EFFECT OF CONCANAVALIN A DOSE, UNBOUND CONCANAVALIN A, TEMPERATURE, Ca²⁺ AND Mg²⁺, AND VINBLASTINE ON CAPPING OF CONCANAVALIN A RECEPTORS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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SUMMARY
The capping of Concanavalin A (Con A) receptors induced by Con A was studied using human peripheral blood lymphocytes. The effects of Con A dose (5–100 µg/ml), pretreatment at 4 °C, unbound Con A, extracellular Ca²⁺ and Mg²⁺ and vinblastine were evaluated using Con A-horseradish peroxidase and electron microscopy. Lymphocytes incubated with Con A at 4 °C and fixed with glutaraldehyde exhibited Con A-horseradish peroxidase around the entire cell periphery. After raising the temperature to 37 °C, the Con A-horseradish peroxidase moved to form a cap at one pole of the cell and subsequently underwent endocytosis. Capping of Con A receptors induced by Con A at 37 °C was observed only at low Con A concentrations in the presence of unbound Con A and extracellular Ca²⁺ and Mg²⁺. Increased capping was found after pretreatment of cells with Con A at 4 °C, removing unbound Con A and/or removing extracellular Ca²⁺ and Mg²⁺, and by treatment with vinblastine. Following removal of both unbound Con A and extracellular Ca²⁺ and Mg²⁺, the percentage of capped cells at 37 °C was the same as on pretreatment at 4 °C under the same conditions. While pretreatment at 4 °C caused the breakdown of microtubules, removal of unbound Con A and/or extracellular Ca²⁺ and Mg²⁺ had no morphological effect on microtubules or microfilaments. Following exposure of lymphocytes to vinblastine and removal of unbound Con A, capping of Con A receptors by Con A was observed in over 90% of cells at all Con A dosages. However, when cells were exposed to vinblastine in the presence of unbound Con A the formation of Con A caps was either partially or completely inhibited.

INTRODUCTION
It has been observed that ligand receptors are randomly distributed on the surface of the cell membrane. The binding and crosslinking of multivalent ligands by lymphocytes and other cell types can lead to the formation of receptor spots or patches. This

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can be followed by energy-requiring receptor movement within the plane of the cell
membrane resulting in the formation of an aggregation or cap at one pole of the cell.
Caps can be endocytosed or shed leaving the cell surface denuded of capped receptors,
which are subsequently regenerated (Ault & Unanue, 1974; Bretscher, 1976; DePetris
& Raff, 1973; Greaves, Bauminger & Janassy, 1972; Gunther et al. 1973; Loor, Forni
& Pernis, 1972; Pernis, Forni & Amante, 1970; Taylor, Duffus, Raff & DePetris,
1971; Unanue, Perkins & Karnovsky, 1972). Based on the fluid mosaic model of cell
membranes (Singer & Nicolson, 1972) several suggestions concerning the modulation
of surface receptors by ligands have been made (Bretscher, 1976; Edelman, 1976;

The plant lectin, Concanavalin A (Con A) has been shown to be capable of inducing
as well as inhibiting the capping of its own receptors (Yahara & Edelman, 1973b,
1975); and also of inhibiting the capping of immunoglobulin receptors (Unanue
& Karnovsky, 1974; Yahara & Edelman, 1972). It has previously been reported using
murine lymphocytes that capping of Con A receptors by Con A at 37 °C is inhibited
unless the cells are first exposed to low temperatures or to antimitotic agents (Unanue
peripheral blood lymphocyte Con A receptors by Con A, we have found that under
appropriate conditions capping of human lymphocyte Con A receptors by Con A can
occur at 37 °C without prior low temperature treatment. Further, it was found that the
increase in capping of Con A receptors induced by vinblastine is blocked in the
presence of unbound Con A.

MATERIALS AND METHODS

Lymphocyte preparation

Heparinized (10 units/ml) venous blood was collected from healthy laboratory personnel.
The mononuclear cell fraction was separated using a Ficoll-Hypaque gradient (Boym, 1968)
and washed once with Hanks' balanced salt solution. The cells (5 × 10⁷) were subsequently
suspending in 40 ml of phosphate-buffered saline (PBS, pH 7·3) with or without Ca²⁺ (CaCl₂,
6·4 × 10⁻⁴ M) and Mg²⁺ (MgCl₂, 6·8 × 10⁻⁴ M) and centrifuged at 200 g for 10 min. The
washing procedure was repeated twice more. Cell number was determined using an Accu-Stat
blood cell counter and concentration adjusted to 1 × 10⁶ cells/ml. Cell viability was greater
than 95 % with or without the addition of 100 μg/ml Con A for 1 h using trypan blue dye
exclusion test. Haemocytometer counts indicated that over 90 % of the mononuclear cell
fraction consisted of lymphocytes.

Con A labelling

Human peripheral blood lymphocytes at a concentration of 1 × 10⁶/ml were incubated with
2·5-100 μg/ml of Con A (Type IV, Sigma) in PBS with or without Ca²⁺ and Mg²⁺. Incubation
was carried out at either 37 °C for 1 h or at 4 °C for 15 min, followed by incubation at 37 °C
for 45-50 min. Excess Con A was removed after 15 min at either 4 or 37 °C by washing the
cells 3 times with PBS with or without Ca²⁺ and Mg²⁺ at the appropriate temperature. In some
experiments, the lymphocytes were treated with 10⁻⁴ M vinblastine (Eli Lilly) at room tempera-
ture for 30 min prior to incubation at 37 °C for 45-50 min.
Horseradish peroxidase labelling of bound Con A

Following incubation with Con A, the cells were washed 3 times with PBS and fixed in 1 % glutaraldehyde-PBS for 30 min. The cells were again washed with PBS and incubated with 50 µg/ml horseradish peroxidase (HRP) (Type VI, Sigma) in PBS for 30 min at room temperature (Bernhard & Avrameas, 1971). Following a further wash, the HRP was localized by the diaminobenzidine reaction (DAB) (3,3'-diaminobenzidine tetrahydrochloride, Sigma) following the procedure of Graham & Karnovsky (1966). Cells were postfixed in 1 % osmium tetroxide at room temperature.

Electron microscopy

Following osmication, the cells were washed 3 times with PBS, centrifuged at 800 rev/min for 10 min and embedded in 4 % aqueous agar. After dehydration with graded ethanol and propylene oxide, the agar blocks were embedded in Epon-Araldite and ultrathin sections cut using a Porter Blum MT-2B ultramicrotome. Sections were examined unstained for analysis of Con A-HRP-DAB labelling, or stained with uranyl acetate and lead citrate to visualize microtubules and microfilaments. Sections were viewed in a Zeiss EM 9S-2 transmission electron microscope.

Analysis of results

In each experiment and at each Con A dose, several blocks were sectioned and a minimum of 100 cells were examined in thin sections. Cells were counted as being capped if the HRP label was confined to one half or less of the cell periphery (Fig. 1, p. 35). Care was taken to eliminate occasional granular leukocytes or monocytes from the count as determined by morphological appearance. Results were expressed as the percentage of cells examined which exhibited caps.

RESULTS

Preliminary studies were conducted with 10 µg/ml Con A in PBS containing Ca²⁺ and Mg²⁺ but without the presence of unbound Con A. Lymphocytes were pretreated at 4 °C with Con A and either fixed at this temperature or incubated at 37 °C for various times prior to fixation. When lymphocytes were pretreated with Con A and fixed at 4 °C, the Con A-HRP-DAB label was present around the entire cell periphery. After raising the temperature to 37 °C the label in many cells moved towards one pole of the cell to form a cap. Capping reached its maximum after 30 min with 50 % of the cells having capped within 10 min. Further studies using 2.5–100 µg/ml Con A under the same conditions showed that the intensity of labelling was dependent upon Con A concentration; the most heavy labelling occurring at 100 µg/ml. At 2.5 µg/ml Con A, the label was quite faint and it proved difficult to judge the extent of capping at this dose. Consequently, further experimentation was confined to Con A concentrations of 5–100 µg/ml.

The influence of Con A dose, pretreatment with Con A at 4 °C, unbound Con A, and Ca²⁺ and Mg²⁺ on the capping of human lymphocyte Con A receptors following incubation at 37 °C is shown in Table 1. It can be seen that regardless of other variables Con A receptor capping was in general progressively inhibited with an increase in Con A concentration. Capping of Con A receptors at 37 °C in the presence of Ca²⁺ and Mg²⁺ and unbound Con A was markedly evident only at the lowest dose
of Con A (Expt. 1). Pretreatment with Con A at 4 °C (Expt. 2) resulted in some increased capping at all Con A doses under the same conditions.

The frequency of capping at 37 °C increased with the removal of unbound Con A (Expt. 3). This increase was most evident at 10 and 20 µg/ml Con A. Pretreatment at 4 °C under the same conditions (Expt. 4), produced essentially the same or a slightly greater effect at the same Con A doses. However, the increased capping associated with lowering the temperature in Expt. 2 was still evident.

Table 1. The effect of Con A dose, temperature, extracellular Ca²⁺ and Mg²⁺ and unbound Con A on human peripheral blood lymphocyte capping of Con A receptors

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Temp., °C*</th>
<th>Ca²⁺ and Mg²⁺</th>
<th>Unbound Con A†</th>
<th>Con A, µg/ml</th>
<th>Percentage of capped cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37</td>
<td>4-37</td>
<td>+</td>
<td>5</td>
<td>72 73 80 94 83 88 92 91</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4-37</td>
<td>+</td>
<td>10</td>
<td>12 28 55 87 51 73 91 91</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4-37</td>
<td>+</td>
<td>20</td>
<td>3 19 38 63 48 48 69 84</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4-37</td>
<td>+</td>
<td>50</td>
<td>9 23 27 41 44 50 49 47</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4-37</td>
<td>+</td>
<td>100</td>
<td>3 9 11 11 2 8 23 38</td>
</tr>
</tbody>
</table>

* Lymphocytes were incubated with Con A for 60 min at 37 °C or for 15 min at 4 °C followed by 45 min at 37 °C.
† Excess Con A was removed after 15 min by repeated washing at the appropriate temperature.

After removal of Ca²⁺ and Mg²⁺ (Expt. 5), capping at 37 °C was markedly increased at 10, 20 and 50 µg/ml Con A. Pretreatment at 4 °C (Expt. 6) under these conditions produced essentially the same effect. Again the increased capping seen following incubation at 4 °C was still present.

In Expts. 7 and 8, both unbound Con A and Ca²⁺ and Mg²⁺ were removed. It can be seen that capping at 37 °C was now greater at all Con A doses than was found when unbound Con A or Ca²⁺ and Mg²⁺ were removed separately. It can also be seen that under these conditions lowering the temperature prior to incubation at 37 °C did not cause any further observable increase in the percentage of capped cells at most Con A doses.

Electron-microscopic examination of at least 100 cells for microtubules and microfilaments in each experiment and at each Con A dose as specified in Table 1 revealed the following: (1) microtubules, but not microfilaments, were absent or reduced in prevalence in the cytoplasm of cells incubated and fixed at 4 °C for 15–30 min (Fig. 2); (2) after increasing the temperature to 37 °C for 30 min, microtubules reappeared; (3) removing unbound Con A and/or Ca²⁺ and Mg²⁺ from the incubation medium had no apparent morphological effects on microtubules or microfilaments in cells incubated at 37 °C (Fig. 3); (4) microtubules and microfilaments were seen in different parts of cytoplasm and adjacent to the cell surface but there was no indi-
Fig. 1. A. A cell incubated with 10 μg/ml of Con A at 4 °C for 15 min, washed 3 times with Ca²⁺ and Mg²⁺-free PBS to remove unbound Con A and fixed with 2 % glutaraldehyde. Con A-HRP-DAB label is seen all over the surface. No staining. × 20000.
B. A cell illustrating a typical Con A cap at 37 °C represented by a dense HRP-DAB reaction product. No staining. × 20000.
Fig. 2. A cell incubated with 10 μg/ml Con A at 4 °C followed by fixation at the same temperature. No intact microtubules are visible. Compare with Fig. 3. Uranyl acetate and lead citrate. x 29000.

cation of their direct association with the cell membrane; and (5) although microtubules were often seen oriented towards the capped pole of the cell, they were occasionally found in other areas of the cytoplasm (Figs. 3–5).

Experiments were also performed to investigate Con A-HRP endocytosis and reappearance of Con A receptors in capped cells. Lymphocytes were preincubated in PBS containing Ca²⁺ and Mg²⁺ with 10 μg/ml Con A at 4 °C for 15 min. Excess Con A was removed by washing and the cells incubated for 30 min to 5 h at 37 °C. In
Fig. 3. Normal reformation of the microtubules in a cell that was incubated with Con A in Ca\(^{++}\)- and Mg\(^{++}\)-free PBS at 4 °C for 15 min and then at 37 °C for 45 min prior to fixation. Microfilaments can be seen interspersed with microtubules. Uranyl acetate and lead citrate. × 40,000.

Fig. 4. Micrograph of a portion of the lymphocyte incubated with 10 μg/ml Con A in PBS containing Ca\(^{++}\) and Mg\(^{++}\) at 4 °C for 15 min and then at 37 °C for 45 min prior to fixation. Microtubules (arrows) are most abundant in the vicinity of the centrioles seen in this portion of the cell. Except for the dense reaction seen at the upper left of the cell (arrowhead), Con A-HRP-DAB label has displaced from this half of the cell. Uranyl acetate and lead citrate. × 31,000.
Fig. 5. The cell seen in this micrograph was incubated with 10 μg/ml Con A at 37 °C for 45 min. Con A-HRP-DAB label has been capped. The microfilaments (arrows), which can be seen adjacent to nucleus, cell membrane or in cellular microextensions, however, are not restricted to the labelled area of the cell membrane. Uranyl acetate and lead citrate. ×40000.
Regulation of Con A capping in lymphocytes

Capped cells, endocytosis was indicated by the presence of the label within the membrane-bound vesicles in the cytoplasm. These vesicles began to appear within 1 h of incubation at 37 °C and were always located in the cap portion of the cytoplasm. Cells which had not capped also showed evidence of label endocytosis around the entire periphery of the cell membrane. In another set of experiments, following varying periods of incubation at 37 °C, 10 μg/ml Con A was again added to the medium for 15 min. This was followed by washing and fixing of the cells. It was then observed that approximately 35% of the cells did not cap and that it required 4-5 h of incubation before the Con A-HRP label could again be seen around the periphery of 90% of the cells examined.

Table 2. The effect of vinblastine in the presence or absence of unbound Con A and Ca^{2+} and Mg^{2+} on human lymphocyte capping of Con A receptors

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Ca^{2+} and Mg^{2+}</th>
<th>Unbound Con A</th>
<th>1st treatment</th>
<th>2nd treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>Con A*</td>
<td>Vinblastine</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>Con A</td>
<td>Vinblastine†</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>Con A</td>
<td>Vinblastine‡</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>Vinblastine</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Con A, μg/ml</th>
<th>Percentage of capped cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
</tr>
</tbody>
</table>

* Lymphocytes were incubated with Con A for 15 min followed by vinblastine (10^{-4} M) for 30 min at room temperature and at 37 °C for an additional 45 min.
† Lymphocytes were incubated with vinblastine (10^{-4} M) for 30 min at room temperature followed by Con A for 45 min at 37 °C.
‡ Lymphocytes were incubated with vinblastine (10^{-4} M) for 30 min followed by Con A for 15 min at room temperature. Excess Con A was removed by washing and the cells incubated at 37 °C for 45 min.
§ Lymphocytes were incubated with Con A for 15 min at room temperature, washed, and incubated with vinblastine for 45 min at 37 °C.

The effects of vinblastine on capping of Con A receptors are shown in Table 2. Comparison of Expt. 1 in Table 2 with Expt. 1 in Table 1 indicates that the addition of 10^{-4} M vinblastine had no effect on the percentage of capped cells in the presence of unbound Con A and Ca^{2+} and Mg^{2+}. Electron-microscopic examination of cells treated with vinblastine under these conditions revealed the absence of microtubules along with the presence of paracrystalline structures. These observations indicate that vinblastine entered the cells despite its failure to increase Con A capping (Figs. 6, 7) (Bensch & Malawista, 1969). As seen in Expt. 2, when lymphocytes were first treated with vinblastine they showed a marked increase in capping. However, capping did not increase above 90% at all Con A concentrations unless unbound Con A and Ca^{2+} and Mg^{2+} had been removed (Expt. 3). Expt. 4 suggests that it was the presence of
Fig. 6. The cell depicted in this micrograph was incubated with 10 μg/ml Con A at room temperature for 15 min in PBS containing Ca²⁺ and Mg²⁺, following which 10⁻⁴ M vinblastine was added to the medium and incubation carried out for 30 min at room temperature and another 45 min at 37 °C. Con A-HRP-DAB label is diffusely dispersed over the entire cell membrane. No staining. ×25,000.

Fig. 7. A portion of the cell treated exactly as in Fig. 6. Stained with uranyl acetate and lead citrate to reveal the vinblastine-induced microtubular paracrystals (arrow). ×65,000.
unbound Con A which inhibited the action of vinblastine on cells previously treated with Con A.

DISCUSSION

Our studies indicate that capping of Con A receptors by Con A could be increased by the pretreatment of cells at 4 °C, or by removing unbound Con A and/or extracellular Ca²⁺ and Mg²⁺. After removal of unbound Con A and Ca²⁺ and Mg²⁺, capping at 37 °C was generally equivalent to that seen following pretreatment of cells at 4 °C under the same condition; i.e. lowering the temperature did not cause any increase in the percentage of capped cells. Several studies have indicated a very low frequency of Con A-capped cells or none at all at 22 or 37 °C even after removal of excess Con A (DePetris & Raff, 1973; Yahara & Edelman, 1973b, 1975). However, other studies have reported that Con A capping occurred at 22 or 37 °C in a high percentage of cells (Greaves _et al._ 1972; Smith & Hollers, 1970; Stackpole _et al._ 1974). These contradictory observations could reflect different experimental conditions, different strains of animals or different animal species used in these studies.

Our studies on human lymphocytes indicate that under certain conditions pretreatment of cells with Con A at 4 °C increased the capping of Con A receptors at 37 °C. Similar findings have been reported earlier in murine lymphocytes (Yahara & Edelman, 1973b). Although more Con A is bound to the cell at 37 than at 4 °C, this temperature-dependent difference in Con A capping is apparently not due solely to the amount of bound Con A (Yahara & Edelman, 1973b). It has been suggested that temperature also affects the mobility of the receptor to which Con A binds. The mobility of Con A and other ligand receptors may in part be controlled by microtubules (Yahara & Edelman, 1973b). The increase in Con A-receptor capping following the pretreatment of cells with Con A at 4 °C has been attributed by some investigators to increased receptor mobility due to microtubule dissociation (Yahara & Edelman, 1973b, 1975). We observed that the percentage of capped cells after removing unbound Con A and extracellular Ca²⁺ and Mg²⁺ was the same as after pretreating cells with Con A at 4 °C under the same conditions. Further, removing unbound Con A and Ca²⁺ and Mg²⁺ did not result in the dissociation of microtubules.

Our findings that the incidence of Con A capping of Con A receptors increased after removal of extracellular Ca²⁺ and Mg²⁺ run counter to studies on the capping of murine immunoglobulin receptors which were not affected by removing extracellular divalent cations (Schreiner & Unanue, 1976; Taylor _et al._ 1971). There is evidence which suggests that membrane bound or intracellular calcium may play an important role in ligand capping. It has been shown by the studies of Freedman, Raff & Gomperts (1975) that the binding of Con A to T-lymphocytes results in a transient influx of extracellular calcium into the cell. A study by Schreiner & Unanue (1976) demonstrated that if intracellular Ca²⁺ was increased, capping of immunoglobulin receptors was inhibited and formed caps disrupted. Ryan, Unanue & Karnovsky (1974) reported that local anaesthetics and tranquillizers inhibit Con A- and immunoglobulin-receptor capping. As one explanation for their observations, these authors...
suggested that the drugs used in their studies prevented the release of intracellular calcium which in turn caused the inhibition of cytoplasmic structures involved in receptor movement. Poste, Papahadjopoulos, Jacobson & Vail (1975) observed that cell aggregation by Con A was increased following the use of local anaesthetics and suggested that this might result from an increase in membrane fluidity due to loss of membrane-bound Ca\(^{2+}\). Whether the presence of extracellular Ca\(^{2+}\) or Mg\(^{2+}\) directly or indirectly influences Con A-receptor movement is unclear. Our studies found that the inhibitory effects of unbound Con A on Con A-receptor capping could be reversed following removal of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) except at the highest Con A dose. This could indicate that the inhibition of Con A-receptor movement by unbound Con A is dependent upon the presence of extracellular divalent cations except at high Con A doses. Extracellular Ca\(^{2+}\) and Mg\(^{2+}\) could also influence the interaction of Con A receptors with cytoplasmic proteins which may restrict receptor movement. However, removal of Ca\(^{2+}\) and Mg\(^{2+}\) may simply reduce the number of Con A molecules bound to the cell surface and thus shift the dose-response curve.

Previous studies have indicated that Con A-receptor capping can be increased by the use of colchicine or vinca alkaloids (Yahara & Edelman, 1973a). We found that increased Con A capping induced by vinblastine is either partially or completely blocked if unbound Con A is present. This apparently is not because vinblastine was prevented from entering the cell. Our electron-microscopic analysis of vinblastine-treated lymphocytes in the presence of unbound Con A showed that microtubules were dissociated and paracrystalline structures formed. This would indicate that the entry of vinblastine into the cells had occurred (Bensch & Malawista, 1969). It has been suggested that the unbound Con A restricts receptor movement by cross-linking mobile receptors with receptors attached to cytoplasmic proteins sensitive to anti-mitotic agents. Treatment of cells with colchicine or vinblastine allows greater receptor mobility because few receptors remain in an attached state (Yahara & Edelman, 1973b, 1975). Our results suggest that unbound Con A may also effect receptor mobility through some other mechanism since vinblastine apparently entered the cells and became bound to the cytoplasmic sensitive proteins but did not induce maximal Con A-receptor movement.

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