THE RELATIONSHIP BETWEEN ENDOCYTOSIS OF CONCANAVALIN A AND PHYTOHAEMAGGLUTININ RECEPTORS AND BLAST TRANSFORMATION, AND DIRECT IDENTIFICATION OF INDIVIDUAL RABBIT LYMPHOCYTES REACTIVE TO BOTH MITOGENS

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SUMMARY

Distribution and modulation of rabbit lymphocyte phytohaemagglutinin acceptors and concanavalin A acceptors during activation of rabbit lymphocytes have been examined by electron microscopy. Two types of cell surface acceptors have been tentatively identified, lectin binding acceptors that do not modulate, and receptors that are endocytosed when blast transformation is stimulated. All of the cells have binding acceptors for both lectins. Endocytosis correlates with early blast transformation and serves as an early marker for lymphocyte activation. When examined after 24 h of culture, those cells that undergo blast transformation contain endocytosed lectin receptors, whereas small untransformed cells do not. Capping prior to endocytosis is rarely observed. The mechanism whereby the signal for transformation is maintained after the reaction of lectin with cell surface receptors and transposed to the nucleus is not known. Although we conclude that endocytosis is an early event required for cell activation, it is possible that endocytosis is secondary to other activation events. By evaluation of sequential endocytosis, individual rabbit lymphocytes that endocytose only concanavalin A, only phytohaemagglutinin, both concanavalin A and phytohaemagglutinin, or neither lectin, have been identified.

INTRODUCTION

The ability of plant lectins such as phytohaemagglutinin (PHA) and concanavalin A (Con A) to stimulate lymphocyte blast transformation has been used to study events leading to cell activation and to identify lymphocyte populations. One of the first things that occurs after the lectin has bound to mouse lymphocyte cell surface lectin-acceptors is the migration of the lectin-acceptor complex to form a cap at one pole of the cell (Greaves, Bauminger & Janossy, 1972; Unanue, Karnofsky & Engers, 1974; Edelman, Yahara & Wang, 1973; Loor, 1974). Capping is apparently mediated by a
submembrane actin–myosin interaction (Ash & Singer, 1976). The translocation of
the lectin–acceptor complexes in the plane of the plasma membrane is thought by
some workers to be a necessary step towards subsequent blast transformation (Taylor,
Duffus, Raff & DePetris, 1971; Bourguignon, Tokuyasu & Singer, 1978). Another
early event after lectin has bound to receptors on the cell membrane is endocytosis of
lectin–lectin acceptor complexes (Linticum, Elson, Mendelsohn & Sell, 1977;
Pauli et al. 1973). In this paper, it is shown that all rabbit blood lymphocytes have
acceptors for PHA and Con A, but only those lymphocytes that endocytose are trans-
formed by the lectins.

Since endocytosis may be used as an early marker for lymphocyte activation, direct
evaluation of cells reactive to lectins may be accomplished. Lymphocyte reactivity is
usually measured by quantifying mitogen-induced DNA synthesis. Estimates of
reactive cells are obtained by comparing the DNA synthesis of one population of cells
with that of a maximally responding population. Because the transformation response
is measured by incorporation of radiolabelled nucleotides into DNA, entire cell
populations, rather than subpopulations or individual cells, are examined.

Some attempts have been made to obtain more precise estimates of responding
lymphocytes by directly determining percentages of cells that transform into blasts
(Sell & Gell, 1965). This can only be done if the starting cell population does not con-
tain dividing cells and no cell loss occurs during the time required for transformation
to occur. In the rabbit, Con A stimulates over 60% of peripheral blood lymphocytes
(PBL) (Sell & Sheppard, 1975; Sell, Sheppard, & Redelman, 1973); PHA stimulates
over 50% of PBL (Sell et al. 1975; Sheppard, Redelman & Sell, 1976). These numbers
indicate that some lymphocytes in rabbit peripheral blood can be stimulated to trans-
form by either lectin. A similar overlap of reactive populations in cells from spleen
(Sheppard et al. 1976), lymph node and thymus suggests that some lymphocytes from
these organs are also capable of reacting with both lectins. However, these studies, as
well as those using incorporation of radiolabelled DNA precursors into DNA, require
the cells to be cultured for several days; during culture, transformation may be induced
by cell–cell interactions or soluble factors, rather than as a direct result of mitogen
binding. In this paper, the ability of individual rabbit lymphocytes to respond to both
PHA and Con A by endocytosis is examined. Since endocytosis is complete within
30 min, the possible effects of cell–cell interactions or soluble factors are minimized
as longer culture times are not necessary. On the basis of direct reactivity to these
mitogens, four subpopulations are identified.

MATERIALS AND METHODS

Preparation of lymphocytes

Peripheral blood lymphocytes were purified from whole blood obtained from the ear arteries
of adult rabbits. The blood was defibrinated and allowed to sediment in 3% pig-skin gelatin
for 30 min at 37 °C (Sell & Gell, 1965; Sell, 1974). The lymphocytes from the enriched super-
natants were washed free of gelatin twice, with warm (37 °C) DME medium, then centrifuged
at approximately 906 g on discontinuous Ficoll–Hypaque gradients (ρ = 1.077 g/ml). Between
90 and 95% of the resulting cells were small lymphocytes; greater than 95% of the cells were
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viable as judged by trypan blue exclusion. Prior to use, the cells were washed three times with phosphate-buffered saline containing 0.2 % bovine serum albumin (PBSA). Suspensions of spleen cells, popliteal lymph-node cells and thymus cells were prepared from tissue taken under sterile conditions from rabbits sacrificed by air embolism. Spleen cells were gently teased from the capsule using stainless steel mesh. After washing in warm DME, the cells were centrifuged on Ficoll-Hypaque gradients as described for PBL. Greater than 95 % of the cells were viable as judged by trypan blue exclusion. Prior to use, the cells were washed three times in PBSA. Cells from six rabbits were examined. There was less than ± 5 % variation among the six rabbits in the percentages of cells reactive in any given set of experiments.

Phytohaemagglutinin-ferritin conjugate (PHA-F)

PHA was covalently coupled to ferritin (F) (EM grade, Polysciences, Inc., Warrington, PA) by glutaraldehyde conjugation. A 50 mg sample of PHA-P (Difco, Detroit, MI) in o·1 M-sodium phosphate buffer (pH 7·2) was combined with 200 mg Fe in the same buffer; (total vol. 4 ml). This mixture was stirred at room temperature and 0·4 ml of 0·5 % glutaraldehyde was added. After 1 h, 0·4 ml of o·1 M-(NH₄)₂CO₃ was added and the mixture was dialysed overnight at 4 °C against phosphate-buffered saline (PBS), then centrifuged at 10 000 rev./min for 30 min in a Beckman SW-50L rotor. The resulting supernatant was centrifuged three times at 31 500 rev./min for 3 h in the same rotor. After each centrifugation, the pellet was resuspended in PBS by gentle mixing. Analysis by immunoelectrophoresis of the resulting solution revealed PHA coupled to F and free F, but no free PHA.

Preparation of Con A

Con A was prepared by elution of Jack Bean meal extracts from a Sephadex column as described previously (Sell & Sheppard, 1975).

Preparation of whelk haemocyanin (WH)

Haemolymph was collected from marine whelks (Busycon canaliculatum; Marine Biological Labs, Woods Hole, MA). The haemolymph was centrifuged at 10 000 rev./min in a Beckman SW-41 rotor; the resulting supernatant was spun three times at 31 500 rev./min for 2 h in the same rotor. After each centrifugation, the pellet was resuspended in 1 % NaCl containing 0·1 % NaN₃. Before use, the WH was dialysed against PBS overnight.

Ultrastructural labelling of lymphocyte PHA receptors

PHA-F conjugate (100 µl) was added to 3 x 10⁶ purified lymphocytes in 12 mm x 75 mm plastic culture tubes (Falcon no. 2052, Oxnard, CA). The cells were incubated at 4 °C for 30 min, then washed three times with ice-cold PBSA + NaN₃ (0·1 %).

Ultrastructural labelling of lymphocytes for Con A receptors

A 100-µl sample of Con A (1 mg/ml) was added to 3 x 10⁶ purified lymphocytes in 12 mm x 75 mm plastic culture tubes. After a 30 min incubation at 4 °C, the cells were washed three times with ice-cold PBSA + NaN₃. The cells were resuspended in 0·2 ml PBSA + NaN₃ and 100 µl of WH (90 mg/ml) was added. The WH, a glycoprotein containing Con A acceptors, bound to Con A on the cell surface during a second incubation at 4 °C for 30 min. The cells were then washed 3 times in ice-cold PBSA + NaN₃.

Endocytosis of lectin-lectin receptor complexes

Lymphocytes were labelled for PHA receptors or Con A acceptors as above and the labelled acceptor allowed to endocytose (Linthicum et al. 1977). No NaN₃ was added to the PBSA used. Labelled lymphocytes were washed in RPMI (Roswell Park Memorial Institute Medium) containing 10 % foetal bovine serum and penicillin (50 units/ml)/streptomycin (50 µg/ml). The cells were sampled into 96-well tissue culture plates (Limbro Scientific, Inc., Hamden CN) (5 x 10⁶ cells/well) in 0·2 ml of the same medium. Con A + WH (25 µl of each) or PHA-F (25 µl) was added to the appropriate wells. Six wells were harvested and pooled at 0 min, 30 min, 3 h, 6 h and 24 h.
Table 1. Mitogenic activity of Con A + WH and PHA-F

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Total counts/10^6 cells</th>
<th>Day 2–3</th>
<th>Day 3–4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A (10 μl)</td>
<td>36697</td>
<td>51373</td>
<td></td>
</tr>
<tr>
<td>Con A + WH (50 μl)</td>
<td>81159</td>
<td>89009</td>
<td></td>
</tr>
<tr>
<td>PHA (1 μl)</td>
<td>40400</td>
<td>51709</td>
<td></td>
</tr>
<tr>
<td>PHA-F (10 μl)</td>
<td>7975</td>
<td>22248</td>
<td></td>
</tr>
</tbody>
</table>

Values represent [3H]uridine incorporation by rabbit PBL incubated for 3 or 4 days with optimal amounts of Con A, PHA, Con A + WH or PHA-F as described in Materials and Methods. The c.p.m. for unstimulated cultures were less than 200 per culture. Each value represents the mean of six separate cultures, which varied less than ±10%.

The cells were washed three times in ice-cold PBSA + NaN₃. For sequential endocytosis experiments, the cells were labelled with a second lectin, and then incubated again for 30 min at 37°C. After transfer to the cold PBSA, the cells were processed for electron microscopy.

Fixation and electron microscopy

Cells were fixed as suspensions in 2% glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.2), for at least 1 h at 4°C. Post-fixation was carried out with 1% osmium tetroxide in the same buffer for 1 h at 4°C. Dehydration was accomplished with a graded series of acetone solutions and final embedding was in Epon 812 resin.

Thin sections (60 nm) were stained with uranyl acetate (1 h at 60°C), post-stained with alkaline lead citrate (3 min at room temperature) and viewed on Zeiss EM-10 electron microscope with an accelerating voltage of 60 keV. Over 100 individual cell sections were examined from each experiment for the presence of lectin–receptor labelling and/or endocytosis.

Because thin sections of cells were examined in gathering the data presented here, portions of a cell containing vesicles not seen in a given section might exist. Therefore, the percentages given should be taken as minimum values.

RESULTS

Mitogenic activity of PHA-F and Con A + WH

By incubating lymphocytes with various amounts of mitogen, the concentration of lectin or labelled lectin stimulating maximum incorporation of [3H]uridine by rabbit PBL was determined. Optimal concentrations of PHA, PHA-F, Con A and Con A + WH occurred as the peaks of bell-shaped curves; incubating the cells with either more or less mitogen gave less stimulation (data not shown). The incorporation of [3H]uridine in lymphocyte cultures exposed to optimal amounts of PHA, PHA-F, Con A and Con A + WH is shown in Table 1. Both PHA-F and Con A + WH stimulate DNA...
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synthesis significantly as compared to unstimulated cultures. PHA–F stimulates 40–45% of the response induced by free PHA when incorporation is assayed from 72 to 96 h of culture. Con A + WH, however, stimulates a greater amount of DNA synthesis than Con A alone. The addition of unconjugated F or WH alone to cell cultures does not stimulate above background levels. The optimal concentration of both Con A + WH and PHA–F identified in these experiments was used in all subsequent experiments.

**Labelling for PHA acceptors and Con A acceptors**

All rabbit PBL have PHA acceptors as demonstrated by the binding of the PHA–F conjugate. A cell labelled for PHA acceptors is shown in Fig. 1. The distribution of F grains on the plasma membrane is continuous and dense. The continuity and density of the labelling is essentially identical for all cells. Con A acceptors are also found on all rabbit lymphocytes from peripheral blood. The labelling of Con A acceptors by Con A + WH is shown in Fig. 2. The distribution of Con A acceptors also is both dense and continuous. Double labelling experiments, in which lymphocytes are labelled sequentially for one lectin acceptor and then for another, result in all cells being densely labelled for both PHA and Con A acceptors. An example is shown in Fig. 3.

Similarly, all lymphocytes from spleen, lymph node and thymus have both PHA and Con A acceptors when labelled with PHA–F or Con A + WH, respectively. The distribution of the label on cells from different organs was indistinguishable. Label was seen coating the entire plasma membrane with uniform density; no area of focal concentration was seen. Addition of unconjugated F or WH alone results in only 2–3% of the lymphocytes bearing the marker. In this case, labelling consists of 3–10 molecules/cell section and, thus, is extremely sparse and discontinuous.

**Endocytosis of PHA and Con A receptors**

To determine the distribution of the labelled lectins, PHA–F and Con A + WH, during the early stages of lymphocyte activation, lymphocytes were examined for the location of labelled lectin–lectin receptor complexes after warming. Cells labelled at 4 °C were incubated at 37 °C with no added NaN₃ for various times to allow modulation to occur. When peripheral blood cells treated in this way are examined by electron microscopy (EM), many have endocytosed the marker (Figs. 4 and 5). Endocytotic vesicles containing the markers varied in size, shape and number per cell section. Vesicles containing PHA–F were generally more electron-dense than those containing Con A + WH, especially after longer incubation times. The amount of surface label, although significant, was considerably less than that seen without incubation. The
percentages of PBL endocytosing lectin acceptors after incubations of 10 min, 30 min and 3 h are shown in Table 2. Although the number of endocytosing cells change between 10 min and 30 min, the number remains constant from 30 min to 3 h.

Endocytosis is also seen in lymphocytes from spleen, lymph node and thymus. Endocytosis occurs randomly around the cell. In some cell sections as many as six vesicles were seen. The size and shape of the vesicle vary. The lectin-reactive cells have no other morphological features that distinguish them from non-reactive cells. By examining cells for the presence or absence of endocytosis, the percentage of cells from each organ reactive with lectin can be quantified (Table 3). The lymph node contains the most Con A-reactive cells, followed by thymus and spleen. Lymph nodes contain the highest percentage of PHA-reactive cells; the spleen has the fewest.

**Cells endocytosing lectin transform into blast cells**

To determine whether endocytosis of lectin acceptors correlates with blast transformation, PBLs were labelled for lectin acceptors, then cultured with the same unlabelled lectin for 24 h. These cells were then examined by EM for blast characteristics and endocytosis of lectin acceptors. Table 4 shows the percentage of blasts and normal cells that had endocytosed the label for both PHA-F and Con A + WH. The percentage of cells undergoing blast transformation at 24 h is essentially equal to the percentage of cells endocytosing the label at 30 min. When cells are examined at 6, 12 and 18 h, the number of cells endocytosing lectin remains constant; and at 36 h, results are not different from those obtained at 24 h. Regardless of the length of incubation, the cells observed appeared viable by ultrastructural criteria whether they had endocytosed the lectin or not.

**Sequential endocytosis**

Because the percentage of cells from the various organs endocytosing each lectin indicate an overlap in lectin-reactive populations, experiments designed to detect cells reactive to both lectins were performed (Fig. 6). The procedure used in the experiments and the possible results are illustrated in Fig. 7. Cells were labelled and incubated with one lectin, then labelled and incubated with the other. The cells were then examined for endocytosis of either lectin, both lectins or neither lectin. Table 5 gives the results of the sequential endocytosis by PBL. Four populations of lymphocytes are seen; cells reactive with Con A or PHA only, cells reactive to both lectins, and cells reactive to neither lectin. The order of lectin labelling does not influence the results. Fig. 6 shows an example of vesicles containing both markers. All of the possible results indicated in Fig. 7 were seen.

These data are consistent with those for the total percentages of PBLs endocytosing...
Table 2. Kinetics of endocytosis by mitogen-treated peripheral blood lymphocytes

<table>
<thead>
<tr>
<th>Label</th>
<th>Incubation time</th>
<th>Cells with endocytosed label (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-F</td>
<td>10 min</td>
<td>28</td>
</tr>
<tr>
<td>Con A + WH</td>
<td>10 min</td>
<td>37</td>
</tr>
<tr>
<td>PHA-F</td>
<td>30 min</td>
<td>48</td>
</tr>
<tr>
<td>Con A + WH</td>
<td>30 min</td>
<td>53</td>
</tr>
<tr>
<td>PHA-F</td>
<td>3 h</td>
<td>42</td>
</tr>
<tr>
<td>Con A + WH</td>
<td>3 h</td>
<td>52</td>
</tr>
</tbody>
</table>

Cells were examined at 10 min, 30 min and 3 h for the presence or absence of endocytotic vesicles containing labelled lectins. A total of 200 individual cells was counted for each determination.

Table 3. Percentage of cells reactive to PHA and Con A in various tissues

<table>
<thead>
<tr>
<th>Label</th>
<th>% Reactive cells in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>Con A + WH</td>
<td>52</td>
</tr>
<tr>
<td>PHA-F</td>
<td>56</td>
</tr>
</tbody>
</table>

The percentage of lymphocytes reactive to lectin was determined by quantifying the number of cells endocytosing labelled lectin-lectin receptor complexes during a 30 min incubation at 37 °C. At least 100 cell sections from each of six rabbits, were examined to determine each percentage. The percentages varied by 5 % or less.

Table 4. Correlation between endocytosis and blast transformation

<table>
<thead>
<tr>
<th>Label</th>
<th>Blasts (%)</th>
<th>Small cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endocytosed label</td>
<td>Label not endocytosed</td>
</tr>
<tr>
<td>PHA-F</td>
<td>52</td>
<td>4</td>
</tr>
<tr>
<td>Con A + WH</td>
<td>58</td>
<td>3</td>
</tr>
</tbody>
</table>

Cells were examined at 24 h for simultaneous endocytosis and blast transformation. Blasts were identified on the basis of a small nuclear to cytoplasmic ratio, a small heterochromatin to euchromatin ratio, and increased amounts of polysomes and rough endocytoplasmic reticulum. At least 200 individual cells were counted for each determination.

each lectin alone. The percentage of cells reactive to PHA is the sum (34 %) of those reactive to PHA alone (14 %) plus those reactive to both PHA and Con A (20 %); 38% of the cells are reactive to PHA-F when used alone. Likewise for Con A, 28% of PBL are reactive to Con A alone, and 20% are reactive to both Con A and PHA. The sum of these, 48%, is quite close to the percentage (52%) seen when Con A–WH is used alone.
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Fig. 7. Sequential endocytosis by a Con A and PHA reactive cell. A cell reactive to both Con A and PHA (1) is first labelled with PHA-F, resulting in a continuous distribution of F grains over the cell surface (2). After an incubation for 30 min at 37 °C, endocytosis of some of the label occurs (3). The cell is then labelled for Con A acceptors with Con A + WH, resulting in a cell with both WH and F on its surface and containing F in intracellular vesicles (4). After a second incubation at 37 °C for 30 min, cells endocytosing both labels may contain individual vesicles with only one marker or vesicles containing both markers (5).

Table 5. Sequential endocytosis by PBL

<table>
<thead>
<tr>
<th>First label</th>
<th>Second label</th>
<th>% Cells reactive to</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-F</td>
<td>Con A + WH</td>
<td>PHA only</td>
</tr>
<tr>
<td>Con A + WH</td>
<td>PHA-F</td>
<td>14</td>
</tr>
</tbody>
</table>

Experimental protocol was as illustrated in Fig. 11. Cells were identified as reactive to the first lectin if they contained only vesicles of the first marker. Cells were identified as reactive to the second lectin if they contained only vesicles of the second marker and/or vesicles of both markers. Cells were identified as reactive to both lectins if they contained vesicles of the first marker and vesicles of the second marker or vesicles of both markers. At least 200 cell sections from each of six rabbits were examined for each sequential endocytosis experiment. The percentages for the six rabbits varied less than ± 4 %.

Table 6 gives the results of sequential stimulation of spleen, lymph node and thymus lymphocytes, respectively. Again, the data in this Table are consistent with those of Table 3. As with PBL, four subpopulations of lymphocytes are found in these organs as well.

**PHA and Con A do not compete for binding sites**

Because the experiments presented involve the sequential addition of lectins, the effect of acceptor binding by the first lectin on the binding of the second lectin was examined. This was done in two ways. First, cells were incubated for 30 min at 4 °C with free, unlabelled lectin and then subsequently labelled for the other lectin's
Table 6. Sequential endocytosis by cells from spleen, lymph node and thymus

<table>
<thead>
<tr>
<th>Cell source</th>
<th>% Cells reactive to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>Spleen</td>
<td>11</td>
</tr>
<tr>
<td>Lymph node</td>
<td>10</td>
</tr>
<tr>
<td>Thymus</td>
<td>34</td>
</tr>
</tbody>
</table>

Experimental protocol as in Fig. 11. Cells classified as in Table 5. At least 200 cell sections from each of six rabbits were examined for each sequential endocytosis experiment. The percentages varied less than ±5% for the six rabbits.

Acceptors. Preincubation with the unlabelled lectin has no effect either on the percentage of cells labelled for the second lectin, or on the distribution of the labels. Secondly, if the cells are labelled for one set of lectin-receptors, and then incubated for 30 min at 37 °C, many functional acceptors for that lectin are cleared from the membrane by endocytosis. To determine if the removal of these acceptors effects labelling with the second lectin, the cells were labelled for the acceptors of the second lectin after endocytosis was induced by the first. Binding of the second lectin after endocytosis was not visibly affected. Also, despite removal of acceptors for the first lectin, the distribution of the second marker was not affected; labelling remained dense and continuous on all cells.

Discussion

All rabbit PBL have acceptors for Con A and PHA but only those that endocytose the lectin–lectin receptor complex appear to be stimulated to transform into blast cells. Earlier studies also demonstrated that all lymphocytes in the mouse have acceptors for both lectins (Greaves et al. 1972; Stobo, Rosenthal & Paul, 1972). In our system the initial distribution of Con A and PHA acceptors is uniformly dense; PHA acceptors on human lymphocytes and Con A acceptors on murine splenic lymphocytes have been shown to be similarly distributed (Greaves et al. 1972). Although the density of 125I-labelled lectin was used to identify three different subpopulations of lymphocytes in the mouse by autoradiography (Taylor et al. 1971), we are unable to identify rabbit lymphocytes subpopulations on the basis of amount of label bound.

The present data suggest that capping is not a prerequisite event for blast transformation. Upon warming labelled cells to 37 °C, capping of Con A or PHA occurs on less than 5% of rabbit PBLs. Because thin sections of cells are examined by electron microscopy some capped cells might be missed due to the plane of sectioning. Using transmission electron microscopy, capping and clustering has been readily identified on lymphocytes of other species treated with ligands that bind to the cell surface (Gonatas, Antoine, Steiber & Avrameas, 1972; Karnovsky, Unanue & Leventhal, 1972; Kourilsky et al. 1972; DePetris & Raff, 1973). It would appear that capping is
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not an important event in the rabbit. On the other hand, an apparent correlation between lectin-induced endocytosis of lectin acceptors and subsequent blast transformation is demonstrated in the present study. Activation of rabbit PBLs with anti-immunoglobulin-surface immunoglobulin interactions has also been correlated to endocytosis and not capping (Linthicum & Sell, 1974; Sell et al. 1980). Thus, endocytosis appears to be an early marker for later blast transformation.

The mechanism by which lectin binding to lectin acceptors leads to blast transformation and mitosis remains unclear. However, any hypothesis devised must take into account the following points: (1) binding of lectin to its acceptor is a necessary step towards blast transformation; (2) migration of lectin–lectin acceptor complexes over relatively large distances in the plane of the plasma membrane, i.e. capping, seems not to be required in all systems, although localized migration into small patches may be important; (3) endocytosis of acceptors correlates well with later blast transformation; and (4) the processes leading to blast transformation can be reversed by competitive inhibitors of lectin binding for 18–24 h after initial lectin stimulation (Sell & Sheppard, 1974, 1975; Stenzel, Rubin & Novogrodsky, 1978).

In order for lectins to stimulate cells to transform, the binding of the lectin to its acceptor on the outside of the cell must be translated into effects inside the cell. Our data suggest that lectin must enter the cell via endocytosis for cell activation to occur. Whether cell activation results from the clearing of lectin acceptors from the plasma membrane or from intracellular effects of the internalized lectin is not clear. The signal may be directly related to the formation of the endocytotic vesicle or endocytosis could be a secondary event. It is possible that stimulation requires the removal of inhibitory cell surface molecules (Sell & Linthicum, 1975; Sell et al. 1980).

The possibility that stripping acceptors off the plasma membrane can trigger blast transformation is supported by the ‘piggy-back effect’ (Sell, Lowe & Gell, 1970). In this system, addition of a second antiserum directed against a first-stimulating antiserum may result in enhanced transformation. Increased transformation is seen despite a qualitative decrease in endocytosis. Even with less observed endocytosis, the label is removed from the surface of the cells. It is presumed that, in contrast to blocking, which results in dissociation of the mitogen from the cell surface receptor or prevention of further interaction of lectin with cell surface acceptor, for the piggy-back effect, the second antibody leads to enhanced cross-linking of the first antibody still bound to the cell surface, causing an increasing modulation of the cell surface receptor leading to enhanced stripping. Thus it appears that extensive cross-linking, such as that seen in piggy-back systems induces stripping of proteins from membranes rather than endocytosis. Endocytosis may occur when extensive cross-linking of the inhibitory surface proteins is not possible. The loss of surface inhibitors by stripping as a cell surface event related to activation of blast transformation is also supported by the finding that lectins bound to Sepharose beads (Greaves & Bauminger, 1972) or plastic dishes (Andersson, Edelman, Moller & Sjoberg, 1972a) can stimulate lymphocytes to proliferate. These bound lectins may induce transformation by stripping inhibitory proteins from the membrane, or the lectin may be endocytosed after being leached off the solid substrate.
Soluble mitogenic factors or interleukins produced by lectin-stimulated cells have been described in both murine (Andersson, Moller & Sjoberg, 1972b; Parker, Fothergill & Wadsworth, 1979; Moller, 1980; Dutton & Swain, 1981; Aarden, Brunner & Cerotinni, 1979) and human (Mackler, Wolstencroft & Drummond, 1972; Smith & Barker, 1972) systems, although some workers report that the mitogenic activity observed is caused directly by the stimulating lectin (Taylor et al. 1971; Sell & Linthicum, 1975; Sell et al. 1980; Elfenbein & Gelfand, 1975). The present results are difficult to explain by the effects of soluble factors and suggest direct cell activation by mitogens. If mitogenic factors were operating to cause endocytosis, they would not only have to be released by one cell and act on another in the short span of 30 min, but also they would have to induce endocytosis of lectin bound to the surface of the cell upon which they were acting.

Experiments described here identify individual lymphocytes reactive to both Con A and PHA in the peripheral lymphoid population by showing that single cells will endocytose both lectins. Because the data obtained with sequential stimulation (Tables 5, 6) correlate well with those obtained for each lectin alone (Table 3), it would appear that passive endocytosis of one label along with the other does not influence these experiments. Many previous methods for examination of lectin-reactive lymphocyte populations have not allowed for examination of individual cells. These methods, including incorporation of radiolabelled nucleotides into DNA and examination of blast cells, have revealed evidence suggesting that single lymphocytes might be stimulated by more than one lectin. In this laboratory, it was found that sequential stimulation of rabbit lymphocytes by two different mitogens resulted in a synergistic response, even when the effect of the first lectin was blocked some time after addition by a competitive binding agent (Sell et al. 1975). Fanger, Reese, Schoenberg & Reese (1974), using anti-thymocyte globulin to block mitogen responses in rabbit lymphocytes also obtained evidence that Con A and PHA stimulate possibly overlapping sub-populations. A subpopulation of T cells reactive to both Con A and PHA has been described in the mouse (Stobo & Paul, 1973), and additional confirmatory evidence for an overlap in cells reacting to different mitogens has also been published (Andersson, Coutinho & Melchers, 1979).

The results presented in this paper indicate that even though PHA and Con A acceptors are both distributed densely and continuously over the surface of all lymphocytes, the functional receptors for PHA and Con A are distinct. It appears that one must distinguish between functional receptors (endocytosis) and non-functional acceptors (binding only). Because pretreatment of cells with one lectin does not block binding of the second, we conclude that PHA cannot block the binding of Con A or vice versa. PHA and Con A also do not compete for binding sites on mouse lymphocytes (Greaves et al. 1972). That cells are capable of endocytosing a second lectin after having done so to the first further suggests that there are also different functional receptors for Con A and PHA.

The technical assistance of R. M. Cheri Lloyd and Roxanne Holdbrook is gratefully acknowledged. This investigation was supported by Grant no. CA 16367, awarded by the National Cancer Institute, DHEW.
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(Received 30 October 1981 - Revised 4 January 1982)