Detection of Both T-Cell and Ia-like Antigens on Cells from Patients with Acute Myelomonocytic Leukemia and Chronic Myelogenous Leukemia in Blast Crisis

Joy K. Anderson, Joseph O. Moore, and Richard S. Metzgar

ABSTRACT

Appropriately absorbed antisera to the lymphoblastoid cell lines HSB and SB detect a human T-lymphocyte-associated antigen (TLAA) and the human Ia-like antigens, respectively. Cells from some patients with acute myelomonocytic leukemia (AMML) and chronic myelogenous leukemia (CML-BC) expressed both TLAA and Ia antigens when tested in a complement-dependent microcytotoxicity assay (>90% lysis with both antisera). When patients were in remission, expression of TLAA and Ia antigens returned to normal values. Quantitative absorption of anti-TLAA serum with increasing numbers of AMML cells showed that these cells could remove reactivity of the serum for both HSB and human thymocytes. Similarly, absorption of anti-Ia serum with AMML cells removed all serological reactivity when this serum was tested on chronic lymphocytic leukemia cells or normal B-cells. These serological findings were confirmed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies using radiolabeled antigens. Cells from an AMML patient were labeled with $^{35}$S using lactoperoxidase; both the TLAA and Ia antigens were precipitated from the resulting solubilized membrane preparation. Leukemic cells from one AMML patient and one patient with chronic myelogenous leukemia in blast crisis were studied for Ia and TLAA antigens with a double fluorescence technique. Over 80% of the cells showed dual fluorescence.

MATERIALS AND METHODS

Antisera. Antisera were prepared in monkeys and rabbits by immunization with HSB and SB tissue culture cell lines and were appropriately absorbed to detect the T-cell and Ia antigen specificities previously described (3, 5). The rabbit and monkey antisera detected the same antigen specificities and immunoprecipitated the same molecules. In some instances, rabbit antiserum prepared to affinity purified Ia antigens from the SB cell line was used in place of absorbed antisera to SB.

For quantitative absorption studies, aliquots of serum were twice absorbed with increasing numbers of cells for 30 min at room temperature. The sera were centrifuged at 25,000 x g for 30 min before being used in cytotoxicity and precipitation studies.

Cells. The leukemic patients studied were in the untreated or relapse stage of their disease and the peripheral blood or bone marrow samples from AMML patients had 80% or more leukemic blasts. Samples from CML-BC patients had 40% or more leukemic blasts. Samples for study were provided by the Hematology Division of the Department of Medicine at Duke University Medical Center.

Cell suspensions were prepared for testing by mixing the heparinized blood or bone marrow with Plasmagel and allowing the erythrocytes to sediment for 20 min at 37°. The supernatant was centrifuged through Ficoll-Hypaque, and the cells at the interface were removed, washed in 0.9% NaCl solution and in Hanks' balanced salt solution, and adjusted to 4 x 10^6 cells/ml.

Cytotoxicity Testing. A modification of the micromethod described by Mittal et al. (16) was used. Antiserum (1 μl) diluted in Hanks' balanced salt solution was mixed with 0.5 μl cells (4 x 10^3/ml) in a Falcon microtiter plate and incubated for 35 min at room temperature. Rabbit complement (5 μl) was added,
and the mixture was incubated for 35 minutes at 37°. Five μl eosin and 2 μl formalin were sequentially added to each well. The plates were read on an inverted phase microscope. The reaction was considered positive if the immune sera gave a 30% increase in lysis over the normal serum controls.

**Rosette Studies.** Details of the methods used in our laboratory to detect cells rosetting with sheep erythrocytes have been reported previously (14). Sheep erythrocytes were washed and adjusted to 0.5% (v/v) concentration in Hanks’ balanced salt solution. Equal volumes of this suspension and leukemic cells were mixed and spun at 100 × g for 5 min. After 1 hr incubation at room temperature or overnight incubation at 4°, the cell button was gently resuspended, toluidine blue was added, and the percentage of rosetting mononuclear cells was determined. At least 200 mononuclear leukocytes were counted. A cell was considered to be E-rosetting if 3 or more sheep erythrocytes were attached to it.

**Fluorescence Studies.** For fluorescence studies, 106 cells were incubated with rabbit anti-TLAA serum for 30 min at 4°. They were washed 3 times with PBS containing 2% bovine serum albumin and incubated with fluorescein conjugate swine anti-rabbit immunoglobulins (Bio-Rad Labs, Richmond, Calif.) for 30 min at 4°. After washing, the cells were incubated with monkey anti-İa serum, washed, and then incubated with rhodamine-conjugated goat anti-monkey IgG (Cappel Labs, Cochranville, Pa.). Control experiments showed that the antiglobulin reagents alone did not react with the cells. The antiseras were used at dilutions previously determined to result in specific reactivity for HSB, SB, peripheral blood lymphocytes, and cells from a patient with chronic lymphocytic leukemia.

Cells were examined with a Zeiss Standard RA phase contrast microscope equipped with IV/F epifluorescence condenser, narrow band fluorescein and rhodamine filters, and ×40 Ph 2 objective.

**Radiolabeling of Cell Surface Antigens.** Cells were labeled with 125I using lactoperoxidase (19). The reaction mixture (1 ml volume) contained 106 cells, 10⁻⁵ M sodium iodide, 40 μg lactoperoxidase (Calbiochem, La Jolla, Calif.), and 1 mCi 125I (Amersham, Arlington Heights, Ill.). Iodination was initiated by adding 10 μl freshly prepared 10⁻² M hydrogen peroxide. At 5-min intervals, additional 5-μl aliquots of hydrogen peroxide were added until a total reaction time of 30 min had elapsed. The cells were then diluted in 15 ml PBS and pelleted. The cells were washed twice in PBS containing NaI (20 g/liter) instead of NaCl and solubilized in 1 ml 10 mM NaCl buffer, pH 8.0, containing 0.5% Triton X-100. The suspension was centrifuged at 100,000 × g for 60 min in a Beckman L-2 ultracentrifuge to remove unsolubilized material.

The supernatant was dialyzed overnight at 4° against 10 mM Tris and 2 mM phenylmethylsulfonyl fluoride (Calbiochem), a protease inhibitor. The dialyzed material was frozen in aliquots at −60°.

**Radiolabeled and SDS-Polyacrylamide Gel Electrophoresis.** Details of these methods have been reported earlier (3–5). Aliquots of radiolabeled antigen were reacted with normal rabbit serum, normal monkey serum, or rabbit or primate anti-TLAA and anti-İa sera for 60 min at 37°. The antigen-antibody complexes were precipitated by addition of the appropriate anti-globulin reagent and by overnight incubation at 4°. Immunoprecipitates were centrifuged at 2200 × g for 10 min and washed 3 times with cold 10 mM Tris:150 mM NaCl buffer containing 0.5% Triton X-100 and once with cold distilled water. The immunoprecipitates were counted with a Beckman Biogamma Counter.

The washed immunoprecipitates were resuspended in 50 μl of a sample reducing buffer containing 1% SDS and 5% 2-mercaptoethanol and heated in a boiling-water bath for 3 min. The samples were layered on precast 10- x 0.7-cm polyacrylamide gels (Bio-Rad Labs) that had been prerun with 0.205 mM Tris:0.205 mM glacial acetic acid:0.1% (w/v) SDS. Electrophoresis was carried out at 60 V until the samples entered the gel and then at 125 V until the tracking dye had reached the end of the gel. The gels were sliced into 1-mm fractions using a Gilson automatic gel fractionator and counted with a Beckman Biogamma Counter.

Two commercial protein mixtures (M.W. 53,000 to 265,000, Gallard-Schlesinger, Carle Place, N. Y.; low-molecular-weight calibration kit, Pharmacia Fine Chemicals, Piscataway, N. J.) were treated as described for the immunoprecipitates, loaded onto separate gels, and electrophoresed simultaneously with the labeled antigens. The gels were fixed for 4 hr in isopropylamine/glacial acetic acid:water (4:1:5) and stained overnight with 0.5% Coomasie Blue in 7% acetic acid. Gels were destained at 37° in 7% acetic acid. The molecular weights of the labeled antigens were estimated from log molecular weight versus mobility curves of the standards.

**RESULTS**

The serological reactivity and diagnosis of the 20 AMML and CML-BC patients studied are given in Table 1. The patients were all adults being seen in the hematology clinic of the Department of Medicine at Duke University Medical Center. All patients classified as AMML had typical myeloblasts as well as cells with monoblastic features. With one exception (Patient 19), all of the CML-BC patients were positive for Ph1 chromo-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Reactivity of cells from AMML and CML-BC patients with anti-TLAA and anti-İa sera</th>
<th>% of lysis at 1:10 antisera dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Anti-TLAA</td>
<td>Anti-İa</td>
</tr>
<tr>
<td>1 WA</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>2 JC</td>
<td>98</td>
<td>80</td>
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<td>3 DEd</td>
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<td>90</td>
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<td>5 D EV</td>
<td>70</td>
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</tr>
<tr>
<td>6 JH</td>
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<td>90</td>
</tr>
<tr>
<td>7 MJ</td>
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<tr>
<td>20 AT</td>
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* NT, not tested.
some. The patients were studied in the untreated or relapse phase of their disease. Peripheral blood samples were tested from all patients except Patients 9 and 16, which were bone marrow specimens. All patients showed a high degree of reactivity with xenoantisera defining the human TLAA and Ia-like antigens. In general, more than 75% of the cells were lysed by the anti-TLAA serum, and 90% or more of the cells were lysed by the anti-Ia serum. The titer of both antisera was generally greater than 1:40 when tested against these cells. In several cases, the cells were completely lysed by both antisera. When E-rosettes were also done on 13 of these patients, the values were low, with only 2 patients having more than 10% E-rosette-forming cells. A heterogeneous group of 8 acute nonlymphocytic leukemia patients showed reactivity only with the anti-la serum (data not shown). Cells from 26 ALL patients reacted with either the TLAA or la antisera but not with both reagents (5).

Cells from one AMML (Patient 4) and one CML-BC patient (Patient 19) were studied using both cytotoxicity assay and immunofluorescence. By cytotoxicity, the cells were strongly reactive with antisera detecting both T-cell and la antigens (Table 1). Cells from both patients formed 5% or less E-rosettes. When the same cells were tested by immunofluorescence using the rabbit anti-T-cell serum plus a fluoresceinated anti-TLA reagent and the monkey anti-B-cell serum plus a rhodamine-labeled anti-TLA reagent, 80% or more of the cells showed dual fluorescence.

Serial studies done on one AMML patient (Patient 10) are shown in Table 2. When first tested, the patient presented an essentially normal serological profile, with 70% E-rosette-forming cells and 90% lysis by the anti-T-cell serum. When the patient relapsed, approximately 2 weeks later, both peripheral blood and bone marrow samples showed a strong dual reactivity. The serological pattern again returned to normal when the patient was in remission several months later. With a subsequent relapse in March 1978, the cells were again reactive with antisera defining both the T-cell and la antigens. At this time, only 1% of the cells formed E-rosettes. Several other AMML patients have shown patterns similar to this. That is, when the patient is in remission, the serological pattern is essentially normal. Approximately 80% of the remission lymphocytes are lysed by the anti-TLAA sera. Bone marrow samples from remission patients or from normal donors are negative when tested with anti-TLAA antisera. When the patient relapses, the cells from peripheral blood or bone marrow are reactive with both the anti-TLAA and anti-B-cell sera.

Aliquots of both the anti-TLAA and anti-la sera were quantitatively absorbed with cells from AMML Patient 14. The results are given in Chart 1. Absorption of the anti-TLAA serum with increasing numbers of AMML cells removed reactivity when the absorbed serum was tested against HSB cells and thymocytes. Similarly, absorption of the anti-la serum with AMML cells removed the reactivity of this antiserum for chronic lymphocytic leukemia cells and peripheral blood B-cells from normal donors. Absorption of aliquots of the monkey anti-TLAA antiserum with peripheral blood lymphocytes or thymocytes showed that 8 x 10^6 cells/ml and 5 x 10^6 cells/ml, respectively, were required to reduce the TLAA to background levels when the antisera were tested by immunoprecipitation and gel electrophoresis against ^125^I-lactoperoxidase-labeled HSB cells. Additional absorption of the monkey anti-TLAA antiserum with 10^6 cells/ml from pooled B-lymphoblastoid cell lines did not reduce the titer when the antiserum was tested by cytotoxicity on normal peripheral blood lymphocytes or T-cell lines. Absorption of the monkey anti-la with 2 x 10^6 pooled cells from B-lymphoblastoid cell lines per ml was sufficient to remove cytotoxic reactivity of the antiserum for B-cell lines (data not shown). Conversely, additional absorption of the anti-la sera with 10^6 cells from pooled T-lymphoblastoid cell lines did not reduce the cytotoxic titer when the antiserum was tested on B-cell lines. Thus, while the quantitative amounts of TLAA expressed on AMML cells may nearly equal that of lymphocytes and thymocytes, the quantity of la-like antigens is at least 25 times less than that expressed by B-lymphoblastoid cell lines.

To confirm the quantitative absorption studies, radioimmunoprecipitation and SDS-polyacrylamide gel electrophoresis were performed with the absorbed sera and various ^125^I-lactoperoxidase-labeled membrane antigen preparations. A high-molecular-weight antigen (M.W. ~170,000) was precipitated from ^125^I-labeled, detergent-solubilized peripheral blood lymphocyte membranes by the anti-TLAA antiserum (Chart 2). This antigen was additionally absorbed with 5 x 10^6 AMML cells/ml and then used for radioimmunoprecipitation and SDS-polyacrylamide gel studies, this high-molecular-weight peak was reduced to background levels.

Similar studies were performed using ^125^I-lactoperoxidase-labeled, detergent-solubilized SB membranes and the monkey anti-la antiserum (Chart 3). The M.W. 35,000 and M.W. 28,000 la antigens which were precipitated from SB membranes by the HSB-absorbed antiserum were reduced to background levels when the antiserum was further absorbed with AMML cells.

To further establish the presence of TLAA and la antigens on AMML cells, cells from one patient (Patient 8) were labeled...
with $^{125}$I using lactoperoxidase, solubilized in Triton X-100, and used in radioimmunoprecipitation and polyacrylamide gel electrophoresis studies. The cell preparation used for labeling contained 5% E-rosette-forming cells and consisted of 98% blasts. These blasts showed strong reactivity for both TLAA and Ia antisera (Table 1). As shown in Chart 4, both the TLAA and Ia-like antigens could be precipitated from this antigen preparation. These reduced precipitates were electrophoresed on 7.5% SDS-gels so that both the TLAA and Ia antigens could be shown. Determination of TLAA molecular weight is not accurate on these gels, and therefore the TLAA value is listed as M.W. >150,000. When similar precipitates were electrophoresed on 4% SDS-gels, the molecular weight of the TLAA was 160,000 to 170,000, as we have reported previously (3, 4). An additional antigen (M.W. 94,000) was precipitated from these cells by absorbed antiserum to HSB. Work from this laboratory (2) suggests that this antigen is similar to the common ALL-associated antigen described by others (6, 20, 25). The antigenic sites on the M.W. 94,000 moiety may be arranged in a fashion which does not result in cytolysis after reaction with antibody and complement, since a number of cells (NALM-1 and some non-B, non-T ALL cells) have been found which are negative by cytotoxicity assay but which possess this antigen by radiolabeling and SDS-gel studies (2). The common ALL antigen has been reported to be present in some cases of acute nonlymphocytic leukemia by other investigators (6, 10).

**DISCUSSION**

In this study, a high percentage of cells from 20 patients with AMML or CML-BC was shown to react with both anti-TLAA and anti-Ia antisera. In most cases where E-rosette studies were done, less than 10% of the target cell suspension formed E-rosettes. Quantitative absorption of the anti-TLAA serum with cells from an AMML donor showed that these cells could remove reactivity of the antiserum for HSB and thymocytes. Similarly, when the absorbed serum was tested by radioimmunoprecipitation and SDS-polyacrylamide gel electrophoresis using $^{125}$I-lactoperoxidase-labeled, detergent-solubilized peripheral blood lymphocytes, the peak at M.W. 170,000 was removed. Thus, by several criteria, the T-cell-associated antigen detected on AMML cells seems to be the TLAA described in our other reports (3–5). Quantitative absorption of the anti-Ia serum with AMML cells followed by either microcytotoxicity assay or radioimmunoprecipitation and SDS-polyacrylamide gel electrophoresis showed that Ia-like antigens were present on these cells. In addition, both TLAA and Ia antigens could be precipitated from radiolabeled, detergent-solubilized leukemic blasts from an AMML patient. Using a double fluorescence technique, over 80% of the blast cells from one AMML patient and one CML-BC patient were shown to express both TLAA and Ia antigens.

While a number of other investigators have reported the presence of T- and B-cell markers on the same cell (9, 11, 21, 23, 24), most of this work has involved isolated case studies of patients with various lymphoproliferative disorders. In most instances, this dual reactivity was based on mixed rosette studies using E-rosettes and C3 rosettes (erythrocytes coated with antibody and complement). In one instance (11), E-rosettes were combined with an assay for surface immunoglobulin.

Ia-like antigens have been reported by other investigators on cells from some acute myelogenous leukemia and CML-BC patients using both heteroantisera (7, 22) and alloantisera (8) to these antigens. Winchester et al. (26) precipitated peaks at M.W. 37,000 and M.W. 28,000 from leukemic myeloblasts using an anti-Ia serum; absorption of the antiserum with leukemic myeloblasts removed serological reactivity when the antiserum was tested on B-cell lines. In addition, normal granulocytic cells have been shown to express Ia antigens at an early stage of differentiation (26). The majority of the positive
cells in normal marrow were myeloblasts with some positive promyelocytes.

In one of the few other studies in which acute nonlymphocytic leukemia and CML-BC cells were tested with anti-T-cell and anti-la sera, Janossy et al. (12) reported that these cells were negative with the T-cell antiserum. However, their T-cell antiserum was prepared in rabbits against monkey thymocytes and absorbed with acute myeloid leukemia cells; thus, the specificity of their reagent may be different from our anti-TLAA antisera.

Antisera raised against non-B, non-T ALL cells have been reported to detect a cALL antigen with a molecular weight near 100,000 (6, 25). While this antigen has been reported chiefly on cells from non-B, non-T ALL patients, there are reports of its occurrence on cells from some acute myelocytic leukemia patients (6, 10) as well as on cells from CML-BC patients (6, 12, 20), some T-cell ALL patients (20), and some T-lymphoblastoid cell lines (6). Data from this laboratory (2) suggest that the antigen near M.W. 96,000 precipitated from the radiolabeled AMML cells by absorbed antisera to HSB is similar to the cALL antigen (Chart 4). However, it may be that there are at least 2 distinct antigens in this region between M.W. 94,000 and M.W. 100,000 and that the tube method does not resolve them. Current radiographic SDS-polyacrylamide gel electrophoresis studies using the discontinuous system of Laemmli (13) and slab gel electrophoresis may help to resolve this point. The possible discrepancy between our data and previously reported data on distribution of the cALL antigen may result from differences in methods, since most earlier work involved immunofluorescence and cytotoxicity assays rather than radiolabeling and gel electrophoresis.

NALM-1 and non-B, non-T ALL cells were negative by cytotoxicity assay with the anti-HSB sera, yet the M.W. 94,000 antigen was detected by radioimmunoprecipitation and SDS-gel studies on these cells. Either the antibodies to this antigen are not cytotoxic or the antigen distribution is such that the cells are not lysed. Additional absorption of the antiserum with 5 x 10⁸ thymocytes/ml removes cytotoxic reactivity against all cells tested, yet the M.W. 94,000 antigen can still be detected by radioimmunoprecipitation and SDS-gel electrophoresis on positive cells. Thus, the absorbed antisera to HSB are serologically T-cell specific, although an additional antigen can be detected on some cells by gel electrophoresis.

Several serological reports have previously appeared from this laboratory on antigens of cells from AMML and CML-BC patients (17, 18). Simian antisera to both lymphocytic and myelogenous leukemia cells were shown to lyse cells from AMML donors. Since all antisera were absorbed with human WBC, any potential TLAA reactivity would have been removed. Some of the leukemia antisera used in these earlier studies may have been detecting la-like antigens or the cALL antigen. However, cross-absorption studies (17, 18) indicate that there may be other antigen(s) detected by these sera which do not fit the pattern of previously described surface membrane antigens. The anti-T- and anti-B-cell sera which were shown to react with AMML and CML-BC patients in an earlier report (17) were different from the ones used in this study. The molecular nature of the antigens detected by these earlier antisera were not characterized by labeling and electrophoresis studies. The earlier report (17) that absorption with AMML or CML-BC cells removed anti-B-cell but not anti-T-cell reactivity may be a reflection of the number of cells used for absorption. Quantitative absorption of anti-TLAA serum with AMML cells required >5 x 10⁸ cells/ml to significantly reduce its cytotoxic titer; in the earlier report, the anti-T-cell serum was absorbed with only 2 x 10⁶ AMML cells/ml.

Our current and past data indicate that cells from most AMML and CML-BC patients express la-like antigens and a T-cell-associated antigen simultaneously (3, 17). Thus, leukemic cells from these patients may be representative of a marrow stem cell with characteristics of both B- and T-cells. The TLAA-la-positive leukemic cells from most AMML and CML-BC patients do not form a high percentage of E-rosettes. However, recent studies by us have demonstrated that 80 to 100% of peripheral blood T-lymphocytes from normal donors when cultured on ‘‘conditioned’’ medium can form E-rosettes and express la antigens as well (15). It may be that some cells have the genetic information for expression of both T-cell and HLA-DR (la) antigens, but the quantitative expression of these antigens is influenced by cellular differentiation events. When these cells are activated or become malignant, they may then by dedifferentiation show quantitative differences in the expression of T-cell or la-associated antigens.

Abramson et al. (1) have already provided evidence that some murine stem cells give rise to both myeloid and lymphoid cells, including B- and T-lymphocytes, while other stem cells are more restricted in their differentiation pathway. Since at least one of these antigens (la) can be considered a differentiation-type antigen and the TLAA shows restricted tissue and cellular distribution, membrane marker studies may help to define the stem cell origin of some of the human leukemias.

REFERENCES

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