-Ig in the presence of NaN_3 and Con A separately; the labeling patterns of the cells by fluorescence microscopy are shown in Fig. 2*B* and *C*. Labeling patterns in the presence of Con A were diffuse or continuous, whereas labeling in the presence of NaN_3 showed patches or discrete distributions. Cells directly labeled with fluorescent Con A (Fig. 2*D*) showed diffuse patterns similar to those seen with fl-anti-Ig in the presence of Con A. These patterns were not changed by the addition of specific rabbit antiserum to Con A.

Reversibility of the effects of Con A and NaN_3 on cap formation

Incubation of cells with Con A, followed by further incubation with fl-anti-Ig, prevented cap formation. When α-methyl-D-mannoside was added to the cell suspensions (final concentration 40 mM), however, cap formation was observed without further addition of fl-anti-Ig (Fig. 6). The rate of cap formation after the addition of α-methyl-D-mannoside was different than that of the release of Con A from the cells, as determined with [^{125}I]Con A (Fig. 5). Moreover, the time of appearance of caps after removal of Con A from the cell surface was longer than that observed when cells were mixed with fl-anti-Ig (Fig. 1).

As can be seen from Fig. 3, 3 mM NaN_3 inhibited for capmation completely at 21°C. When cells were incubated with fl-anti-Ig in PBS–BSA containing 3 mM NaN_3, then transferred to PBS–BSA without NaN_3, cap formation occurred rapidly (Fig. 6). The rate of cap formation after the removal of NaN_3 was distinctly different from that observed after the release of Con A. These findings and the labeling patterns strongly indicate that the mechanisms of inhibition of cap formation by NaN_3 and Con A are different.

DISCUSSION

Several observations are essential to the interpretation of the effects of Con A on cap formation by Ig receptors: (*a*) Cap formation occurs after a short time lag, whereas the bind-

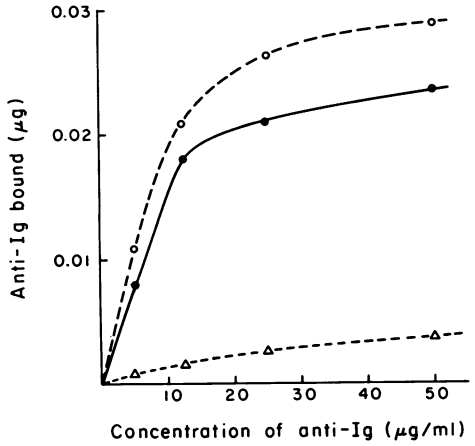

FIG. 5. Effect of Con A on binding of anti-Ig to cells: 1×10^7 cells/ml were incubated at 21°C for 30 min with various concentrations of ^{125}I-labeled anti-Ig (7.6×10^4 cpm/μg) in 0.5 ml of PBS–BSA with (●——●) or without (O- - -O) Con A (100 μg/ml). They were then washed on filters with PBS–BSA, and the radioactivity was determined. Binding of [^{125}I]Ig from an unimmunized rabbit, (Δ- - -Δ).

ing of anti-Ig to the lymphocytes is very rapid. (*b*) Fluorescent Con A itself does not lead to cap formation and shows diffuse binding without evidence of patches, despite the fact that Con A is tetravalent (11). Moreover, addition of antibodies to Con A does not alter this binding pattern. (*c*) Cells treated with fl-anti-Ig in the presence of Con A show diffuse labeling patterns, whereas in the presence of NaN_3 they show patches (Figs. 2*B* and *C*). (*d*) Con A inhibits cap formation reversibly; recovery after the release of Con A from the cells occurs slowly after a lag of 5 min. (*e*) NaN_3 also inhibits cap formation reversibly, but the reversal after removal of NaN_3 from the medium occurs within 1 or 2 min at 21°C.

Based upon the above findings, a model for cap formation and for the effect of Con A on the cell membrane can be formulated (Fig. 7). Addition of anti-Ig leads to three successive processes: (*a*) binding of divalent antibodies to Ig molecules on the cell surface, (*b*) formation of patches of antibody-Ig receptor complexes, and (*c*) cap formation over one pole of the cell. Processes (*a*) and (*b*) are not inhibited by NaN_3, since binding is unaffected by NaN_3 and patches can be observed in its presence. We infer that NaN_3 inhibits process (*c*). The rate of cap formation after the removal of NaN_3 from

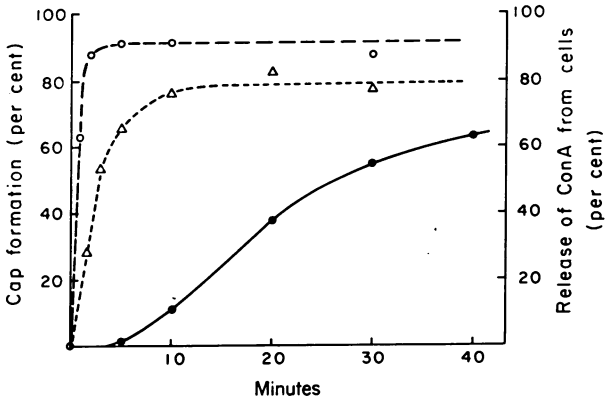

FIG. 6. Cap formation after release of Con A from cells (●——●): 2×10^7 cells/ml were incubated with Con A (100 μg/ml) at 0°C for 10 min in PBS–BSA and fl-anti-Ig (80 μg/ml) was added to the mixture. The mixture was then incubated at 0°C for 30 min and centrifuged. The cells were washed with PBS–BSA and resuspended in the same volume of PBS–BSA at 21°C, to which α-methyl-D-mannoside was added to give a concentration of 40 mM. Aliquots were pipetted at various times after the addition of α-methyl-D-mannoside, the cells were washed, and cap formation was determined.

Rate of release of [^{125}I]Con A from cells after the addition of α-methyl-D-mannoside (O– – –O): 2×10^7 cells/ml were incubated with 100 μg/ml of [^{125}I]Con A (2.4×10^4 cpm/μg) at 21°C for 10 min in PBS–BSA. Unlabeled anti-Ig was added to this mixture to give a final concentration of 80 μg/ml. After incubation at 21°C for 30 min, the mixture was centrifuged and the cells were resuspended in PBS–BSA. Aliquots of the mixture were transferred to small tubes containing α-methyl-D-mannoside in PBS–BSA (final concentration, 40 mM) and were incubated at 21°C for various times, then the radioactivity was determined.

Cap formation after removal of NaN_3, (Δ– – –Δ); 2×10^7 cells/ml were incubated with fl-anti-Ig (80 μg/ml) for 30 min at 21°C in PBS–BSA containing NaN_3 (3 mM). Cells were collected by centrifugation and resuspended in PBS–BSA at 21°C with a decrease in NaN_3 concentration to 30 μM. Cells were washed, and at various times cap formation was determined.

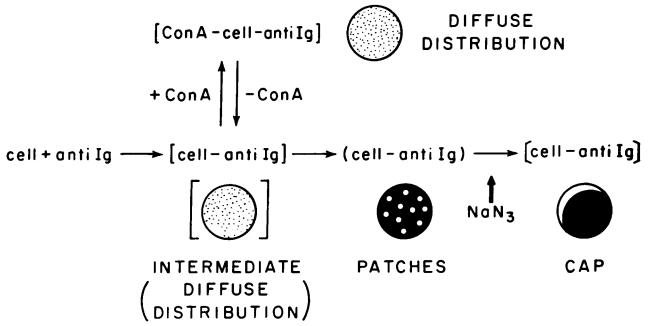

FIG. 7. A model for patch and cap formation and their inhibition by Con A.

the medium indicates that cap formation usually occurs within, at most, 10 min after the formation of patches at 21°C. Cap formation, therefore, appears to be dependent on cell metabolism, and possibly requires a certain concentration of ATP. These conclusions are in accord with those of Taylor *et al.* (7).

Con A inhibits both cap formation and patch formation (Fig. 2*C*), and it also slightly inhibits the binding of anti-Ig to cells. Release of Con A from cells to which fl-anti-Ig had already been bound led to cap formation without further addition of fl-anti-Ig. Moreover, the extent of binding of fl-anti-Ig in the presence of Con A at saturation is sufficient for cap formation. Consequently, we conclude that Con A inhibits process (*b*) or patch formation.

Patch formation appears to result from free diffusional motion of Ig receptors, which form larger aggregates after binding with divalent antibody. The resultant patches do not then diffuse at appreciable rates. A calculation from the known dimensions of Con A and the location of its binding sites (11), as well as the results of titration (5) of the number of receptors on lymphocytes (1.4×10^6/cell), indicates that at saturation Con A occupies no more than 1% of the cell surface, in a diffuse distribution. Inasmuch as the Ig receptors (about 4×10^4/cell) also occupy no more than 1% of the surface, binding of Con A cannot directly block the diffuse movement of Ig receptors. We therefore propose that Con A binding leads to a change in the cell surface or the cell membrane that results in alteration of either the anchorage or the free path of Ig receptors. This change could be aggregation of intramembranous protein particles linked to Con A receptors, either with formation of a tortuous path for motion of Ig receptors or, more likely, the binding of Ig receptors to particle-associated structures. Alternatively, binding of Con A to cell surface glycoproteins may result in secondary interactions of the Con A with the membrane and a change in membrane fluidity. The first mechanism is consistent with observations using freeze–fracture techniques (12) and electron microscopic labeling techniques (13), which suggest that Con A is bound in small aggregates on the cell surface. The second mechanism could be tested by measurement of relaxation times of spin-labeled molecules (14) in the presence and absence of Con A.

Con A binds to various cells from different tissues (5), and it is important to know whether the effects observed in lymphocytes also occur in other cells. Intermixing of cell surface antigens has been observed to occur rapidly when different types of cells were fused (15). This process was not inhibited

by NaN_3, dinitrophenol, inhibitors of protein synthesis, or 6-diazo-6-oxonorleucine, but only by low temperature. Movement of cell surface antigens seems to be similar to process (*b*) (Fig. 7), since patch formation can also be inhibited by cold. We therefore suggest that intermixing of cellular antigens may also be inhibited by prior binding of Con A to fused cells.

The alteration of the lymphocyte cell membrane by Con A seen in the present studies may shed light on the process of mitogenesis by this lectin. If this process is the same as that caused specifically by antigens, it does not seem likely that patch formation is required for the specific stimulation of immunocytes.

NOTE ADDED IN PROOF

Independent observations on inhibition of patch and cap formation by Con A have been made by Loor, F., Forni, L. & Pernis, B. "Dynamic State of Lymphocyte Membranes: Factors Affecting the Distribution and Turnover of Surface Ig," *Eur. J. Immunol.*, in press.

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