CONCANAVALIN A RECEPTORS, IMMUNOGLOBULINS, AND θ ANTIGEN OF THE LYMPHOCYTE SURFACE

Interactions with Concanavalin A
and with Cytoplasmic Structures

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ABSTRACT
The effect of concanavalin A (Con A) on the capping of mouse lymphocyte surface immunoglobulin (surface Ig), cross-linked by rabbit anti-mouse Ig antibody, and on the capping of mouse thymocyte θ antigen, cross-linked by anti-θ alloantibody and rabbit anti-mouse Ig antibody, has been studied by immunofluorescence, using fluorescein conjugated Con A and rhodamine-conjugated anti-mouse Ig antibody, and by electron microscopy, using native or fluorescein-conjugated Con A and ferritin-conjugated anti-mouse Ig antibody.

Prior incubation of the cells with Con A inhibited only partially capping of surface Ig, whereas it blocked almost completely capping of θ antigens. Both on cells with rings and on cells with caps the staining for surface Ig or θ antigen was superimposed to the staining for Con A. When Con A receptors on spleen cells were capped by Con A at concentrations of 10 μg/ml or higher, and the distribution of surface Ig was examined under noncapping conditions, all detectable surface Ig were found in the caps. As shown by electron microscopy, surface Ig remained dispersed in a layer of Con A. The ability of Con A to cap surface Ig was not altered by the presence of colchicine or vinblastine. These results suggest that surface Ig are cross-linked by Con A to other Con A receptors. In these conditions surface Ig behave essentially as Con A receptors, as for example, in their sensitivity to cytochalasin B during inhibition or reversal of capping induced by this drug. The behavior of surface Ig parallels that of Con A receptors also in the presence of vinblastine. It is concluded that in the presence of Con A, antimitotic drugs do not modify directly the interaction between Con A receptors and surface Ig, but probably influence the capping ability of the Con A receptors or, more in general, affect the ability to elicit movements over the cell surface. The role in capping of cytochalasin-sensitive and vinblastine-sensitive structures is discussed. Both types of structures appear to play an active role in the formation of a cap, although the former probably corresponds to the main mechanical system responsible for the active displacement of cytoplasmic and surface material.
The recognition that individual macromolecular components of the plasma membrane of lymphocytes and other cells are capable of translational movement in the plane of the membrane and that they can be segregated from other components when cross-linked by a multivalent ligand \((38, 8, 21, 41, 18)\) has provided a useful tool for studying their mutual physical relationship. In particular, when one individual component, suitably cross-linked and labeled by a specific ligand, forms a “cap” at one pole of the cell, it is possible to establish, using a second specific label, whether another recognizable component of the membrane has moved together with the first into the cap, or, alternatively, has been unaffected by the movement of the first. In this way, for example, it has been demonstrated that lymphocyte surface immunoglobulin (surface Ig) \(^{1}\) of different classes and H-2 and HL-A histocompatibility antigens in mouse and man, coded by different loci or alleles of the same genetic locus, behave as independent molecules \((38, 2, 24, 29)\), whereas human HL-A and \(\beta_2\) microglobulin are associated in the same “mobile unit” \((28, 35)\). I have used this approach to study the relationship between receptors for concanavalin A (Con A) and surface Ig on mouse lymphocytes and \(\theta\) antigens on mouse thymocytes. A similar study has been recently published by Loo Loo (20).

Surface Ig of peripheral mouse lymphocytes, cross-linked by anti-lg antibody, and similarly, \(\theta\) (Thy 1.2) antigen of mouse thymocytes \((31)\), cross-linked by a double layer of mouse anti-\(\theta\) and rabbit anti-mouse Ig antibodies, can cap in a few minutes at \(20^\circ\) or \(37^\circ\) C in metabolically active cells \((38)\). It has been found, however, that when the cells are first exposed to Con A, a lectin which binds to mannoside residues in the carbohydrate side chains of glycoproteins (and possibly, glycolipids), capping of surface Ig or \(\theta\) antigens is severely inhibited \((21, 44, 46)\). The inhibition of surface Ig capping was obtained only when tetravalent Con A was used \((44)\), and it could be relieved by treating the cells with microtubule-dissociating drugs like vinblastine and colchicine \((13, 45)\). On the basis of these observations, Edelman et al. \((13)\) and Yahara and Edelman \((45)\) suggested that the inhibition was probably indirect and occurred through the activation, by the cross-linking of the Con A receptors by Con A, of an inhibitory cytoplasmic system consisting of microtubules or microtubule-like proteins. This system would have, in turn, prevented the redistribution of surface Ig. In the presence of Con A, a microtubule-like system could therefore control or “modulate” the capping of surface Ig which otherwise would normally occur without the involvement of these structures \((8, 38)\). On the basis of preliminary experiments, Edelman et al. \((13)\) seemed to exclude the possibility that the interaction of Con A receptors and surface Ig could have been mediated by direct cross-linking of the two components by Con A. Conclusive proof of this point, however, was not provided, since those authors did not make use of two different fluorochromes to label simultaneously surface Ig and Con A receptors. Independence of surface Ig and Con A/Con A receptor complexes was also reported by Karnovsky and Unanue \((16)\).

The results of the present study are consistent with the main observations of Yahara and Edelman \((45)\), but indicate that surface Ig and probably \(\theta\) antigen are directly cross-linked by Con A to other Con A receptors, and suggest that in this experimental system the behavior of surface Ig reflects essentially the behavior of the Con A receptors themselves, in particular in regard to their interaction with cytoplasmic structures. Moreover, these studies provide some direct evidence that among these structures both microtubules and microfilaments have an active and synergic role in cap formation.

**MATERIALS AND METHODS**

**Cells**

3-4 mo old Balb/c and in a few experiments CSW mice were used as a source of spleen cells, and 4-6-wk old C3H mice were used as a source of thymus cells carrying the \(\theta\)C3H antigen (Thy 1.2) \((31)\). Cell suspensions were prepared by teasing the organs in cold Dulbecco’s physiological saline containing Ca\(^{++}\) and Mg\(^{++}\) and 0.1 or 0.2% bovine serum albumin (PBS/BSA). The cells were filtered through glass wool to remove aggregates, and washed twice in cold PBS/BSA. In most

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\(^{1}\) Abbreviations used in this paper: BSA, bovine serum albumin; Con A, concanavalin A; \(\beta\)-Con A, fluorescein-conjugated concanavalin A; Ig, immunoglobulin; PBS, Dulbecco’s complete physiological saline; RaMlg, rabbit anti-mouse immunoglobulin; RaMlg-FT, ferritin-conjugated rabbit anti-mouse immunoglobulin; rh-RaMlg, rhodamine-conjugated rabbit anti-mouse immunoglobulin; SS, standard salt solution.
experiments, to reduce agglutination by Con A, the red
cells of spleen preparations (in 0.1 ml) were lysed by
adding, for 30–60 s, 1 ml of distilled water or 1 ml of 10
mM Tris-HCl buffer, pH 7.3 (46); in some experiments
the red cells were removed together with several
granulocytes by sedimentation (10 min at 2,000 g)
through a layer of 16% urografin (Schering, Berlin,
Berkamen). No difference was noted between the results
obtained with cells treated in these different ways
and untreated cells. The cells were washed two times
more with PBS/BSA and once with Leibovitz’s tissue culture
medium L15 (Flow Laboratories, Irvine, Scotland)
containing 0.2% BSA (L15/BSA). In a few experiments
the cells were prepared at room temperature, without any
substantial difference in the results. All subsequent steps
(incubation, staining) were carried out in L15/BSA,
unlike previous indications. Medium L15 contains
galactose, but no glucose, and is supplemented with
sodium pyruvate, making less likely the possibility of
secondary effects due to the impairment of sugar
transport in experiments with cytochalasin B (17, 23).
In some of these experiments sugar-free PBS/BSA was
used without any change in the results (e.g., Fig. 28).

Fluorescent Reagents

**CONJUGATION OF CON A:** The Ig fraction (0.5
mg/ml) of a polyvalent rabbit anti-mouse Ig anti-serum
(RaMlg), conjugated to tetrathiodamine isothiocyanate
according to the method of Cebra and Goldstein (6),
was generously provided by Miss Luciana Forni and
used as standard fluorescent anti-mouse Ig reagent
(rh-RaMlg).

Fluorescein-conjugated Con A (fi-Con A) was
prepared using commercial lyophilized Con A (Miles
Laboratories, Inc., Miles Research Div., Kankakee, Ill.
or Sigma Chemical Co., St. Louis, Mo.). The Con A was
dissolved (at 10 mg/ml) in a cold standard salt solution
(SS) made of: 1.0 M NaCl, 0.25 mM CaCl₂, 0.25 mM
MnCl₂, 0.1 M glucose, and brought to pH 7.2 with 10
mM of Na-phosphate buffer. Any undissolved material
was removed by centrifugation. After 16 h in the cold,
the solution was dialyzed against SS, buffered with 0.1 M
carbonate buffer, pH 9.3–9.4, for about 6 h and then
overnight at 4°C against 10 vol of the same solution
containing 250 µg/ml of fluorescein-isothiocyanate (ca.
25 µg/mg Con A inside the dialysis bag). The low
fluorochrome concentration was employed to reduce the
number of fluorochrome groups bound per Con A
molecule, and, therefore, possible alterations in the
molecule. If Con A was dissolved directly in the
conjugation buffer at pH 9.4, a large part of the Con A
was inactivated (cf. 26) and the peak of absorbance of the
bound fluorescein usually shifted from 492–493 to
496–500 nm, suggesting that the fluorochrome groups
had become exposed to a more hydrophobic environment
(11). Preformation of functional tetramers and high ionic
strength are probably protective factors when Con A is
brought to high pH.

The conjugate was extensively dialyzed against SS, pH
7.2, without glucose, and purified by affinity
chromatography on a column of Sephadex G-50. Almost
all of the conjugate bound to the Sephadex. The column
was washed overnight with buffer and then eluted
stepwise with 0.01 and 0.10 M glucose in buffer. Only the
more strongly bound fraction, eluted with 0.10 M
glucose, which contained most of the conjugate, was used
in the experiments reported here. The eluate was
centrifuged at 3 mg/ml by vacuum dialysis, dialyzed
against 0.9 M NaCl, 2 × 10⁻⁴ M CaCl₂, 1 × 10⁻⁴ M
MnCl₂, 20 mM Na-phosphate buffer, pH 7.0, and stored
at 2°C. This stock solution was diluted before use with 5
vol of water and then with PBS as required (final
dilutions of 1:30–1:300). The ratio of the optical densities
OD₄₉₆/OD₆₃₀ was 0.25. This preparation, used in most
experiments, gave a visible ring staining at a protein
concentration of >5 µg/ml. Other preparations with an
OD₄₉₆/OD₆₃₀ ratio of 0.93 and 0.77, prepared at pH
9.2–9.3 by dialysis against 500 µg/ml of fluorescein-isothiocyanate
and eluted in one step with 0.10 M glucose or
α-methylglucopyranoside, were used in a few experi-
ments with comparable results although they were
slightly less efficient in capping Con A receptors (see Fig.
32).

Immunofluorescence

**(g) PREINCUBATION:** Most experiments were per-
formed at 37°C (cf. 13, 46). The cells were preincubated
20–60 min in L15/BSA at 37°C before labeling. In
experiments in which vinblastine (Velbe, Ei Lilly & Co.,
Indianapolis, Ind.) or cytochalasin B (Imperial Chemical
Industry) were used, 0.1 or 1.0 ml of cells (2–4 x 10⁸
cells/ml) were preincubated for variable times (30–120
min for vinblastine; 10–30 min for cytochalasin B) at the
appropriate concentration of the drugs. Samples with
cytochalasin contained 0.125–0.25% dimethylsulfoxide,
final concentration, which had no effect on the phenom-
ena under investigation.

**(b) CAPPING OF fi-Con A:** 10 µl of fi-Con A in
PBS/BSA at the appropriate concentration were added
to 80 µl of cell suspension and 10 µl of PBS containing no
additive or the various drugs at the appropriate concen-
tration. The cells were incubated for 10 or 15 min
(exceptively up to 2 h) at 37°C. In most cases, cells
incubated with the lightly labeled fi-Con A at ≤50 µg/ml
were not washed, but examined directly at room temper-
ature (22°–24°C) to avoid the excessive agglutination
caused by centrifugation. The fluorescent background
did not seriously interfere with the observations.

**(c) CAPPING OF rh-RaMlg:** 20 (or 40) µl of rh-
RaMlg conjugate were added to 25 (or 50) µl of cells and
5 (or 10) µl of PBS containing the appropriate additives.
After incubation for 10–15 min or more at 37°C, the cells
were washed with 50–100 vol of PBS/BSA or L15/BSA,
suspended in fresh medium, and immediately examined
at room temperature.
(d) COCAPPING OF f1-CON A AND RH-RaMlg:
Cells were first exposed to f1-Con A as in (b). To 50 ~l of this suspension, 40 ~l of rh-RaMlg and 10 ~l of PBS with fl-Con A and the appropriate additives were added either immediately or after 5 min. The incubation was continued for 15 min more at 37°C. The cells were washed once with 80–100 vol of medium and examined at room temperature.

(e) CAPPING OF SURFACE lg BY fl-CON A: The cells were first incubated as in (b) with fl-Con A for 10 min at 37°C, and then put on ice. All subsequent steps were done at 0–4°C in the presence of 0.2% azide (38). The cells were washed once, stained with rh-RaMlg for 30 min, washed, and examined on a refrigerated microscope stage (at 0°C; the actual temperature on the slide was about 4°C) in the presence of azide. Either fl-Con A caps were scored first and then examined for the distribution of rh-RaMlg, or vice versa. In some experiments the cells were treated and examined in the same way but without azide, with identical results.

(f) INHIBITION OF CAPPING AND COCAPPING OF lg ANTIGEN AND fl-CON A ON THYMOCYTES:
Small aliquots of cells (25 ~l) were incubated for 15 min at 37°C with 10 ~l of anti- lg C3H antisemur which by itself is unable to induce capping of lg antigens (38, 10). The cells were washed and incubated for 15 min at 37°C either with rh-RaMlg alone, or with fl-Con A followed after 2 min by rh-RaMlg. The cells were washed and examined at room temperature. Parallel experiments were carried out in the presence of vinblastine. In some experiments the cells were incubated with anti-lg antisemur, followed by rh-RaMlg in the cold, washed, and warmed up to 37°C, while fl-Con A was added either simultaneously or after some minutes. After 15 min incubation, the cells were washed and examined. In preliminary experiments to study capping of lg antigens by fl-Con A, a procedure similar to that described in (e) was employed. The cells were incubated with fl-Con A at 37°C, washed, and incubated with anti-lg antisemur and rh-RaMlg in the cold in the presence of azide. At the end of this lengthy procedure, practically none of the few Con A caps initially formed were present, probably due to a slow cap reversion in the cold (see Results).

(g) IMMUNOFUORESCENCE MICROSCOPY: All observations for fluorescein and rhodamine staining were carried out using a Leitz Orthoplan microscope with Opak-Fluor vertical illumination and standard equipment.

Electron Microscopy

For the study of capping of surface lg by Con A, spleen cells were incubated for 10 min at 37°C with native Con A or fl-Con A at various concentrations. The cells were then cooled down to 0–4°C, washed by centrifugation, and stained with anti-lg antibody conjugated to ferritin (RaMlg-FIT) for 30 min at 0°C as previously described (8). In some experiments, part of the samples were exposed to an anti-Con A antibody-ferritin conjugate (the anti-Con A antisemur was kindly provided by Dr. F. Loor). The antibody-ferritin conjugates were prepared as described. After washing, the cells were fixed with 3% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.3, postfixed in 1% OsO4 and 0.5% uranyl acetate in distilled water, dehydrated, and embedded in Araldite (8). Thin sections were stained with uranyl acetate and lead citrate in order to make visible the unconjugated Con A. In the experiments with fl-Con A, small aliquots of cells were examined by immunofluorescence, after fixation with glutaraldehyde, to determine the percentage of fl-Con A caps.

RESULTS

Capping of Con A Receptors by Con A in Splenocytes and Thymocytes

When spleen cells, preincubated 5–60 min at 37°C, were incubated with 20–100 ~g/ml of fl-Con A, Con A caps formed in about 50% of the cells (32–64%, average 52%, in 15 experiments) within 5–10 min at 37°C. The same results were obtained in a few experiments at temperatures between 30° and 37°C. At concentrations of fl-Con A of 5–10 ~g/ml, capping was reduced and comparatively slower, although in some experiments up to 45% caps were obtained after 15 min. The caps persisted for at least 90 min at 37°C, although the percentage often fell slightly to 30–40% after prolonged incubation. Precise determinations after prolonged incubation were difficult because most cells agglutinated in large clumps at Con A concentrations of 10 ~g/ml or higher.

The presence of 10–4 M vinblastine had either no effect on the percentage of caps or increased it by 10–20%. This increase was more appreciable when the percentage in the controls was less than 50%. Therefore, in the present system, vinblastine appeared to have the effect of raising the percentage of caps towards some maximum values (50–65%), which were reached only in part of the samples in the absence of the drug.

The percentage of caps induced in thymocytes by 50 ~g/ml of Con A at 37°C was much lower, varying between 3 and 12% in five experiments, and was only marginally increased, and never above 20%, by preincubation with 10–4 M vinblastine or by prelabeling the cells with Con A at 0°C (44). It was not increased by cross-linking further the Con A with anti-Con A antibody.

Capping of Surface lg in the Presence of Con A

In the absence of Con A, 80–95% of the lg-positive cells incubated with anti-lg antibody
formed caps in 15 min at 37°C. Preloading the spleen cells with Con A has been shown to inhibit capping of surface Ig (44, 46). With the present fl-Con A preparations, the inhibition was only partial and varied between 10 to 40%, with little variation in the range of Con A concentrations between 20 and 100 μg/ml. An example is shown in Table I. Although in every experiment the percentage of surface Ig caps formed in the presence of fl-Con A (55-85%) in the subpopulation of Ig-positive cells was higher than the percentage of the fl-Con A caps in the entire population, the absolute number of the Ig caps was always less than that of Con A caps, the fraction of cells with detectable surface Ig being only 40-50% of the total population (e.g., Table II). When cells labeled simultaneously at 37°C with fl-Con A, 20-100 μg/ml, and rh-RaMlg were examined individually it was found that all the rh-RaMlg caps were superimposed to a fl-Con A cap. The same result was obtained in the presence of 10^{-4} or 10^{-5} M vinblastine (Figs. 1, 2). The cells with double cap represented about 75% (71-77%) of the total number of fl-Con A caps, the remaining 25% being surface Ig-negative cells. Conversely, all the remaining surface Ig positive cells, which had rh-RaMlg rings, corresponded to cells with fl-Con A rings. This result indicates that fl-Con A caps tended to form preferentially on the subpopulation of Ig-positive lymphocytes (B lymphocytes), while only a minor fraction of Ig-negative cells were able to cap (Table II), accounting for the difference in the percentages of rh-RaMlg and fl-Con A caps. Moreover, the superimposition of rh-RaMlg and fl-Con A caps (Figs. 1, 2) suggested that surface Ig and Con A receptors were linked and moved together on the cell surface. This point was proved in the following experiments which showed that fl-Con A alone was able to cap all surface Ig.

Capping of Surface Ig by fl-Con A in the Absence or Presence of Vinblastine

fl-Con A caps were induced at 37°C. Then the cells were cooled down to 0°C (in the presence or absence of azide) to prevent further capping (38), stained with rh-RaMlg, and examined at ≤4°C. Control experiments showed that under these conditions there was no further capping of rh-RaMlg. The samples were scored at random for green fl-Con A caps and then examined for the distribution of the red rh-RaMlg label, or vice versa. Similar observations were made on cells with green or red rings. The results were unambiguous. A representative example is shown in Table III. At 20, 50, or 100 μg/ml of fl-Con A, all the Ig-positive cells with a fl-Con A cap had also a rh-RaMlg cap exactly superimposed to the fl-Con A cap (as in Figs. 1, 2). In only 2% of the cells at 20 μg/ml of Con A the mixed cap was accompanied by a thinner Con A or RaMlg ring, indicating incomplete capping. As in previous experiments, about one-quarter of the fl-Con A caps had no rh-RaMlg label. Conversely, none of the cells with fl-Con A rings had a rh-RaMlg cap, but as before, about 15-25% of the cells had rh-RaMlg rings, the remainder being surface Ig negative. At a fl-Con A concentration of 10 μg/ml, in two experiments the results were identical with those obtained at higher concentrations; in a third experiment, although all the rh-RaMlg caps were superimposed to a fl-Con A cap, in about one-half of these cells the fl-Con A cap was accompanied by a thinner fl-Con A ring surrounding the remaining part of the cell. Thus, in these cells the capping of fl-Con A was only partial, but the capped fraction had capped all the

### Table I

| Sample | Con A concentration (μg/ml) | rh-RaMlg caps (of the Ig-positive cells) | Inhibition |
|--------|-----------------------------|-----------------------------------------|-----------|
| 1      | 0                           | 94                                      | -         |
| 2      | 20                          | 63                                      | 33        |
| 3      | 100                         | 56                                      | 41        |

### Table II

| Percentages of Ig-Positive Cells, Ig Caps, and Con A Caps in a Typical Experiment | % |
|---------------------------------------------------------------------------------|---|
| 1) Ig caps/total Ig-positive cells (no Con A)                                    | 96|
| 2) Ig-positive cells/total Con A-positive cells                                   | 42|
| 3) Ig caps/total Ig-positive cells (50 μg/ml of Con A)                           | 86*|
| 4) Con A caps/total Con A-positive cells                                         | 47|
| 5) Ig caps/total A-positive cells                                                | 361|
| 6) % of Ig caps corresponding to Con A caps                                     | 100|
| 7) % of Ig rings corresponding to Con A caps                                    | 0 |

* 10% inhibition with respect to (2).
† This figure can be determined experimentally, but in this case was calculated from (2) and (3).

Spleen cells were incubated at 37°C with both fl-Con A and rh-RaMlg, as described in the text. The control (1) contained no Con A; all the other figures refer to a sample which contained 50 μg/ml of Con A.
TABLE III
Capping of Surface Ig by Con A (20 μg/ml) in the Presence or Absence of Vinblastine

| Sample          | rh-RaMlg caps | rh-RaMlg rings | Ig-negative cells | rh-RaMlg caps | rh-RaMlg rings | Ig-negative cells |
|-----------------|---------------|----------------|------------------|---------------|----------------|------------------|
| 1) No vinblastine | 37            | 0              | 10               | 0             | 39             | ND               |
| 2) 10^-4 M vinblastine | 70            | 0              | 20               | 0             | >30            | ND               |

The cells were incubated with fl-Con A (20 μg/ml) at 37°C for 15 min, and then stained with rh-RaMlg in noncapping conditions, as described in the text. The figures indicate the number of cells examined individually and include cells scanned either way: first for fluorescein staining, then for rhodamine staining, or vice versa. Con A caps and Con A rings were scanned sequentially and therefore the figures are not proportional to their relative frequency in the population. The relative frequency of Ig caps among Con A caps (as reported in the text) in this and similar experiments was calculated from the number of caps scored first for fluorescein staining.

ND, not determined.

detectable surface Ig. This indicates that only a part of the Con A receptors are actually associated with surface Ig, which is consistent with the fact that there are much less Ig molecules than Con A receptors on the lymphocyte surface (12, 30). In agreement with this point, when surface Ig were capped with rh-RaMlg at 37°C and the cells were then stained with fl-Con A in noncapping conditions (0°C, in the presence of azide), rh-RaMlg was found to have capped on almost all cells and was in fact mostly pinocytosed, but fl-Con A was still distributed as a smooth ring. Thus, the distribution of at least part of the Con A receptors is not modified by the capping of surface Ig.

These results indicate that in the presence of Con A, surface Ig are somehow linked to a fraction of the Con A receptors. Since it has been suggested that an indirect link between Con A receptors and surface Ig might involve microtubule or microtubule-like proteins (13, 45), some of the experiments reported above were repeated in the presence of 10^-4 M vinblastine. The results of three experiments, at a fl-Con A concentration of 20 μg/ml, were identical with those in the absence of vinblastine (Table III). About 75% (73, 78, 73) of the Con A caps were coincident with surface Ig caps showing that vinblastine did not affect the capping of surface Ig by Con A.

**Morphological Characteristics of Surface Ig and Con A Caps of Spleen Lymphocytes**

The appearance of the capped Ig was considerably different in the presence or absence of Con A. When surface Ig were incubated with rh-RaMlg alone at 37°C the few rings and the caps were mostly spotty and in fact most of the labeled surface appeared to be internalized in pinocytic vesicles within 10 min (the rate of internalization varies, however, with different antisera) (Figs. 3, 4). On the contrary, when rh-RaMlg was added after fl-Con A, or simultaneously with it, most of the label remained on the surface, superimposed to the fl-Con A label, and formed smooth caps or smooth rings with much reduced pinocytosis (Figs. 2, 5), which paralleled the slow pinocytosis of the fl-Con A itself. At 37°C the majority of the mixed caps were on cells with elongated shapes or with marked uropods (Figs. 6, 9), whereas the cells with rings remained spherical. Not infrequently the cap on elongated cells was located on the thicker part of the cell (Figs. 8, 9). The different distribution of rh-RaMlg suggests that, in the presence of Con A, surface Ig does not segregate into homogeneous patches (46), but remains dispersed over the areas covered by Con A. This was confirmed by electron microscope observations of spleen cells labeled with Con A, or fl-Con A, and rabbit anti-mouse Ig antibody coupled to ferritin (RaMlg-FT).

**Electron Microscope Observations**

Native Con A or fl-Con A can be detected on the surface of lymphocytes even without the help of an additional electron-dense marker as a fuzzy layer 11–15 nm thick (Figs. 10–12). This layer is easily detected on cells incubated with high concentrations of Con A (50 μg/ml or more), but is visible also at lower concentrations (20 μg/ml), although it appears more irregular and loose. This layer can be specifically labeled with anti-Con A antibodies coupled to ferritin (Fig. 15), but not by nonspecific antibody conjugates (cf. Fig. 11).
FlCURE 1 Spleen cells, labeled with fl-Con A (80 µg/ml) for 5 min at 37° C, then with rh-RaMlg for 10 min more at 37° C in the presence of 5 x 10^-6 M vinblastine. Distribution of fl-Con A staining.

FIGURE 2 Same cells as in Fig. 1. Distribution of rh-RaMlg staining.

FIGURE 3 Spleen cells incubated with rh-RaMlg for 10 min at 37° C. Phase contrast.

FIGURE 4 Same field as in Fig. 3. rh-RaMlg staining. All cells, but one, are labeled. Most of rh-RaMlg is endocytosed.

FIGURE 5 Cap of rh-RaMlg on spleen cell in the presence of 92 µg/ml of fl-Con A (10 min at 37° C). Almost no endocytosis of rh-RaMlg (compare with Fig. 4).

FIGURES 6–9 Caps on spleen cells incubated with fl-Con A and F(ab) - rh-RaMlg (superimposed to fl-Con A). In Figs. 6 and 7, the cap is on the uropod and encompasses the central constriction. In Figs. 8 and 9 the cap is located on the widest part of the cell.
When the spleen cells were incubated with high doses of native Con A or fl-Con A, cooled to 0°C, and labeled with RaMlg-FT in noncapping conditions, 40–50% of the cells were labeled with ferritin. The ferritin dots were not grouped in patches, as usual (8), but appeared to be dispersed at random as single molecules or very small clusters over the layer of Con A (Fig. 13). The same disperse distribution of ferritin molecules over the areas of the membrane covered by Con A was also obtained on cells incubated with lower concentrations of fl-Con A (20 μg/ml) (Fig. 14), despite the fact that the fuzzy layer of fl-Con A was much looser and uneven, and obviously considerably less Con A was bound to the surface (cf. 44). In several cells, the Con A layer formed a continuous, uninterrupted thickening (a cap) over about one-half of the cell surface, while the remaining surface was free of Con A (Figs. 10, 11, 13). In other cells corresponding to the rings of light microscopy, the layer covered most of the surface and was interrupted in some points by gaps of unlabeled cytoplasmic membrane. The regions of unlabeled membrane often corresponded to protrusions or microvilli. These gaps were usually too small and the fraction of surface area covered by Con A too high for the unevenness to be detected under the fluorescence microscope, where the rings around the maximum circumference of the cell appeared, as a rule, smooth and uniform. On both cells with caps and cells with rings, the RaMlg-FT molecules were absent from the areas of the membrane free of Con A indicating that surface Ig did not form small patches "trapped"
FIGURE 11 Detail of Fig. 10. Area opposite to the cap. No fuzzy layer or ferritin molecules are present on this part of the surface. The bar represents 0.1 μm. × 82,000.

FIGURE 12 Detail of the cap of Fig. 10, formed by an almost continuous fuzzy layer, about 15 nm thick, of Con A. Virtually no ferritin molecule is bound to this cell. Some transversely cut microtubules are visible near the perinuclear space. The bar represents 0.1 μm. × 82,000.

FIGURE 13 Detail of a cap of Con A from the same sample as in Figs. 10–12. In this cell several RaMlg-FT molecules are dispersed over the Con A layer, none on the areas free of Con A. The bar represents 0.2 μm. × 57,000.
between Con A patches, but were actually dispersed inside the Con A patches themselves. On visual inspection, the number of RaMIg-FT molecules on the Con A layer appeared to be less than that normally found on caps of RaMIg-FT alone, especially in samples at high Con A concentration, in agreement with the decreasing intensity of rhodamine fluorescence in similar conditions. This suggests that Con A at high concentration (>50 μg/ml) might sterically interfere with the binding of anti-Ig antibody.

No ferritin molecules, or only occasional ones, were found on the fuzzy layer of the remaining cells (Fig. 11). Nonspecific labeling due to the interaction of membrane-bound Con A with the Ig in the incubation medium was therefore negligible. Some nonspecific staining was noted only when the labeled antibody contained some aggregates not completely removed during the purification of the conjugate.

The same distribution was obtained when the cells were incubated and fixed at 37°C in the presence of both fl-Con A and RaMIg-FT. In this case Con A caps or mixed caps were found on typical uropods (Fig. 16), which were usually located on the side of the cell containing the centrioles.

Effect of Cytochalasin B and Vinblastine on Capping of Surface Ig and Con A Receptors

The results presented above show that on cells double-labeled with fl-Con A and anti-Ig antibody at sufficiently high concentrations of Con A (>10 μg/ml), the distribution of surface Ig is superimposed to that of the Con A receptors and has the morphological characteristics of the latter. The following experiments show that in the presence of Con A surface Ig indeed behave as Con A receptors in the way in which their ability to redistribute is affected by cytochalasin B and vinblastine.

Several groups (38, 21, 36, and unpublished...
results) have found that cytochalasin B, at doses of 10 μg/ml or more, can inhibit consistently, although only partially, capping of surface Ig. With the antiserum used in this study (rh-RaMlg), the level of inhibition varied between 10 and 70% and in most cases was about 30%. Contrary to an earlier report (38), but in agreement with later experiments done in more suitable experimental conditions, and at the higher resolution of the electron microscope (de Petris and Raff, unpublished data), cytochalasin B did not appear to inhibit pinocytosis of the labeled membrane, although the process may be slightly delayed. In the present experiments, pinocytosis was marked at 37°C, and many surface Ig caps obtained after 20 min in the presence of 10–20 μg/ml of cytochalasin B consisted largely of labeled intracellular vesicles accumulated at one pole of the cell, similar to those observed in the controls (Fig. 2). Microtubule-affecting drugs, like colchicine, Colcemid, and vinblastine (4, 22), had no inhibitory effect on capping of surface Ig (38, 44), even at doses of 10^-5 M, apart from a slight toxic effect at the highest doses of vinblastine (cf. Table IV). As described in detail elsewhere (7), however, when spleen cells were incubated with both cytochalasin B, at 10 or 20 μg/ml, and vinblastine or colchicine, at 10^-4–10^-5 M, capping of surface

| Sample | Con A caps* | rh-RaMlg | fl-Con A |
|--------|-------------|----------|----------|
| 1) Control | 0 | 92 | -- |
| 2) Vinblastine (10^-5 M) | 0 | 78 | -- |
| 3) Cytochalasin B (10 μg/ml) | 0 | 66 | -- |
| 4) Vinblastine (10^-5 M) + cytochalasin B (10 μg/ml) | 0 | 7 | -- |
| 5) Cytochalasin B (10 μg/ml) | 45 | <1 | 0 |
| 6) Vinblastine (10^-5 M) + cytochalasin B (10 μg/ml) | 45 | 0 | 0 |

*Percentage of caps in the subpopulation of Ig-positive cells. 
†The slight inhibition by vinblastine in this experiment was probably due to a slight toxic effect.
lg was virtually completely and reversibly inhibited, even at 37°C (Table IV). The inhibited cells maintained a spherical shape. The fluorescent antibody on the inhibited cells formed discrete spots distributed at random over the cell surface (Fig. 17). These spots were more numerous and smaller the higher the concentration of the microtubule-affecting drug.

On the contrary, capping of the fl-Con A preparation used in this study was completely and reversibly inhibited (98–100%) by 10 μg/ml of cytochalasin B alone, either in the presence or in the absence of vinblastine (7) (Table IV). The fluorescent Con A remained distributed as a smooth ring on a spherical cell (Fig. 18), although sometimes some pinocytotic vesicles could still accumulate at one pole of the cell (Fig. 19).

When surface lg were labeled at 37°C with rh-RaMlg on cells previously or simultaneously labeled with fl-Con A, their capping was completely inhibited in the presence of 10 μg/ml of cytochalasin B alone, in the same way, that is, as the capping of fl-Con A itself (Table IV) and rh-RaMlg remained distributed over the entire cell as a uniform ring (Fig. 21, 22).

**Reversal of Capping by Cytochalasin B**

It has been recently found that under suitable conditions Con A and anti-lg caps can be reversed into rings by cytochalasin B (7). The reversal can
be obtained, of course, only if the capped molecules are not so extensively cross-linked by the ligand as to form one single large complex. The extent of reversal is usually different for fl-Con A and rh-RaMlg caps (7).

With the fl-Con A preparations used in this study, addition of 10 μg/ml of cytochalasin B to a cell suspension in which Con A caps had been induced by incubation with 50 μg/ml of fl-Con A for 10 or 15 min at 37°C caused an almost complete reversal of caps into rings in about 30 min (Fig. 27). In many cells, the reversal was very rapid (Fig. 27; inset), the rate of reversal being comparable to the rate of cap formation (38), but in other cells it required at least 30 min at 37°C, or it was incomplete (Fig. 23). The staining of the "reverted" cells was fairly smooth, but less uniform than in cells in which capping had not occurred or had been inhibited by cytochalasin. This may be attributed to the formation of larger patches as a result of capping and to the presence of some pinocytosed materials, which remained at one pole of the cell. The reversal occurred either in the presence or in the absence of vinblastine (Fig. 27), which by itself was unable to cause any reversion. Reversion of fl-Con A caps could also be induced by depriving the cells of metabolic energy, for example, by adding 0.2% sodium azide to the cells suspended in PBS/BSA. The reversion at 37°C was comparable to that obtained with cytochalasin B (Fig. 28 A), although it seemed to be slower at lower temperatures (Fig. 28 B).

On the contrary, there was little or no reversion by cytochalasin B of surface Ig caps formed by rh-RaMlg at 37°C (Fig. 29). This did not appear to be, however, an intrinsic characteristic of surface Ig, but rather a characteristic of the surface Ig-anti-Ig complexes formed with the particular antisera used in this study, which induced pinocytosis and interiorization of most of the labeled membrane in 10 min at 37°C before the addition of cytochalasin B (cf. Fig. 4), leaving relatively little material on the surface. In fact, when pinocytosis was reduced, although not abolished, and true surface caps were formed, the latter could be reversed in about 60% of the cells by the addition of 15 or 20 μg/ml of cytochalasin B. This was obtained by incubating the cells with rh-RaMlg at lower temperature (23°C) and in the presence of 10^-5 M vinblastine (Fig. 29) (7). In the reverted cells the distribution of the fluorescent stain was more irregular and spotty than on cells labeled

![Figure 17](image17.png) Spleen cells incubated with rh-RaMlg in the presence of 20 μg/ml of cytochalasin B and 1 × 10^-5 M colchicine (preincubation 45 min) for 15 min at 37°C. Cells washed and kept for about 3 h at room temperature. Patchy rings.

![Figure 18](image18.png) Spleen cells incubated for 10 min at 37°C with 50 μg/ml of fl-Con A in the presence of 10 μg/ml of cytochalasin B. Uniform rings.

![Figure 19](image19.png) Spleen cell incubated for 15 min at 37°C with 45 μg/ml of fl-Con A in the presence of 10 μg/ml of cytochalasin B. Ring of fl-Con A with polar pinocytosis.

![Figure 20](image20.png) Spleen cell incubated with rh-RaMlg for 10 min at 37°C in the presence of 10 μg/ml of cytochalasin B (same experiment as in Figs. 3 and 4). Most of the stain is pinocytosed at one pole of the cell but a few spots remain scattered over the surface.

![Figure 21](image21.png) Spleen cells incubated with 50 μg/ml of fl-Con A for 5 min at 37°C, followed by rh-RaMlg for 10 min more at 37°C, in the presence of 20 μg/ml of cytochalasin B. Rings of fl-Con A.

![Figure 22](image22.png) Same field as in Fig. 21. Ring of rh-RaMlg (superimposed to a ring of fl-Con A of Fig. 21).

![Figure 23](image23.png) Spleen cells, incubated with fl-Con A (80 μg/ml) and rh-RaMlg for 15 min at 37°C, in the presence of 5 × 10^-4 M vinblastine. Reversion of caps by addition of 20 μg/ml of cytochalasin B; incomplete reversion of two fl-Con A caps after 20 min at 37°C.

![Figure 24](image24.png) Same field as in Fig. 23. Partial reversion of rh-RaMlg caps (superimposed to the fl-Con A caps of Fig. 23).

![Figure 25](image25.png) Spleen cells, incubated with rh-RaMlg 20 min at 23°C, in the presence of 5 × 10^-4 M vinblastine. A typical cap with little pinocytosis.

![Figure 26](image26.png) Same preparation as in Fig. 25, 35 min after addition of 20 μg/ml of cytochalasin B.
with fl-Con A, and considerable material, mostly
pinocytosed, still accumulated at one pole of the
cell (Figs. 25, 26).

When mixed surface Ig/Con A caps were
formed in the presence of fl-Con A and rh-RaMLg
at 37°C, these could be reverted in a virtually
complete way by adding 10 μg/ml of cytochalasin
B, either in the presence or in the absence of vinblastine (Fig. 27), and the rh-RaMLg stain
remained superimposed to fl-Con A (Figs. 23, 24).
These mixed caps behaved therefore as fl-Con A
caps. The rate of reversal of surface Ig caps
paralleled that of the fl-Con A caps themselves
(Fig. 27).

**Capping of θ Antigen and Con A Receptors on Thymocytes**

Mouse anti-θ antibody alone is unable to cap θ
antigen, but can do so when further cross-linked by
anti-mouse Ig antibody (RaMLg) (38, 10). In the
conditions of the present experiments, about two-
thirds of the thymocytes formed caps, whereas the
remaining cells showed patchy rings (Fig. 30). In
agreement with the results of Yahara and Edelman
(46), this capping was almost completely inhibited
when the cells were first incubated with 50 or 75

![Figure 27](image_url)

**Figure 27** Reversal of fl-Con A and mixed fl-Con A/rh-RaMLg caps by cytochalasin B. Spleen cells
incubated with 50 μg/ml of fl-Con A for 15 min at 37°C, in the presence (open circles) or absence (open
triangles) of 5 × 10⁻⁴ M vinblastine. To part of the sample with vinblastine, rh-RaMLg was added after 5
min and the cells were incubated for 10 min more (open squares). At the points indicated (arrows) 10 μg/ml
of cytochalasin B were added and the incubation was continued at 37°C. Control (−cy B) without
cytochalasin B. Inset: initial kinetics of reversion. Fl-Con A caps were induced as above, without
vinblastine, then 10 μg/ml of cytochalasin B were added (t = 0). 20-μl samples were taken at intervals and
diluted in 1 ml of 1% glutaraldehyde in PBS and fixed for 5 min at 24°C and 10 min at 4°C (washing). Cells
suspected in PBS and counted. Some autofluorescence may have led to a slight overestimate of the
percentage of rings.

![Figure 28](image_url)

**Figure 28** Reversal of fl-Con A caps with 30 mM sodium azide or 20 μg/ml cytochalasin B at 37°C and
30°C. Throughout these experiments (capping with 50 μg/ml of Con A for 10 min at 37°C, and reversal) the
medium was PBS containing 0.1% BSA. Solid line: reversal by cytochalasin; broken line: reversal by azide.
µg/ml of fl-Con A. In these conditions the cells labeled by θ/rh-RaM1g had the appearance of smooth uniform rings, superimposed to identical rings of fl-Con A (Table V; Fig. 31). Addition of 10⁻⁴ M vinblastine relieved the inhibition of capping in only a minority of cells (<20%); in these cells θ/rh-RaM1g caps were coincident and superimposed to fl-Con A caps (Figs. 32, 33).

**FIGURE 29** Reversal of rh-RaM1g caps. Spleen cells incubated with rh-RaM1g for 20 min at 23°C, in the presence or in the absence of 5 × 10⁻⁴ M vinblastine, then washed once. Cytochalasin B (20 µg/ml) added to both samples (arrow). Medium: L15/BSA. Similar results were obtained in PBS/BSA. The appearance of the caps before the addition of cytochalasin is shown schematically on the right.

**FIGURE 30** Thymus cells incubated with anti-θ antibody (15 min at 37°C) and rh-RaM1g (15 min at 37°C). Caps and patchy rings.

**FIGURE 31** Thymus cells incubated with anti-θ antibody (15 min at 37°C) and fl-Con A (80 µg/ml, 2 + 13 min at 37°C) plus rh-RaM1g (13 min at 37°C). Smooth rings of θ/rh-RaM1g. The distribution of fl-Con A (not reported here) was identical with that of θ/rh-RaM1g.

**FIGURES 32 and 33** Thymus cells treated as in Fig. 31, but in the presence of 10⁻⁴ M vinblastine. Two partial caps. Fig. 32: fl-Con A distribution. Fig. 33: θ/rh-RaM1g distribution. The fl-Con A preparation used in this experiment had an OD ratio of 0.77 (see Materials and Methods).
**TABLE V**

Inhibition of O Antigen/rh-RaMlg Capping by fl-Con A

| Exp. | Sample | θ/rh-RaMlg caps | fl-Con A caps | Type of θ/rh-RaMlg staining | Type of fl-Con A staining |
|------|--------|-----------------|--------------|-----------------------------|--------------------------|
| I 1) Control (no fl-Con A) | 70 | — | Caps and patchy rings | — |
| 2) fl-Con A (80 µg/ml) applied 2 min before θ/rh-M1g | <1 | 1 | Uniform rings | Uniform rings |
| 3) As no. 2, but with 10⁻⁴ M vinblastine | 19 | 19 | Caps and uniform rings | Caps and uniform rings |
| II 1) Control (no fl-Con A) | 69 | — | Caps and patchy rings | — |
| 2) θ/rh-RaMlg at 0°C, cells brought to 37°C with simultaneous addition of fl-Con A (50 µg/ml) | 8 | 4 | Caps* and patchy rings | Caps* and uniform rings |
| 3) θ/rh-RaMlg at 0°C, fl-Con A (50 µg/ml) added 3 min after the cells were brought to 37°C | 52 | 4 | Caps and patchy rings | Caps and uniform rings |

In exp. I, the cells were incubated at 37°C with anti-θ antiseraum (Ig fraction), washed twice at room temperature, and stained with rh-RaMlg for 15 min at 37°C, washed, and examined at room temperature. In exp. II the cells were labeled with anti-θ and RaMlg at 0°C, and fl-Con A (50 µg/ml) was added to the cells at 37°C, as indicated in Table V.

* All fl-Con A caps superimposed to θ/rh-RaMlg caps. θ/rh-RaMlg caps either superimposed to fl-Con A caps or accompanied by fl-Con A rings.

† fl-Con A caps corresponding, but not superimposed, to θ/rh-RaMlg caps.

fl-Con A inhibited capping almost completely even when the cells were first labeled in the cold by anti-θ and rh-RaMlg and then warmed to 37°C at the same time as fl-Con A was added: in this case anti-θ/rh-RaMlg label had formed discrete patches, on which was superimposed a continuous uniform ring of fl-Con A (Table V). In the case of θ antigen, therefore, preformation of patches of anti-θ/rh-RaMlg was not sufficient to overcome to any significant extent the inhibition caused by fl-Con A. In the few cells that had capped under these conditions, fl-Con A and rh-RaMlg labels were not always superimposed. When fl-Con A was added 3 min after the cells, prelabeled with anti-θ/rh-RaMlg in the cold, had been warmed to 37°C, 52% of the cells had rh/RaMlg caps, accompanied in more than 80% of the cases by uniform rings of fl-Con A. Obviously in these cells the capping of θ/rh-RaMlg shortly before or at the same time as the addition of Con A had no appreciable stimulatory effect on the capping of fl-Con A.

Attempts to induce capping of θ antigen by fl-Con A alone at 37°C, followed by labeling of θ antigen in noncapping conditions, as it was done in the case of surface Ig-positive spleen cells, were not conclusive in view of the small number of fl-Con A caps obtained and their tendency to slowly revert to rings during the two subsequent labeling steps with anti-θ and rh-RaMlg in noncapping conditions. The few observations made suggested, however, that Con A by itself was indeed capable of capping θ antigen, as in the case of surface Ig, although some of the θ antigen may have trailed behind the fl-Con A. This point is still under investigation.

**DISCUSSION**

**Relationship Between Con A Receptors and Surface Ig or θ Antigen**

The present experiments demonstrate that when Con A caps Con A receptors on the surface of Ig-positive spleen cells, surface Ig move into the cap together with the Con A receptors. At concentrations of 20 µg/ml or higher, and in most cases also at 10 µg/ml, Con A was able to cap all the detectable surface Ig, which were found closely
should not be surprising, considering that Con A by Con A than some of the other receptors. This surface Ig may thus be cross-linked more readily to another surface molecule carrying Con A receptors, although their concentration could be below the limit of detection of the methods employed.

Similar interpretation probably applies also to θ antigens on thymocytes, although the evidence is not as compelling. As can be judged from the immunofluorescence experiments, θ antigens (plus rh-RaMlg) appear also to be interspersed among Con A receptors and they appear to be completely superimposed to Con A, when they cocap at 37°C. This suggests that they too are a class of Con A receptors which can be cross-linked by Con A to other Con A receptors. The second possibility is the most likely since it has been shown that 8S surface Ig of mouse lymphocytes are, like other immunoglobulins, glycoproteins with mannose-containing carbohydrate side chains (1). In these conditions, surface Ig not only cannot exhibit their characteristic capping properties, but they behave essentially as Con A receptors. In the present experiments, this was expressed in the partial inhibition of Ig capping, the remarkable reduction of pinocytosis, and the different sensitivity to cytochalasin B in the inhibition and reversal of capping, all phenomena in which the behaviors of surface Ig on Con A-labeled cells paralleled that of the Con A receptors.

The cross-linking of surface Ig by Con A was apparently complete at all concentrations at or above 10 μg/ml. At 10 μg/ml, in some experiments and in part of the cells not all the Con A receptors to which Con A was bound were able to cap, but some were left behind to form a thin ring. The Con A receptors which were capped appeared to contain, however, all the detectable surface Ig. In this case the inability of some Con A receptors to cap was probably due to an insufficient degree of cross-linking. Considered as Con A receptors, surface Ig may thus be cross-linked more readily by Con A than some of the other receptors. This should not be surprising, considering that Con A receptors are probably a heterogeneous population, in valency and perhaps affinity (19), which may include different glycoproteins and possibly glycolipids. The dimeric surface Ig would be expected to be at least divalent with respect to Con A and to be easily cross-linked by a tetravalent Con A molecule. Monovalent receptors, for example, would be "trapped" in patches only if the degree of cross-linking were very high, as apparently it was in the present experiments. Even in this case some would probably remain isolated and would be left "behind" during capping, although their concentration could be below the limit of detection of the methods employed.

Effect of Vinblastine on Con A Capping

As surface Ig or θ antigens appear to be cross-linked by Con A to other Con A receptors, the partial or complete (44, 46) inhibition of capping of these molecules after prior exposure to Con A, most likely reflect the inability to cap of
the Con A receptors themselves. Vinblastine does not dissociate the movement of surface Ig from that of the Con A receptors. In the presence of vinblastine surface Ig can still be capped by Con A and in general behave as Con A receptors, for example in the response to cytochalasin, as they do in the absence of vinblastine. In this experimental system, therefore, vinblastine appears to counteract the inhibition of surface Ig capping by Con A by increasing the capping of Con A receptors with which surface Ig remain associated. This effect was not very marked in these experiments, but probably it was qualitatively similar to the more striking effect observed by Edelman et al. (13) and Yahara and Edelman (45). The reason for the quantitative differences in these two sets of experiments, done in similar conditions, is unknown. It may be due to differences in the Con A preparations, in particular in the ratio divalent/tetraivalent molecules, or may reside in some still undefined differences in the experimental conditions. From both sets of experiments it seems clear, however, that microtubule-affecting drugs, even at high doses, are unable to induce capping of Con A (and surface Ig) in all spleen cells (usually, in fact, in not more than 60%) or in the majority of thymocytes. Most B lymphocytes, but only a minority of T lymphocytes and very few thymocytes, are apparently able to cap spontaneously or to be released from inhibition by vinblastine. Other factors besides the presence of microtubules or microtubule-like protein, as, for example, structural differences in cells of different subpopulations, differences in types, valency, and concentration of Con A receptor-carrying molecules, in the degree of cross-linking, etc., must also be involved (see also Discussion, the last section).

Mechanism of Capping: Effect of Cytochalasin B

The capping characteristics of Con A receptors and surface Ig and their different response to various inhibitors can give some indication about the cellular structures involved in capping. The mechanism of this process is still unclear. The polar segregation of cross-linked molecules from other unlabeled membrane components apparently involves a countercurrent movement of the segregating molecules in the plane of the membrane, without any essential contribution of intracellular transport of membrane components (apart from the end process of pinocytosis), or formation of new membrane (8, 9). The characteristics of the capping process indicate that it does not occur by simple diffusion of the membrane macromolecules in a completely "fluid" membrane, but must involve the interaction, direct or indirect, of some of the segregating components with cytoplasmic structures which cause their displacement (8, 9).

The effect of cytochalasin B and vinblastine on inhibition and reversal of capping suggests that these structures include both contractile elements, presumably a system of filaments of actomyosin type, and microtubules.

Thin actinlike microfilaments, 40–60 Å thick, are the only structural elements clearly detectable underneath the lymphocyte membrane (Fig. 35), and at least part of them are able to bind heavy meromyosin (unpublished data). It is not known whether they are organized in more than one different functional system. It has been suggested that these microfilaments are part of the structural system which provides the mechanical force for the displacement of membrane components (8, 9). Cytochalasin B apparently impairs the function of the microfilament system(s) (42), although it does not seem to affect dramatically its structural integrity (14). Cytochalasin B has also other effects on cellular functions and in particular can inhibit the transport of sugars across the membrane (17, 23), affecting indirectly the energy metabolism of the cell. This cannot account, however, for the effects of cytochalasin in the present experiments, since identical results were obtained also in media not containing sugars (e.g., Fig. 28) or containing alternative metabolites. In earlier experiments (38, 21) cytochalasin B was found to inhibit capping of surface Ig, but in most cases the effect was only partial, and was not considered significant by some investigators (40). Cytochalasin B inhibits cell movement and cytoplasmic streaming (5, 43), phenomena which involve a coordinate and massive displacement of cellular material; it seems likely, however, that the block of the cytochalasin-sensitive structure is never complete and might still allow some limited movement of surface material, which could explain the variable
and partial effects observed in capping. The present experiments (together with data published elsewhere [7]) strongly support the conclusion that cytochalasin-sensitive structures are involved in capping and give some hints on the sites of action of this drug.

When the capping of β-Con A on a stationary spleen cell is followed under the light microscope, it often appears to proceed as is schematically indicated in Fig. 34. A small unlabeled projection, or "pseudopod," starts to form at one point of a previously uniformly labeled cell. This projection, which is usually connected to the cell body through a constriction, progressively enlarges as more and more material (including eventually the nucleus) accumulates in the projection and as the constriction moves relatively backwards. At the same time the labeled membrane concentrates progressively towards the rear of the cell, which is relatively stationary, and which may eventually form a uropod. Sometimes the labeled membrane may also encompass the constriction (Fig. 7). Some stages of this process are illustrated in Figs. 6-9. This sequence of events, which requires metabolic energy, is similar to that described by Loo (20) although in somewhat different terms. Loo considered the formation of a pseudopod as due to formation of new membrane, a process which does not seem necessary for capping to occur (8, 9). The morphological characteristics of capping, in particular the observation that cell elongation is mainly due to forward displacement of unlabeled material, suggests that interaction of membrane with cytoplasmic structures is involved both in this forward displacement and in the "anchoring" of the cross-linked patches which prevents them from following this movement. In the presence of cytochalasin B, no pseudopod is formed, the cell remains round, and the Con A cap does not form. If cytochalasin B is added after a cap has been allowed to form, the pseudopod retracts, the constriction, if present, disappears, and the cell returns to a spherical shape, and elements of the cap can mix again, at least partially, with the unlabeled membrane. Thus cytochalasin-sensitive structures seem to be involved both in the formation of the "constriction ring" and the active forward displacement of the unlabeled membrane, and also in the "anchoring" of the labeled membrane in the cap. The labeled patches can apparently be held together in the cap even when they are not cross-linked by the ligand into a single complex, as a fully cross-linked cap would be virtually irreversible. Although the formation of a single interconnected network of cross-linked molecules seems unnecessary for cap formation, the formation of patches of some adequate size is probably required, as is suggested by the dependence of capping on the receptor (antigen)-ligand ratio (38, 37).

The rate of cytochalasin-induced reversion of many Con A caps, (Fig. 27, inset) is surprisingly high and is dubious whether it could be accounted for entirely by free diffusion of molecules and patches in a stationary lipid bilayer. The sudden release of the cell from an asymmetrical shape might induce flow in the cytoplasm and passive formation of streams in the adjacent unlabeled membrane, which could accelerate the mixing. The reversion does not require, however, metabolic energy, but on the contrary can be induced by metabolic inhibitors (Fig. 28), as observed previously by others (34). In some cases Con A caps can apparently reverse spontaneously (20). The less efficient reversion of surface Ig caps as compared with that of Con A or mixed Con A/surface Ig caps may be accounted for by a higher degree of cross-linking and more accentuated pinocytosis of the labeled membrane. When the latter process is suitably reduced, a partial reversion can be observed.

The difference in effectiveness of cytochalasin B in inhibiting capping in different systems3 can be explained if this drug is in general unable to block completely limited surface movements, but is able to inhibit the relatively large displacements of cellular material which may be required to cap some of the surface components, such as the Con A receptors. The different requirements could be determined by differences in the interaction of the various surface molecules with cytoplasmic structures, or by quantitative factors, such as the degree of cross-linking, the size of the patches, and fraction of total surface area occupied by them. It should also be noted that in systems in which there is marked pinocytosis, as for example in the case of the anti-Ig antisera used in these experiments, but

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3This variability is demonstrated also by the fact that cytochalasin B failed to affect the capping of β/μ-RAIg on thymocytes, and was only partially effective when used in combination with vinblastine (unpublished data). These results may depend on the fact that "caps" on thymocytes may form by two different processes (36), indistinguishable at the light microscope level, of which only one corresponds to the mechanism of capping discussed in this paper.
FIGURE 34 Schematic diagram of the formation of a fl-Con A or a mixed fl-Con A/rh-RaMlg cap. For the explanation see text. Arrows indicate that unlabeled membrane is actively displaced forwards. Stippled areas with a bar perpendicular to the membrane represent cross-linked patches, directly or indirectly anchored to cytoplasmic structures. In reality, the cell shape is often more irregular and sometimes the process outlined in the figure stops at intermediate stages and the forward protrusion may be retracted. Although the cell elongates in the forward direction, capping does not require actual movement with respect to the substrate.

not in the case of fl-Con A, a polar accumulation of stain might result either from true cap formation followed by pinocytosis, or by nonlocalized pinocytosis followed by intracellular transport to the same pole. These two processes are difficult to distinguish, especially if both are fast, although the first seems to be more important. Cytochalasin B may affect the two processes to a different extent, although both surface and intracellular movements seem to be drastically reduced by the combination of cytochalasin B and vinblastine or cholechicine (7).

Microtubules and Capping

The role of microtubules in capping is less obvious and it became apparent in the surface Ig system only when the function of other structures was partially impaired by cytochalasin B. A controlling role of microtubule-like proteins in the inhibition of capping by Con A was suggested by Edelman et al. (13) and Yahara and Edelman (45). The synergic inhibitory effect of vinblastine and cytochalasin B on capping of surface Ig (7) is the first evidence indicating that microtubules may also contribute actively to the displacement and separation of membrane components. Microtubule contribution is not indispensable, since capping can occur also in the absence of microtubules, indicating that the capability of inducing some polarized displacement of membrane components may also reside in other structures. When microtubules are disrupted, however, the polarity of the cell is affected to some extent, e.g., the alignment of the cap with the centrioles is sometimes lost (unpublished observations). There is no unambiguous evidence that microtubules interact directly with the membrane, although in lymphocytes they are often found to run parallel to it (Fig. 36) underneath the layer of microfilaments, in particular along the sides of the cell, whereas they are absent from the frontal region of the pseudopod. It is possible that they interact with filaments, providing a kind of structural framework, with respect to which microfilaments and membrane components are relatively displaced (9). Since microtubules probably form a largely interconnected network, through their connection with the centrioles, they may be involved in the coordinate movement in one direction of multiple points of the surface with respect to other stationary points. In the absence of microtubules, these movements would become uncoordinated (e.g., occur en masse [3]), and could be further impaired by the action of cytochalasin B. Capping probably involves the triggering of coordinate relative displacements of surface and cytoplasmic material from some resting position, in which some surface points may be connected to internal structures, possibly microtubules, as suggested by Edelman and his associates (13, 45), and Ukena and Berlin for a different system (39). As microtubules seem also to participate actively in the process of capping, it is conceivable that, as this process is triggered, they pass from a stationary to a dynamic state in their interactions with the membrane. Apart from the possible connections of some receptors with cytoplasmic structures, surface Ig and Con A receptor (+ surface Ig) patches may differ in several respects, which may influence their ability to trigger these movements and may modify their capping characteristics. For example: (a) Ig patches are formed by a homogeneous population of surface molecules, Con A receptor patches are almost certainly heterogeneous (19); it is even possible that the average composition of the latter changes in different experimental conditions (e.g., low or high temperature; low or high Con A concentration), depending on the affinity, valency, and accessibility of the various subpopulations of molecules. (b) Ig patches are probably more stable, i.e., more efficiently cross-linked, because surface Ig, unlike most other surface antigens so far studied, is multivalent with respect to most anti-Ig antiserá; this is consistent with the fact that surface Ig is practically the only antigen which can be capped efficiently without the help of a second cross-linking antibody (37, 38). The valency of
the Con A receptors probably varies in different receptor molecules. Moreover, as deduced from a comparison with model systems, the association constant of an anti-Ig antibody site with a surface Ig determinant should be at least of the order of \(10^{-4}\) mol\(^{-1}\) (15, 25), whereas the association constant of a Con A-binding site with specific sugars is of the order of \(10^{-4}\) mol\(^{-1}\) (27). This would facilitate exchange of Con A receptor molecule of low valency bound to the individual Con A molecules, even if the latter were further cross-linked by anti-Con A antibody. (c) Whereas most anti-Ig antibodies are attached directly to the protein molecule, Con A binds to the carbohydrate chain(s), a type of binding which may cause less perturbation in the membrane structure. (d) At least in the case of some surface Ig classes (8S IgM molecules), other membrane molecules, such as the Fc receptors, may become specifically associated with the cross-linked surface Ig patches, but not with dispersed surface Ig molecules, possibly modifying the biological activity of the cross-linked surface Ig (B. Pernis, personal communication).
The inhibition of capping of surface Ig and Con A receptors by Con A could then express the inability of the Con A patches, unlike the homogeneous surface Ig patches formed in the absence of Con A, to trigger surface movements. An alternative possibility, more in line with the suggestions of Edelman et al. (13), is that the simultaneous binding to, and cross-linking of multiple points on, the surface by Con A actively paralyses the complete interconnected internal network, preventing coordinate surface movements of Con A patches and other molecules. The two alternatives are not mutually exclusive, as microtubule-disrupting drugs are unable to release all the cells from the inhibition. The second alternative is supported by the recent experiments of Rutishauser et al. with Con A-derivatized fibers, where there was no extensive cross-linking of surface Ig with Con A receptors (33). Even in this case, however, it seems likely that the inhibition of movement of surface Ig and other molecules is not caused by direct interaction of all these surface molecules with microtubules or microtubule-like structures, but probably occurs by a block of any active coordinate movement on the surface, i.e., of labeled as well as unlabeled surface areas, which would indirectly prevent the segregation of any surface molecule.

Irrespective of the precise mechanism of the inhibition, when colchicine and vinblastine disrupt the internal microtubule framework and by removing a physical constraint, and/or by favoring triggering, release the cells from the inhibition, they would induce an uncoordinated movement and allow capping of both Con A and surface Ig, which would occur with mechanical characteristics not completely identical to those taking place in the absence of the drugs.

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