Receptor Sites for Concanavalin A on Human Peripheral Lymphocytes and on Lymphoblasts Grown in Long-Term Culture

Louis De Salle, Nobuo Munakata, Richard M. Pauli, and Bernard S. Strauss

Department of Microbiology, The University of Chicago, Chicago, Illinois 60637

SUMMARY

Human lymphoblasts in long-term culture are specifically agglutinated by concentrations of concanavalin A that do not agglutinate normal human lymphocytes. The agglutination is inhibited by α-D-methyl mannoside. Lymphoblast lines kept in long-term culture bound 2 to 3 times more concanavalin A per cell than did normal lymphocytes. Normal lymphocytes and lymphoblasts bound approximately equivalent amounts of concanavalin A per unit surface area.

INTRODUCTION

Cells transformed with the DNA viruses SV40 or polyoma agglutinate on treatment with conA (11, 14, 21), a protein with specificity for α-glucosides (12). Normal cells are not agglutinated by conA, at least not at the low doses that agglutinate transformed cells, and it has been suggested (13) that agglutinability measures a change in surface distribution of receptors necessarily associated with both transformed and naturally malignant cells (3, 6, 14, 21). However, not all viral transformations need yield agglutinable cells, since Moore and Temin (18) have reported that cells transformed with RNA tumor viruses need not show increased agglutinability.

In these experiments, we have studied the agglutinability and ability to bind conA of isolated human peripheral blood lymphocytes and of long-term lymphoblast cultures derived from leukocyte cultures. Since such lymphoblast cultures are very probably the result of "transformation" of lymphocytes by association with or activation of the Epstein-Barr virus, this comparison may well be equivalent to a comparison of normal and transformed human cells. It had previously been observed that human lymphatic leukemia lymphocytes were more agglutinable by conA than were normal lymphocytes (15) and that mouse lymphoid leukemia cells bound more conA than do normal lymph node cells (17). Our experiments confirm these observations. However, we find that, per unit surface area, lymphoblast lines kept in long-term culture may have somewhat fewer binding sites than do normal cells.

MATERIALS AND METHODS

Lymphocytes were isolated from freshly drawn human peripheral blood by a modification of the method of Böyum (5), as previously described (19). Preservative-free heparin (10 units/ml; Nutritional Biochemicals Corp., Cleveland, Ohio) was present in each wash to facilitate resuspension. This procedure results in a concentration of approximately 0.5 unit of heparin per ml in the final preparation. This method of isolation gives a preparation of lymphocytes (plus monocytes) that is virtually free of erythrocytes and granulocytes.

Long-term cultures were established by means of a modification of the method of Choi and Bloom (7, 8). Heparinized blood was layered above a dextran-Hypaque mixture, and the erythrocytes were allowed to sediment for 30 min at 37°C (4). The leukocyte-rich plasma was removed, and the cells were collected by centrifugation, washed twice in medium, and resuspended at an initial concentration of 0.5 to 1.0 X 10^6 cells/ml. Thirty ml of this solution were added to each culture flask. One ml of a lysate (that had been prepared by freezing and thawing of a concentrated suspension of cells of a previously established lymphoblastoid line) was added to each flask. One-half of the medium was replaced every 5 days for 12 weeks, or until growth (signaled by clumping and increased acidity) was initiated. Cell line UM-3 was established in the laboratory of Dr. A. Bloom and was generously made available to us. All other long-term lines used were established in this laboratory from blood drawn from normal, healthy donors. All lines had a normal, diploid karyotype when first established.

Freshly isolated lymphocytes were cultured in Medium M199 (Grand Island Biological Co., Grand Island, N.Y.), which contained 20% fetal calf serum (Grand Island Biologicals) and penicillin-streptomycin, at a cell concentration of 0.8 X 10^6 cells/ml.

Long-term lymphoblastic lines were established and maintained in Roswell Park Memorial Institute Medium 1640 (Grand Island Biologicals) plus 20% tissue culture select fetal calf serum (Baltimore Biological Co., Cockeysville, Md.) and antibiotics, in 250-ml tissue culture flasks (Falcon Plastics, Los Angeles, Calif.). All of the sera were heated at 56°C for 30 min before use. Cells were cultured at 37°C in an atmosphere of 95% air and 5% CO₂.

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2Present address: Division of Biology, The University of Texas at Dallas, Box 30365, Dallas, Texas 75230.

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4The abbreviations used are: conA, concanavalin A; PBS, phosphate-buffered saline; MAM, α-D-methyl mannoside.

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For agglutination, either lymphoblastic cells or peripheral lymphocytes kept in culture for at least 24 hr were harvested by centrifugation and were washed 3 times with calcium- and magnesium-free PBS (10). Cells were resuspended to a final concentration of 4 to 5 × 10⁶/ml. Equal volumes of cells and agglutinin were mixed in 25-mm Petri dishes (for large volumes) or in Falcon microtest plates (for small volumes). The conA was obtained from Calbiochem (Los Angeles, Calif.). Each plate or well was scored after 15 min of incubation at room temperature, as follows: —, no agglutination; +/−, equivocal agglutination; +, many clumps of 3 to 5 cells; or ++, many small clumps and some clumps of 10 to 20 cells. The specificity of agglutination was determined by inhibition, with a final concentration of MAM, 10 mg/ml (Sigma Chemical Co., St. Louis, Mo.) (Table 1).

Cell diameter was determined by measurement with an ocular micrometer that was calibrated by means of a stage micrometer.

Iodination of conA with Na¹²⁵I (Amersham-Searle Corp., Arlington Heights, Ill.) was carried out by a modification of the lactoperoxidase method of Marchalonis (16). After iodination, the conA (Calbiochem) was purified by adsorption onto a column of Sephadex G100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The column was washed with 6 column volumes (about 200 ml) of 0.15 M NaCl, and then the adsorbed conA was eluted with 0.2 M α-D-methyl glucoside (Sigma) in 0.15 M NaCl (2). The eluate was dialyzed to remove the glucoside and unbound iodide, and the concentration of protein was determined spectrophotometrically, with the use of an extinction coefficient of 11.4 at 280 nm for the calculation (1). We determined the radioactivity of this isotope in a scintillation counter, using a cocktail made up as follows: Permablend (Packard Instrument Co., Downers Grove, Ill.), 5 g; toluene, 667 ml; and Triton X-100 (Emulsion Engineering, Elk Grove, Ill.), 333 ml. Comparative measurements indicate that we realized about 77% of the counts obtainable with a γ counter for a total counting efficiency of about 36%.

Binding experiments were carried out as follows. Freshly isolated lymphocytes were incubated for at least 1 day in tissue culture medium plus fetal calf serum before use, to allow adherent cells to attach. Long-term lymphoblast cultures were maintained in tissue culture medium and were tested 3 days after transfer. Cells were harvested by centrifugation, washed in PBS Solution A (10), resuspended in PBS Solution A, and distributed into 10-ml conical centrifuge tubes containing labeled conA. The final concentration was about 2 × 10⁶ cells/ml. In addition, control samples contained 0.3 M α-D-methyl glucoside. Samples were incubated for 1 hr at 25°C. The cells were pelleted by centrifugation at 400 × g for 3 min and then were resuspended and washed three times in 3 ml of PBS Solution A. The washed cells were solubilized with 0.2 ml of 0.3 M KOH, and the solution was poured into counting vials. The tubes were rinsed with two 5-ml aliquots of scintillation fluid, which was poured into the vials. The scintillation

Table 1
Agglutination of lymphocytes and long-term lymphoblasts by conA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MAM</th>
<th>5 µg/ml</th>
<th>50 µg/ml</th>
<th>100 µg/ml</th>
<th>500 µg/ml</th>
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<tbody>
<tr>
<td>Normal lymphocytes</td>
<td>A¹</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>UM-3</td>
<td>A</td>
<td>−</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RMP-E01</td>
<td>A</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RMP-RL3</td>
<td>A</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RMP-RL1</td>
<td>A</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RMP-ES1</td>
<td>A</td>
<td>±</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>RMP-ES2</td>
<td>A</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RMP-ES3</td>
<td>A</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RMP-ES4</td>
<td>A</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

¹ A, absent; P, present.
cocktail was then neutralized with 1.0 ml 5% trichloroacetic acid, and the mixture was counted in a Packard Tri-Carb scintillation spectrometer.

RESULTS

Long-term lymphoblast cultures were agglutinated by concentrations of conA which had no effect on normal lymphocytes in our test system (Table 1). The agglutination was reversed on addition of MAM indicating its specificity (12, 22). There are approximately 3 to 20 cells in the agglutinated clumps (Fig. 1). Our findings are comparable to those previously obtained for lymphatic leukemia cells (13, 15). Agglutination of normal cell suspensions can be observed when concentrations of conA that are greater than 500 µg/ml are used, when very high effective cell concentrations are used (for example, when mixtures of cells and conA are centrifuged and resuspended), or when cells held in medium for 2 days or longer are used (see also Ref. 3).

Fresh lymphocytes, stimulated with conA (10 µg/ml, 2.5-ml cultures, 0.8 to 1.0 x 10⁶ cells/ml) and incubated for 42 hr, were assayed for agglutinability after reversal of the initial binding of conA by incubation for 6 hr in MAM; next, they were washed with MAM and then were washed again thoroughly with PBS Solution A. These cells were agglutinable (+ on our scale, with 100 µg of conA per ml). However, proliferation alone cannot completely account for the agglutinability of lymphoblasts in long-term culture, since lymphoblasts grown to saturation in cultures in which proliferation had stopped also were agglutinable.

Binding studies were carried out with 2 long-term lymphoblast lines and with a series of normal cell preparations isolated at different times. We define the amount of conA specifically bound by cells as the fraction of the total that can be removed by competition with 0.3 M α-D-methyl glucoside.

A summary of all our data is given in Table 2; all values are...
Table 2
Binding of conA

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Experiment</th>
<th>Cell no. (x 10^6)</th>
<th>Intercept: 1/μg bound/10^6 cells</th>
<th>Maximum μg conA bound/10^6 cells</th>
<th>Av. μg bound/10^6 cells</th>
<th>Av. no. of molecules bound/cell a</th>
<th>μg bound/sq μm (x 10^3)</th>
<th>Av. μg bound/sq μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1</td>
<td>2.00</td>
<td>1.176</td>
<td>0.850</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>2</td>
<td>2.20</td>
<td>1.810</td>
<td>0.552</td>
<td></td>
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<tr>
<td></td>
<td>3</td>
<td>2.37</td>
<td>2.950</td>
<td>0.339</td>
<td>0.663</td>
<td>5.8</td>
<td>1.9</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.88</td>
<td>1.550</td>
<td>0.644</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.02</td>
<td>1.076</td>
<td>0.929</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoblast</td>
<td>UM-3</td>
<td>1</td>
<td>2.03</td>
<td>0.577</td>
<td>1.732</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UM-3</td>
<td>2</td>
<td>2.00</td>
<td>0.454</td>
<td>2.200</td>
<td>1.921</td>
<td>17</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>UM-3</td>
<td>3</td>
<td>2.05</td>
<td>0.534</td>
<td>1.830</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoblast</td>
<td>RMP-EO1</td>
<td>1</td>
<td>1.50</td>
<td>0.698</td>
<td>1.433</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RMP-EO1</td>
<td>2</td>
<td>1.93</td>
<td>0.770</td>
<td>1.299</td>
<td>1.366</td>
<td>12</td>
<td>2.6</td>
</tr>
</tbody>
</table>

a Assuming a molecular weight for conA of 68,000 (1). The experiments with normal lymphocytes were carried out with cultures of the following age: Experiments 1, 3, and 5, 1 day after isolation; Experiment 2, 2 days after isolation; Experiment 4, 3 to 4 hr after isolation of normal lymphocytes.

Based on least-squares calculations. The mean cell diameter was used to calculate the surface area based on the approximation that the cells are spheres. A normal frequency distribution was obtained for fresh lymphocytes and for cell line RMP-EO1. Line UM-3 had a proportion of giant cells that skewed the distribution slightly (Chart 4).

DISCUSSION

Long-term lymphoblast cultures contain cells that are larger than normal lymphocytes and that bind more conA per cell. However, when the binding is compared on the basis of unit area, there is no significant difference between the 2 types of cell; if anything, the lymphocyte may have slightly more binding sites per unit area. This means that the difference in agglutinability of long-term cells and fresh lymphocytes cannot be accounted for by an increased number of binding sites. In addition, observations by Mr. P. Higgins (in this laboratory), with the use of fluorescein-labeled conA, indicate that all cells in a normal lymphocyte preparation are able to fluoresce after incubation with this substance, indicating that essentially all cells in a normal population have conA binding sites. Transformation may result in a geographic redistribution of constant numbers of binding sites which in turn is associated with an increase in agglutinability (9, 20, 21, 23).

This hypothesis was developed as a result of observations of mouse cells transformed with small DNA viruses. Our experiments indicate that the agglutinability of human lymphoblast lines associated with a large DNA (Epstein-Barr) virus is not correlated with an increased number of binding sites per unit area. If the hypothesis of geographic redistribution is correct, a similar redistribution occurs in lymphocytes stimulated with mitogen, since such cells do agglutinate.

We wish to comment on the variability of the results obtained with fresh lymphocytes as compared with the relative uniformity of our results on the binding of conA to long-term cultures (Table 2). The lymphocyte preparations come from different populations of cells and, in addition, probably contain small but variable percentages of different cell types. On the other hand, the lymphoblast cultures, although not cloned, may well represent the progeny of only 1 or at most few cells that have been further selected by continued growth to give a uniform population.
REFERENCES


Fig. 1. Agglutination of lymphoblasts by conA. Top, lymphoblast strain UM-3; middle, lymphoblast strain RMP-EO1; bottom, normal lymphocytes; left, plus conA, 100 μg/ml; right, plus conA and MAM, 10 mg/ml; center no addition.
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