Characterization of Non-Human Primate Antisera to Acute Lymphoblastic Leukemia (ALL): Evidence for Unique Antigen(s) on Childhood ALL of "T" Phenotype

By T. Mohanakumar, Terry W. Coffey, Michael P. Vaughn, Edward C. Russell, and Daniel Conrad

A non-human primate antiserum was prepared to acute lymphoblastic leukemia of T-cell phenotype (T-ALL) and, after absorptions with normal blood elements, reacted by immunofluorescence and microcytotoxicity to all the T-ALL tested. In addition, the antiserum reacted with cells from about 70% of the common ALL studied and immunoprecipitated the common ALL antigen of 100,000 daltons. However, when the anti-T-ALL serum was absorbed with lymphoblasts from common ALL, it failed to react with common ALL lymphoblasts, yet reacted significantly with cells from patients with T-ALL phenotype and defined a 100,000-dalton membrane component not found on common ALL lymphoblasts. In addition, sequential immunoprecipitation of 125I-labeled T-ALL membranes by anti-common-ALL serum followed by anti-T-ALL serum detected the T-ALL membrane component of 100,000 daltons that was not found on common ALL. Thus, our results demonstrate the presence of a unique human T-ALL antigen present on all T-ALL distinct from the common ALL antigen.

Previous studies utilizing heteroantisera by Greaves and others have defined an antigen associated with cells from many patients with acute lymphoblastic leukemia (ALL), as well as some patients with chronic myelocytic leukemia in blast crisis and non-Hodgkin's lymphomas. This antigen (CALLA) was found to be a single glycosylated polypeptide with an apparent molecular weight of 100,000 daltons. Recently, a monoclonal anti-CALL (J-5) has been produced and shown to present concordant reactivity with the rabbit anti-CALLA. Both sera appear to bind to the same molecular species (gp 100) detected on leukemic cells. These reagents are now the routine diagnostic reagents of choice for common ALL (CALL) in many laboratories.

Although leukemia-associated antigens of clinical importance have been thus defined for one subgroup of childhood ALL (CALL), similar studies on other well-defined ALL subgroups (T-ALL, B-ALL, pre-B-ALL) are not yet available. Although Deng et al. have described a monoclonal antibody (CALL2) that detects a cell surface antigen unique to T-ALL, no biochemical characteristics of this antigen have been presented. In this report, by using non-human primate antisera to CALL and T-ALL, we present evidence for the presence of a unique T-ALL-associated antigen of 100,000 daltons. By absorption analysis, this T-ALL-associated antigen was neither detected on normal thymic lymphocytes nor on other normal or neoplastic cells studied.

Materials and Methods

Target Cells

Mononuclear cells were prepared from heparinized (25 U/ml) blood using Ficoll-Hypaque density gradient centrifugation at 400 g for 20 min as described previously. Leukemic cells were obtained from diagnostic bone marrow aspirations at initial diagnosis or at relapse. All samples studied contained >90% leukemic cells. Standard morphological and cytochemical methods were used to establish the diagnosis of ALL, acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), or chronic lymphocytic leukemia (CLL).

The characteristics of the cultured cell lines (NALM-1, REH, RPMI-8402) used in these studies have been described by Minowada et al. These cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml at 37°C in humidified 5% air, 5% CO2.

Anti-ALL Antiserum

Two anti-ALL sera were produced by immunization of monkeys (M. speciosa) with viable leukemic cells emulsified in Freund's complete adjuvant. The first antiserum (VAN) was prepared against null ALL cells (E-rosette 0%, slg-,-, CALL Ag+) and the second (OST) against T-ALL lymphoblasts (E-rosette >90%, slg-, CALL Ag-). Each animal received the cells from one patient only.

Details of preparation of a monkey antileukemia serum has been reported previously.

For absorption, the sera were heat-inactivated at 56°C for 30 min, incubated with washed pooled human erythrocytes, and then with pooled platelets and tonsillar lymphocytes until the sera were nonreactive against a panel of normal lymphocytes by both immunofluorescence and microcytotoxicity assays.

Microcytotoxicity Assay

The Amos modified microtechnique described earlier was employed, and an antibody-mediated cytotoxicity was considered significant when the number of dead cells exceeded the normal control serum values by 20% or greater.

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Supported in part by ACS Grant 1M190, NIH Grant CA27416, and the Association for the Study of Childhood Cancers, Richmond, Va. T.M. is a recipient of American Cancer Society Faculty Research Award 246.

Submitted February 23, 1982; accepted August 6, 1982.

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Blood, Vol. 61, No. 1 (January), 1983
**Immunofluorescence**

The reactivity of antisera to different target cells was determined by indirect immunofluorescence. Cells (2 x 10^6) were incubated at 4°C for 30 min with the test antisera or with control preimmune sera. Target cells that may express human surface immunoglobulin were first pretreated with an optimal concentration of rabbit anti-mouse immunoglobulin. Unbound antibody was removed by washing and the sensitized cells were then incubated for 30 min at 4°C with 50 μl of fluorescein isothiocyanate-conjugated F(ab')2 fragments of rabbit antisera to mouse immunoglobulin. After the incubation and washing, at least 200 cells incubated with either immune or control sera were examined under a Zeiss photomicroscope III equipped with mercury lamp and a dark field condenser.

**125I Labeling, Immunoprecipitation, and Polacrylamide Gel Analysis**

Iodination of cell surface proteins was done according to the method of Conrad and Froese. Labeled cells (3 x 10^7) were lysed with 1 ml of 0.5% Nonidet P40 nonic detergent in phosphate-buffered saline (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride. The solubilized material was spun in an Eppendorf 5412 centrifuge at 12,900 g for 5 min and the supernatant was recovered for immunoprecipitation.

Immunoprecipitation of 125I-labeled antigens was performed by mixing 0.5 ml of cell extract and 20 μl of antisera or control serum. These mixtures were incubated for 1 hr at 25°C, and then a predetermined optimal amount of goat anti-monkey immunoglobulin was added. Incubation was continued for 1 hr at 25°C and then for 24 hr at 4°C. Immune precipitates were washed 3 times with phosphate-buffered saline (pH 7.4) containing 0.5% Nonidet P40 and then washed once with a 0.0625 M Tris (pH 6.8) before resuspending in a 0.062 M Tris (pH 6.8) containing 2.3% sodium dodecyl sulfate, 5% mercaptoethanol, and 10% glycerol. After heating for 90 sec at 100°C, the sample supernatants were electrophoresed through 10% 10 cm polyacrylamide disc gels prepared according to the procedure of Laemmli. After electrophoresis, the gels were darkened proteins could be estimated by their position relative to the marker proteins.

**RESULTS**

**Serologic Characterization of Monkey Anti-ALL**

The results of microcytotoxicity testing of the normal B lymphocyte and platelet-absorbed antisera to CALLA-positive null ALL (VAN) and E-rosette-positive T-ALL (OST) with subpopulations of normal lymphocytes and different leukemic targets are given in Table 1. The results of the reactivity of the antiserum to CALLA-positive ALL (VAN) are in general agreement with previously reported results for common ALL antigens on ALL lymphoblasts. Most of the "null" ALL (26/29 patients) reacted with the anti-null-ALL antiserum. In addition, cells from 2/8 T-ALL and 1/3 B-ALL were also lysed by this reagent, which indicates the expression of CALLA antigens on lymphoblasts of some T- and B-ALL patients. The antiserum to T-ALL (OST) also reacted with most of the null-ALL (21/29) and all of the T-ALL (8/8) tested. Also, cells from one of three B-ALL that were lysed by the VAN were also lysed by the anti-T-ALL serum (OST). Thus, the direct testing data suggest that the OST may have cross-reactive antibodies to null-ALL, as well as possible unique antibodies reactive to T-ALL, since 6 of 8 T-ALL that reacted with the OST failed to react with the VAN.

In order to delineate the serologic specificities of these reagents, quantitative absorptions of VAN and OST were performed by using different target cells. Absorptions with increasing numbers of B lymphocytes did not reduce the percent immunofluorescence of either VAN or OST against ALL lymphoblasts (data not presented). B-lymphocyte-absorbed antisera failed to react with any of the CLL lymphocytes tested. Furthermore, absorptions of VAN or OST with thymocytes or lymphocytes from CLL donors did not have any significant effect on their reactions to each other. Null-ALL or T-ALL tested (Table 2). Absorptions of VAN with null-ALL lymphocytes removed the binding to both subtypes of ALL lymphoblasts. However, the antibody activity of OST to T-ALL could be removed only by absorptions with T-ALL (previously shown by immunoprecipitation to be CALLA-positive) but not with CALLA-positive null-ALL. OST absorbed with null-ALL lymphoblasts, although failing to react with null-ALL lymphoblasts, bound significantly to T-ALL lymphoblasts (Table 2). The absorp-

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Table 1. Serologic Reactions of Monkey Antisera to ALL

<table>
<thead>
<tr>
<th>Antiserum to</th>
<th>Normal Lymphocytes</th>
<th>Mitogen</th>
<th>Stimulated T Cells</th>
<th>Null</th>
<th>T</th>
<th>B</th>
<th>CLL</th>
<th>AML</th>
<th>CML</th>
</tr>
</thead>
<tbody>
<tr>
<td>CALLA-positive ALL (VAN)</td>
<td>0/25*</td>
<td>0/18</td>
<td>0/3</td>
<td>26/29</td>
<td>2/8</td>
<td>1/3</td>
<td>0/19</td>
<td>0/13</td>
<td>1/10</td>
</tr>
<tr>
<td>E-positive ALL (OST)</td>
<td>0/25</td>
<td>0/18</td>
<td>0/3</td>
<td>21/29</td>
<td>8/8</td>
<td>1/3</td>
<td>0/15</td>
<td>0/13</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*Number reactive versus number tested. Results were confirmed by immunofluorescence in several patients.

†Normal blood lymphocytes were stimulated either with phytohemagglutinin (Burroughs Wellcome, Greensville, N.C.) or concanavalin-A (Calbiochem, San Diego, Calif.) for 3 days, and the lymphoblasts were used as targets.
Antigenic structures expressed on most of the gp 100 expressed on CALLA-positive null-ALL. This is strongly indicated by the existence of a 100,000-dalton component both from T-ALL and CALLA-positive null-ALL, as presented in Table 2 and confirmed by immunoprecipitation studies previously employed monkey anti-null-ALL serum (VAN) compared to SDS-PAGE analysis. As shown in Fig. 1A, such absorbed anti-T-ALL serum failed to precipitate any membrane components from CALLA-positive null-ALL. On the contrary, gp 100 as well as an occasional gp 55 was still immunoprecipitable from several T-ALL studied. Sequential immunoprecipitation analysis of $^{125}$I-labeled T-ALL first with VAN, followed by OST, also gave the results as shown in Fig. 1B. Thus, the absorption analysis presented in Table 2 as well as immunoprecipitation studies presented in Fig. 1 strongly indicate the existence of a 100,000-dalton membrane component present on T-ALL lymphoblasts which apparently is distinct from the gp 100 expressed on CALLA-positive null-ALL.

**DISCUSSION**

Antisera raised in rabbits against null-ALL cells detect antigenic structures expressed on most of the null-ALL cells, a small proportion of T-ALL cells, as well as some CML cells in blastic crisis and non-Hodgkin’s lymphomas.$^{3,12,14-16}$ Immunochemically, it has been shown to precipitate a leukemia-associated antigen with a molecular weight of approximately 100,000 daltons. The findings presented in this study employing monkey anti-null-ALL serum (VAN) confirm other reports of the definition of a 100,000-dalton antigenic structure on lymphoblasts from null-ALL patients.$^{7,13}$ Although only a small proportion of T-ALL was found to be cytotoxic with the monkey antisera to null-ALL (VAN), the 100,000-dalton component is identifiable on a greater percentage of T-ALL when studied by immunoprecipitation analysis. Furthermore, absorption of anti-T-ALL antisera (OST) with either T-ALL or null-ALL cells completely removed its reactivity to null-ALL (Table 2), thus indicating the presence of common ALL antigens on the T-ALL used for absorption and antibodies to common ALL antigen in our anti-T-ALL serum (OST).

In addition to the antibodies in OST that are shared for common ALL antigen, there are several observations suggestive of additional antibodies selectively reactive with T-ALL. (1) All the T-cell ALL so far studied reacted with OST (8/8), whereas only 2/8 gave significant reactions with VAN (Table 1). (2) Although absorptions of VAN with null-ALL lymphoblasts removed all of its antibody specificity to all ALL cells, similar absorptions of OST resulted in different findings. OST absorbed with T-ALL removed all of its binding ability to both T-ALL or null-ALL targets, but absorptions with null-ALL removed antibodies reactive only to null-ALL, not to T-ALL (Table 2). Since a twofold increase in absorbing cells did not result in any change in the reaction pattern, it cannot be explained as a quantitative discrepancy.

Although OST absorbed with cells from CALLA-positive null-ALL patients failed to immunoprecipitate any membrane components from null-ALL, OST precipitated a gp 100 component from all T-ALL studied (Fig. 1). Sequential immunoprecipitation of $^{125}$I-labeled T-ALL membrane with VAN followed by OST confirmed the absorption data. Thus, at least two antigens are detected by OST, one shared between null and T-ALL and another unique to T-ALL. A similar finding for the presence of unique antigens on T-ALL

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**Table 2. Absorption Analysis of Monkey Antiserum to T-ALL (OST)**

<table>
<thead>
<tr>
<th>Target</th>
<th>Platelets</th>
<th>Platelets + CLL</th>
<th>Platelet + Thymic Lymphocytes</th>
<th>Platelet + Null-ALL (CALL+)</th>
<th>Platelet + T-ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-ALL</td>
<td>70 (12)</td>
<td>70 (12)</td>
<td>70 (12)</td>
<td>50 (12)</td>
<td>0</td>
</tr>
<tr>
<td>Null-ALL</td>
<td>80 (48)</td>
<td>80 (48)</td>
<td>80 (48)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate titer.

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**Polyacrylamide Gel Analysis of Leukemia-Associated Antigens Defined by Monkey Antisera to Null-ALL and T-ALL**

In an attempt to determine the number of antigenic specificities detected on null-ALL and T-ALL by our non-human primate antisera, cells from several leukemic cells and established cell lines were labeled with $^{125}$I and immunoprecipitated with either normal monkey serum or anti-ALL serum and subjected to SDS-PAGE analysis as detailed above. Antisera VAN and OST, after absorption with platelets and normal B cells, were able to immunoprecipitate components of approximately 100,000 and 55,000 daltons from several null-ALL lymphoblasts and certain reactive T-ALL lymphoblasts.

Since OST was able to immunoprecipitate a 100,000-dalton (GP100) component both from T-ALL and CALLA-positive null-ALL, we set out to define whether the gp 100 of T-ALL and null-ALL are the same or different components of similar molecular weights. Towards this purpose, OST was further absorbed with a reactive CALLA-positive, E-rosette-negative ALL and then used for immunoprecipitation and SDS-PAGE analysis. As shown in Fig 1A, such absorbed anti-T-ALL serum failed to immunoprecipitate any membrane components from CALLA-positive null-ALL. On the contrary, gp 100 as well as an occasional gp 55 was still immunoprecipitable from several T-ALL studied. Sequential immunoprecipitation analysis of $^{125}$I-labeled T-ALL first with VAN, followed by OST, also gave the results as shown in Fig. 1B. Thus, the absorption analysis presented in Table 2 as well as immunoprecipitation studies presented in Fig. 1 strongly indicate the existence of a 100,000-dalton membrane component present on T-ALL lymphoblasts which apparently is distinct from the gp 100 expressed on CALLA-positive null-ALL.
Fig. 1. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of immunoprecipitate formed by monkey anti-T-ALL (OST) absorbed with a reactive CALLA-positive ALL cell against two T-ALLs (Δ—Δ) and one null-ALL (○—○). (B) SDS-PAGE of sequential immunoprecipitate formed by monkey anti-T-ALL after precleaning with monkey anti-null-ALL against T-ALL (●—●) and null-ALL (○—○). Molecular weight standards are: (1) C₉Δ—110,000; (2) C₉β—70,000; (3) bovine liver catalase—55,000; (4) ovalbumin—44,000; (5) chymotrypsinogen—25,000 daltons.

has been recently reported employing antisera to T-ALL prepared in rabbits.¹⁷ The relationship of the T-ALL-associated antigen defined by OST to other normal alloantigens remains to be determined. Since absorptions of OST with normal human thymocytes did not reduce its reactivity to T-ALL cells, it does not appear to be a normal thymic lymphocyte-associated antigen (Table 2). It is of interest that OST shares some of the serologic and biochemical characteristics noted for the monoclonal antibody OKT-9¹⁸,¹⁹ since it failed to react with normal thymic lymphocytes and blood T lymphocytes, bound only to leukemic T lymphoblasts, and immunoprecipitated a glycoprotein of approximately 100,000 daltons. However, in contrast to the OKT-9, the anti-T-ALL reagent (OST) failed to react with PHA- and CON-A-activated normal T lymphoblasts. In spite of it, the relationship of OST to OKT-9-reactive structures needs further study. Also, the relationship between the monoclonal antibody (CALL2), which reacts with an antigen unique to T-ALL, and the gp 100 detected by the heteroantisera (OST) needs examination. Pesando et al.¹⁵ have recently reported the presence of a family of 100,000-dalton glycoproteins that are present on a variety of normal and transformed cells, and they possess common as well as unique antigenic regions. In addition to the antigenic determinant designated as common ALL antigen detected primarily on non-T, non-B ALL, there may be another unique 100,000-dalton glycoprotein selectively expressed on T-ALL. Whether the gp 100 expressed on T-ALL are structurally similar to the common ALL antigen with minor differences brought about possibly by different glycosylation or whether the antigens are completely different with coincidently similar molecular weights remains to be resolved.

ACKNOWLEDGMENT

The excellent secretarial assistance of Rae Spivey and Fay Akers is greatly appreciated.

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