Peripheral Blood Lymphocyte Receptors for B-Lymphoblastoid Cell Lines (B-LCL)

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Because interactions between B cells and T lymphocytes are of fundamental importance in the generation of the immune response to most antigens, we attempted to identify the cells capable of binding B-lymphoblastoid cell lines (B-LCL), their tissue distribution, and their presence in other species. Cells bearing a surface receptor for B-LCL were found in human peripheral blood, tonsil, and bone marrow, as well as mouse and rat spleen. Binding cells are phenotypically heterogeneous. The majority are T cells as defined by their ability to bind sheep red blood cells (E-rosettes). However, a subpopulation of non-T-lymphocytes were capable of binding B-LCL. This was demonstrated by depleting T cells with an E-rosette centrifugation technique and then performing a double rosette assay. The wide distribution of T lymphocytes with receptors for B-lymphoblastoid cells within peripheral lymphoid organs and their presence in several species suggest that these surface molecules may represent one of the means by which T cells and B cells interact in the induction of the immune response to T-dependent antigens.

Human lymphocytes can be phenotypically distinguished on the basis of characteristic surface markers. B cells express surface membrane immunoglobulin and have receptors for the Fc fragment of IgG as well as the third component of complement. Peripheral blood T cells possess receptors for sheep erythrocytes (SRBC). More recently, distinct subpopulations of T cells have been identified that express receptors for either the Fc fragment of IgM (Tμ) or IgG (Tγ).

In 1975, Jondal et al. reported on a new lymphocyte marker: surface receptors for B-lymphoblastoid cell lines (B-LCL). These receptors were demonstrated by rosette techniques and were present on virtually all peripheral blood T lymphocytes. Since these rosettes may be an in vitro representation of an important in vivo interaction, we attempted to elucidate the cell populations capable of binding B-LCL, their tissue distribution, and their presence in other species. This study demonstrates that although the majority of rosetting lymphocytes are T cells, subpopulations of non-T-lymphoid cells are capable of binding B-LCL. Furthermore, cells with surface receptors for B-LCL are widely distributed in peripheral lymphoid organs and are expressed on murine lymphocytes.

MATERIALS AND METHODS

Purification and Fractionation of Lymphocytes

Peripheral blood lymphocytes (PBL) were purified from heparinized blood by density flotation on Ficoll-Hypaque (Ficoll 400, Pharmacia AB, Uppsala, Sweden; Hypaque sodium 50, Winthrop Laboratories, N.Y.) as previously described. Phagocytic cells were removed by incubation with iron carbonyl powder followed by exposure to a magnet. The recovered cells consisted of >95% lymphocytes and less than 2% esterase-positive cells.

For depletion of T cells we used the procedure described by Wybran et al. The interface cells contained less than 4% SRBC-rosette-forming cells (E-RFC) and consisted primarily of B cells, null cells, and cells with a receptor for the Fc fragment of IgG (Fc-R cells). Cells recovered from the pellets consisted of over 90% E-RFC.

Preparation of Mouse and Rat Spleen Cells and Thymocytes

Animals were sacrificed by cervical dislocation, and a single-cell suspension of spleen cells or thymocytes was prepared by expression of the organ through a stainless steel mesh.

Cell Lines

A variety of human cell lines, derived from normal individuals as well as patients with hematologic neoplasms, were studied. Murine lymphoblastoid cell lines were also employed. All cell lines were grown as suspension cultures in RPMI 1640 medium with 10% fetal calf serum.

LCL-Rosette Formation

The method used has been described by Jondal et al. Briefly, peripheral blood lymphocytes and LCL were suspended in a concentration of 1.5 x 10⁷/ml and 5 x 10⁵/ml in RPMI 1640 with 10% fetal calf serum. Lymphocytes (0.1 ml) were mixed with different cell lines (0.1 ml) in culture tubes (Pyrex, 50 mm x 6 mm, VWR). After centrifugation at 600 rpm for 6 min, the pelleted cell mixture was incubated for 1 hr at 4°C. The supernatant was removed and the pellet was then resuspended gently, placed on a glass slide, and covered with a coverslip. A rosette consisted of a readily distinguishable large cell (LCL) surrounded by 3 or more lymphocytes.

Double Rosettes

These rosettes were performed to determine the percentage of lymphocytes bound to LCL that had surface receptors for SRBC (E) or the Fc fragment of IgG (EA). Lymphocytes and LCL were mixed in a ratio of 5:1 (using 5 x 10⁵ LCL). This ratio was used because it contains the same number of lymphocytes (2.5 x 10⁵) that are used in the standard E or EA rosette assay. After forming rosettes with LCL (method described above) 50 µl of E or EA preparation (10⁵/ml) and 50 µl of MEM were added and the cells centrifuged at...
600 rpm for 6 min and incubated for 1 hr at 4°C. The cells were then resuspended and after counting 200 bound lymphocytes, the percentage of E and EA-RFC that were also bound to LCL was determined. Trypsinized SRBC were employed in the EA preparation to exclude binding to SRBC receptors.

**Antithymocyte Serum Treatment of Murine Cells**

Murine T cells were lysed by incubation of 10^7 cells/ml in a 1:45 dilution of absorbed rabbit anti-mouse thymocyte serum (Microbiological Associates) for 30 min at 4°C. The cells were centrifuged and incubated at the same concentration in a 1:15 dilution of agarose-absorbed guinea pig complement for 30 min at 37°C. This concentration killed 100% of thymocytes, less than 10% of bone marrow cells, and approximately 50% of spleen cells.

**Antithymocyte Serum Treatment of Human Cells**

Rabbit anti-human thymocyte serum was prepared as described previously. Briefly, rabbits were inoculated subcutaneously with human thymocytes and boosted on days 8 and 36. The animals were bled on days 43, 49, and 75 and serum was harvested. Serum was determined. Trypsinized SRBC were employed in the EA preparation to exclude binding to SRBC receptors.

**Generation of T Lymphoblasts**

After purification by flotation of Ficoll-Hypaque, human peripheral blood mononuclear cells were suspended in RPMI 1640 media with 10% fetal calf serum at a concentration of 10^6/ml. Phytohemagglutinin (PHA) (Wellcome Reagents Limited, Research Triangle Park, N.C.) was added to several cultures in a dilution of 1/100. Concanavalin A (Sigma, St. Louis, Mo.) was added to other cultures in a dilution of 2 μg/ml. Some cultures, without added mitogens, were initiated as controls.

The mixed lymphocyte reaction (MLR) was initiated by stimulating peripheral blood lymphocytes with mitomycin-C-treated Raji B-lymphoblastoid cells. Respamer lymphocytes (10^6/ml) were mixed with Raji lymphoblasts (10^6/ml) at a responder: stimulator ratio of 1:1 in 1640 media with 10% fetal calf serum. Flasks were kept in a humidified incubator with a constant supply of 5% CO2 in air at 37°C for various lengths of time. Cultures were then harvested, washed twice, counted, and added to B-LCL for the standard rosette assay.

### RESULTS

**Rosette Formation Between Peripheral Blood Lymphocytes (PBLs) and B-LCL**

Peripheral blood lymphocytes obtained from 25 healthy individuals formed rosettes with a large variability of B-LCL derived from normal and leukemic individuals. As shown in Table 1, this interaction is specific for LCL with B-cell characteristics, since PBLs did not rosette with T-LCL (derived from patients with acute lymphocytic leukemia) or melanoma cell lines growing in suspension.

The number of blast cells rosetted depended on the ratio of PBLs to lymphoblastoid cells employed. Table 2 indicates that high ratios of PBLs:B-LCL enables rosetting of virtually all lymphoblastoid cells present. However, under the best experimental conditions, the percentage of PBLs capable of binding LCLs reaches a plateau at 30%-50%. Table 3 indicates that an attempt to bind increasing numbers of PBLs by decreasing the PBL:LCL ratio resulted in only 30% of PBLs rosetted. In ideal circumstances, the maximal percentage of PBLs rosetted was never greater than 50%.

The antigenic determinants recognized by human PBLs on lymphoblastoid cells have been detected on all B-LCL examined thus far, although the surface density of these determinants seems to vary between cell lines (Table 4). Lymphocytes from the same donors mixed with several B-LCL on the same day resulted in percent rosettes that varied widely from one B-LCL to another.

### Characterization of the Cells Rosetting With B-LCL

PBLs were pretreated with rabbit antiserum that had been prepared against human thymus and...
absorbed with B-LCL, rendering it specific for peripheral blood T cells. As shown in Table 5, pretreatment of PBLs resulted in significant inhibition of B-LCL and E-rosette formation. The inhibition was dependent on antiserum concentration and followed parallel curves for the two types of rosettes.

To characterize the binding cell further, we performed double rosette experiments in which PBLs were first rosetted with B-LCL and then with either sheep erythrocytes (E-rosettes) or with the latter coated with rabbit anti-SRBCs (EA rosettes). Of PBLs rosetting with B-LCLs, 65%-85% also formed E-rosettes, indicating they were T cells. Although an increase in the ratio of SRBC to lymphocytes significantly increased the percentage of E-rosettes obtained using standard methodology, it had minimal effects on the percentage of T cells bound to B-LCL. Similarly, pretreatment of SRBCs with Víbrio cholerae neuraminidase had only mild effects on the number of T cells bound to B-LCL.

Using the double rosette technique, we were able to demonstrate that a small percentage of bound lymphocytes (4%-8%) were Fc-receptor-bearing cells (Fc-R cells). While increasing the ratio of EA to lymphocytes resulted in an increase in the percentage of Fc-R cells, it did not affect the percentage of Fc-R cells bound to B-LCL.

T-cell depletion using the E-rosette centrifugation technique of Wybran et al. confirmed and expanded the above observations. As shown in Table 6, unfractionated PBLs bound 18% of lymphoblastoid cells when combined at a ratio of 10:1. The T-depleted population (interface) had a 50% reduction in LCL-rosette formation (9%), whereas the enriched T-cell population (pellet) showed a pronounced increment in LCL-rosette formation (40%). Of the interface cell population, markedly depleted of T cells (4%) and enriched for Fc-R cells, only 5% of lymphocytes bound to lymphoblastoid cells were T cells (E-RFC). These studies clearly demonstrate that Fc+, E− peripheral blood lymphocytes can rosette with B-LCL.

T cells with surface receptors for the Fc fragment of IgM (Tα) also rosetted with B-LCL. In experiments using the double rosette technique, we were able to show that Tα lymphocytes rosetted with 3 different types of B-LCL (data not shown). While Tα represented 70% of all T cells, it comprised only between 20%-40% of the bound T cells, indicating that only a subgroup of Tα was capable of binding under the conditions employed.

Lymphocytes with surface receptors for B-LCL were found in tonsil, peripheral blood, and the mononuclear fraction of bone marrow cells (Fig. 1). Cryo-
preserved (>80% viable) human thymocytes, obtained from 3 children during open heart surgery, were incapable of forming rosettes at various thymocyte:LCL ratios. By contrast, cryopreserved human PBLs did not lose their ability to rosette. Two subjects were tested at a 20:1 ly:LCL ratio and gave 44% and 59% rosettes before and 42% and 60% after cryopreservation. Using a fixed LCL:PBL ratio, peripheral blood contained higher percentages of rosette-forming cells than tonsil or bone marrow. There was no correlation between percentage of B-LCL rosettes and percentage of E-rosettes from individual lymphoid organs.

Studies in Murine Systems

We tested the ability of PBLs to rosette with a variety of murine tumor cell lines (Table 7). Only one of the cell lines rosetted with human PBLs, and the percent rosettes was quite low (10%). Changes in the ratio of PBL:LCL did not increase percent rosettes. Rosette formation with human B-LCL was also seen with murine lymphocytes (Table 8). As shown, lymphocytes with receptors for B-LCL were found in nonimmune mouse and rat spleen. In contrast, thymocytes from either species lacked this binding ability. No significant differences were noted in percent rosettes formed when splenocytes from immunized mice were assayed. Treatment of spleen with antithymocyte serum and complement resulted in a pronounced decrease in the percent of rosettes, indicating that in mice the binding cells are primarily T cells. Pretreatment with antiserum alone reduced rosette formation, whereas complement alone had mild or no effect at all.

Presence of B-LCL Receptors on T Lymphoblasts

T lymphoblasts, generated by stimulation of human PBLs with PHA or alloantigens, retain the surface receptors that bind LCLs (Fig. 2). Cells harvested 3 days after mitogen stimulation (90% E-RFC) or 5 days after in vitro culture with allogeneic mitomycin-C-treated lymphocytes, rosetted with B-LCL. The percent rosettes formed by the transformed cells were higher than those obtained using unstimulated cells. Lymphocytes cultured alone for 24–48 hr showed no change in their ability to rosette. At 3 days, however, a progressive decline was noted, and by day 7, percent rosettes formed was very small (less than 10%). Con-A-stimulated lymphoblasts, on the contrary, have a reduced capacity to rosette with B-LCL (Fig. 2). As indicated, rosetting with Con-A-stimulated lymphoblasts gave smaller percentages than those obtained with PHA-stimulated lymphoblasts.

**DISCUSSION**

Peripheral blood lymphocytes have receptors for B-lymphoblastoid cell lines. These receptors are specific for B lymphoblasts and will not rosette with T-LCLs. The determinants that are recognized appear to be universal to all B-LCLs. There is no difference between percent rosettes formed with B-LCL derived from normal or leukemic patients. Furthermore, rosette formation is equally present with immunoglobulin secreting and nonsecreting cell lines. Jondal et al. demonstrated weak binding between T cells and normal B lymphoblasts obtained after pokeweed mitogen stimulation. This would indicate that these antigens are expressed on normal B cells. The percent rosettes varied widely between cell lines when the same lymphocyte donor was used. This suggests that

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**Table 7. Rosette Formation Between Human PBL and Murine Tumor Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type</th>
<th>Percent Rosettes</th>
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<tbody>
<tr>
<td>EL-4</td>
<td>T cell</td>
<td>02</td>
</tr>
<tr>
<td>LA-5</td>
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<td>B cell</td>
<td>01</td>
</tr>
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<td>LA-51b</td>
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<td>07</td>
</tr>
<tr>
<td>LA-54a</td>
<td>B cell</td>
<td>10</td>
</tr>
</tbody>
</table>

*EL-4 is a common T-cell lymphoma derived from C57BL/6 mice. All B-cell murine lines are derived from the spontaneous reticulum cell sarcoma arising in SJL/J mice (see references 11, 12, 13).*
the density of the recognized surface determinant may vary among cell lines. Alternatively, the expression of this determinant may depend on the phase of the proliferative cycle of the B lymphoblast. This explanation could account for varying percent of rosettes between different B-LCLs assayed on the same day.

The cells binding B-LCL were phenotypically heterogeneous. The majority of bound cells are T cells as demonstrated by the rosette inhibition with rabbit antithymus serum and by the double rosette experiments. T-depletion experiments, however, reveal that non-T, EA-rosette-forming cells are capable of binding LCLs. Furthermore, since in the T-depleted population, EA-rosette-positive cells account for only approximately 50% of the cells binding B-LCLs, other yet unidentified lymphocytes (E−, Fc-R−) have the ability to bind to these cells.

Heterogeneity was also seen among the T-cell population that binds LCLs. T cells with surface receptors for the Fc fragment of IgM (Tμ) formed rosettes with B-LCL. Although representing 70% of all T cells, Tμ only comprised between 20% and 40% of bound T cells, indicating that at least two subpopulations of T cells, i.e., E+, μ+, and E+, μ−, have the surface receptor required for binding.

Cells with receptors for B-LCL were found in tonsil and bone marrow as well as peripheral blood, indicating a wide distribution in peripheral and central lymphoid organs. Under identical experimental conditions lymphocyte populations derived from peripheral blood gave the highest number of rosettes. The percent of rosettes did not correlate with relative or absolute numbers of T cells (detected by E-rosettes) present in the various tissues, suggesting that the blood contains a larger percentage of the subpopulation of T cells that express these receptors.

T cells with receptors for B-LCL are not restricted to man. Murine theta-positive splenocytes were capable of binding human B-LCL. However, murine thymocytes, as well as human thymocytes, did not express these surface receptors, suggesting that these surface receptors become expressed during T-cell differentiation.

Surface receptors for B-LCL were retained by lymphocytes activated and transformed by polyclonal mitogens or alloantigens. In fact, T cells stimulated with PHA for 72 hr formed a higher number of rosettes than fresh cells or the same cells cultured without mitogen. This increment in rosette formation was in part due to an enrichment of T cells (90%) seen after 72 hr with PHA. It could also be argued that small amounts of PHA, present during the rosette assay, caused increased nonspecific binding through lectin receptors. We consider this unlikely, since PHA-activated cells still retain their specificity for B-LCL. In control experiments, they were unable to bind T-LCL (data not shown).

In contrast to an increase in rosette formation after activation of PBLs with PHA, Con-A-stimulated cells formed a smaller number of rosettes. This observation supports the notion that PHA and Con-A activate different subsets of cells, the former stimulating T lymphocytes carrying the receptor for B-LCLs and the latter stimulating subsets that lack the receptor. Alternatively, Con-A-stimulated T cells may experience a loss of these receptors.

Fan et al.10 have reported that human peripheral blood monocytes can also bind LCL. This interaction was similar to the one between PBLs and LCLs in that it was specific for LCLs of B-cell origin. We could not sufficiently enrich our cell suspensions for monocytes to confirm this observation. It is unknown whether the B-LCL determinant(s) recognized by T cells in this study is identical to the determinant(s) recognized by the rosetting monocytes described by Fan et al.

The biologic significance of the interaction between T cells and B-LCL is intriguing. It may represent one of the mechanisms by which T and B cells interact and communicate in vivo. The responsibility for regulating immune responses appears to reside in the T-cell compartment. The regulatory control of B-cell responses might operate through the above-described T-B interactions. Thus, changes in immunoglobulin synthesis or in B-cell growth kinetics might occur as a result of in vitro rosetting.

Alternatively, this interaction may be a mechanism by which immunocompetent T cells respond to foreign allogeneic lymphocytes. The binding of peripheral blood lymphocytes to B-LCL is not cytolytic to either of the cells involved, in agreement with the data reported by Jondal et al.4 The possibility remains, however, that cytolysis might be delayed, and assays employing incorporation of 3H-TdR or BUDR are indicated. Preliminary experiments in our laboratory indicate that T-cell activation, manifested by increased protein synthesis, release of lymphokines, and increased DNA synthesis, occurs after T-cell rosetting with B-LCL (manuscript in preparation). It is possible that the signal for proliferation and clonal expansion in a mixed lymphocyte reaction is provided through interaction of T cells and B-LCL via the surface molecules identified in this rosette assay.

Whether interactions via these surface molecules are important in the course of B- or T-cell mediated immune responses remains to be determined. The demonstration of a common surface receptor on T cells
of lower species, its persistence through evolution, and the expression of the determinant on normal B cells clearly suggests an important biologic and immunologic function.

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REFERENCES

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