Immunofluorescent Method for Positive Identification of Null-Cell Type Acute Lymphocytic Leukemias: Use of Heterologous Antiserum

By Marshall E. Kadin and Ronald J. Billing

Acute lymphoblastic leukemia (ALL) patients could be subclassified into two groups depending on whether or not their leukemia cells expressed a B-lymphocyte antigen. The antigen was detected by an indirect immunofluorescence test using rabbit antisera. In the positive group, consisting of 26 of 32 patients, the leukemia cells were of the "null"-cell type, i.e., they did not appear to express currently recognized T- and B-cell markers. Absorption studies indicated that the positive null-cell group expressed a common antigen which was not expressed on the negative group. Of the 6 negative cases, 5 expressed complement receptors and 3 expressed T-cell markers. The negative group was also characterized by high white cell counts and the presence of a mediastinal mass.

Cell surface markers enable distinction between two major subpopulations of normal peripheral blood lymphocytes. Bone marrow-derived (B) lymphocytes are recognized by the presence of surface membrane immunoglobulins (SmIg) and complement (C3) receptors, whereas thymus-derived (T) lymphocytes are distinguished by their ability to form rosettes with sheep erythrocytes (E rosettes). Recent studies have shown that approximately 20%-25% of children with acute lymphoblastic leukemia (ALL) have blast cells which form E rosettes. This largest group has been referred to as having "null"-cell type ALL. Recently two studies have emphasized the clinical importance of determining cell surface markers in ALL. It has been suggested that patients with ALL expressing T-cell characteristics have a more aggressive form of leukemia than the majority of ALL patients. Such a distinction has the possibility of leading eventually to a more effective treatment of leukemia patients.

In the present study we describe rabbit antisera to human B cells that react with blasts from the majority of null-cell ALL. The antigen being detected appears to be part of the common region of B-cell (Ia-like) alloantigens that are expressed on B lymphocytes but not T lymphocytes. Here we correlate the presence of the B antigen with other T- and B-cell markers and with certain clinical features of ALL.

From the Cancer Research Institute and Hematology Division, Departments of Medicine and Laboratory Medicine, University of California, San Francisco, and the Department of Surgery, School of Medicine, University of California, Los Angeles, Calif.

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Address for reprint requests: Ronald Billing, Ph.D., Department of Surgery, School of Medicine, University of California, Los Angeles, Calif. 90024.

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Patients

We studied 32 consecutive patients with ALL, 3 with diffuse lymphoblastic lymphoma (DLL), and 1 with neuroblastoma. All ALL patients studied were untreated except for patients 14 and 18 (Table 1), who were studied at the time of relapse. ALL patients ranged in age from 2 to 39 yr and included 27 children less than age 16 and 5 adults more than 16 yr of age. One adult (patient 7, Table 2) with diffuse lymphoma also had leukemia at the time of diagnosis and one child (25, Table 1) with lymphoma involving the liver later developed 90%, lymphoblasts in the bone marrow. The third patient with diffuse lymphoma (8, Table 2) was a child with massive involve-

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*PB, peripheral blood; BM, bone marrow.
1 Mediasl mass.
ND, not determined.
G Granulocytes.
1 This patient had DLL.
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*PB, peripheral blood; BM, bone marrow.
†Mediastinal mass.
ND, not determined.
§DLL with leukemia.
ment of the mediastinum who has had normal bone marrow and peripheral blood for 18 mo fol-
lowing diagnosis. One child (9, Table 2) was referred with a diagnosis of ALL but was found to
have neuroblastoma diffusely infiltrating the bone marrow.

Clinical diagnoses of ALL were confirmed by Romanowsky-stained peripheral blood and bone
marrow aspirate smears and bone marrow biopsy. A diagnosis of DLL was established in 3
patients by biopsies of the liver (25, Table 1), mediastinal mass (8, Table 2), or by bone marrow
and lymph node biopsy (7, Table 2). The diagnosis of neuroblastoma was confirmed by tissue cul-
ture of the bone marrow and laparotomy with biopsy of a retroperitoneal mass. In some cases
myeloperoxidase, periodic acid Schiff, and neutral lipid (Sudan black) stains were done to con-
firm the diagnosis rendered on Romanowsky-stained smears.

A n t i s e r a

Antisera with B-cell specificity were produced by immunization of rabbits with papain digests
of cell membranes of involved spleens from patients with histiocytic lymphoma. The method of
antigen solubilization has been described before. Briefly, fresh or frozen spleen tissue was
cut into small pieces with scissors and then homogenized with a vortex homogenizer. Large cellular
debris was removed by a low-speed centrifugation step (1500 g). Membrane fragments were re-
covered from the supernatant by ultracentrifugation at 80,000 g for 90 min (Beckman Spinco
no. 40 rotor). The crude membrane pellet was washed with 0.15 M NaCl, 10 mM Tris, pH 7.2
(Tris NaCl buffer), and then resuspended in water at 30 mg protein/ml. This suspension was then
digested with an equal volume of crude papain (Sigma Chemical Co., St. Louis, Mo.) at 0.5 U/mg
protein in 0.28 M NaCl, 20 mM Tris, pH 8.6, containing 50 mM cysteine. After incubation at
37°C for 1 hr, a 0.5 M iodooacetic acid solution (neutralized to pH 7.6) was added to a final
concentration of 0.1 M. The undigested membrane was removed by ultracentrifugation at
80,000 g for 60 min. The supernatant was dialyzed against Tris-NaCl buffer and stored at
–90°C.

The soluble membrane extract was emulsified with complete Freund’s adjuvant and injected in-
tradermally into the back and subcutaneously into the foot pads of New Zealand white rabbits.
A booster injection was given 4 wk later and after 2 more weeks the animal was sacrificed. Char-
acterization of the antisera has previously been described.

The anti-B-cell antisera was purchased from Alpha Gamma Laboratories, Sierra Madre, Calif.

C e l l s

Leukemia cells were isolated from heparinized peripheral blood by Ficoll-Hypaque density
gradient centrifugation. Bone marrow cells were not further separated after 0.83% NH4Cl lysis
of erythrocytes. Lymphoma cells were prepared from involved tissues by gentle scraping with
an 80-mesh wire cloth in Hanks’ balanced salt solution without calcium and magnesium. Cell
clumps and debris were removed by aspiration of the cell suspensions through no. 25 gauge
needles followed by sedimentation at 1 g.

Only cell suspensions showing at least 90% viability
as determined by trypan blue dye exclusion were studied by rosetting and immunofluorescence.

I m m u n o f l u o r e s c e n c e

Immunofluorescence studies were done by the indirect technique. SmIg was detected with a
polyvalent heavy-chain antisera (Pentex, Miles Laboratories, Kankakee, Ill.) and specific k and
λ light-chain antisera (Meloy Laboratories Inc., Springfield, Va.). Rabbits were immunized with
papain digests of cell membranes from involved spleens of patients with histiocytic lymphoma.

To detect B-cell membrane antigen, 2 x 10⁶ target cells were incubated at 0°C for 30 min
with the B-cell antisera diluted 1:200 in phosphate-buffered saline (PBS). After washing three
times in PBS supplemented with 1% fetal calf serum (FCS), the cells were reincubated for 30 min
with 100 μl fluorescein-conjugated goat anti-rabbit IgG (Meloy) at dilutions of 1:100. After
washing three times to remove the excess fluorescein reagent, the cells were examined with a
Leitz Orthoplan microscope with the Ploem incident fluorescence illuminator and an HBO 200-watt
mercury vapor lamp. (Filters used were K490 exciter, K530 and BG38 suppressor, KG1 heat,
K480 quenching, and S525 selective for fluorescein.)

Under ultraviolet light, positive cells showed bright membrane immunofluorescent staining,
whereas negative cells appeared completely dark (Fig 1). Controls of the goat conjugate alone and
IDENTIFICATION OF NULL-CELL ALL

Fig. 1. Peripheral blood of patient 20 (Table 1). (A) Shown by phase microscopy. (B) Same field shown by immunofluorescence. The leukemic blast (arrow) with a prominent nucleolus shows bright membrane fluorescent staining with the rabbit B-cell antiserum; nonleukemic cells are not stained. x252.

Preimmunized rabbit serum were negative. Bright membrane fluorescence was observed with 6%-15% peripheral blood lymphocytes (PBL) from normal donors, 80%-95%, PBL from chronic lymphocytic leukemia (CLL) donors, and most peripheral blood monocytes. No fluorescence of normal peripheral blood T lymphocytes or thymocytes obtained from infants at cardiac surgery was observed. In addition, when experiments were done in which PBL were examined simultaneously by anti-human gamma globulin and the anti-B antisera it was shown that the anti-B antisera stained SmIg-positive cells and also a population of non-T SmIg-negative cells. The anti-B antisera also appeared to react with myeloblasts and some erythroid normoblasts in normal bone marrow.

It has been observed that nonspecific binding of antibody molecules to B cells can occur through Fc receptors. In order to eliminate the possibility that this kind of binding was involved, immunofluorescent staining studies using F(ab')2 fragments of this antiserum were also performed. The results showed that the percentage of positive cells using F(ab')2 fragments was similar to that obtained with a whole antiserum, indicating that binding through the Fc receptor was not involved. The finding that the antibody could not be removed by washing at 37°C, which removes Fc-bound molecules, also supported this conclusion.

Rosetting Studies

T-cell receptors were detected by spontaneous rosette formation with unsensitized (E) and neuraminidase-treated (EN) sheep erythrocytes. E were prepared by washing sheep erythrocytes in 10% FCS previously absorbed at room temperature for 1 hr with an equal volume of sheep erythrocytes. To prepare EN, 2 x 10⁹ E/ml were washed in HBSS, pH 6.5, and incubated with an equal volume of 2 x 10⁻³ U/ml neuraminidase (Clostridium perfringens type V, Sigma) for 1 hr at 37°C. For rosetting, equal volumes of white cells (5 x 10⁶/ml) and E or EN (2 x 10⁹/ml) were incubated together at 37°C for 15 min in plastic tubes. Following incubation the cell mixture was centrifuged at 200 g for 10 min and stored overnight at 4°C. EN rosettes were also examined at 2 hr.
Rosette cells were gently resuspended by inverting the vial six times. Two drops were removed with a Pasteur pipette and added to an equal volume of 0.1% trypan blue in PBS. Two hundred viable cells were counted in a hemocytometer chamber; a rosetted cell had three or more attached E or EN. The morphology of the rosetted cell preparations was assessed from Shandon cytocentrifuge preparations stained with Wright-Giemsa. Normal lymphocytes had small nuclei with coarsely clumped chromatin and no conspicuous nucleoli, whereas leukemic blasts had large nuclei, delicate chromatin, and one or more prominent nucleoli.

For detection of the receptor for complement, white cell suspensions were incubated with E which had been sensitized with rabbit anti-E, IgM (19S EA), and activated mouse complement (19S EAC). To prepare EA, 2 x 10⁸ E washed in gelatin veronal buffer were incubated at 37°C for 30 min with rabbit anti-E, IgM diluted 1:250 (Cordis Laboratories, Miami, Fla.). The resulting 19S EA were incubated further with fresh-frozen mouse serum diluted 1:20 at 37°C for 15 min. EAC, washed three times in gelatin veronal buffer, were stored for up to 48 hr at 4°C. Then 0.25 ml of EAC (2 × 10⁶/ml) were incubated with an equal volume of white cells (5 × 10⁶/ml) at 37°C for 30 min with rotation. Rosette formation and phagocytosis of EAC was assessed as above for E and EN.

Absorption Studies

In these studies 150 µl of anti-B-cell antiserum at a dilution of 1:200 (2 dilution steps before the end point) was absorbed with 5 x 10⁶ or 15 x 10⁶ ALL cells. Then 20 µl of absorbed antiserum was tested against a different null-cell ALL by immunofluorescence. T-cell ALL and null-cell ALL were used for absorption but the fluorescence was read blindly, i.e., it was not known in advance which sera were absorbed by which cells.

RESULTS

Thirty-two patients with ALL characterized by lymphoblasts in the peripheral blood and bone marrow were studied. Patients could be divided into two groups depending on whether or not their blast cells reacted with the rabbit B-cell antisera. A positive group consisting of 26 (81%) ALL patients is presented in Table 1 and a negative group of 6 (19%) ALL cases is given in Table 2.

There were certain features that further distinguished the two groups (Tables 1, 2, and 3). With respect to cell markers, the positive group consisted entirely of null-cell type ALL, i.e., they did not express SmIg or complement receptors, and they did not form E rosettes. In contrast, in the negative group three of six cases expressed T-cell characteristics (E rosettes) and five of six expressed complement receptors. In two cases in the negative group (patients 4 and 6, Table 2) the leukemic blasts expressed both complement receptors and T-cell markers. The presence of both these markers on the same cells was confirmed by simultaneous double rosettes consisting of E rosettes and complement-coated zymosan rosettes.¹⁵

Table 3. Comparison of B-Antigen-Positive and -Negative ALL

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>15/11</td>
<td>4/2</td>
</tr>
<tr>
<td>Median age (yr)</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>T-cell marker</td>
<td>0/26</td>
<td>3/6</td>
</tr>
<tr>
<td>Complement receptors</td>
<td>0/26</td>
<td>5/6</td>
</tr>
<tr>
<td>Mediastinal mass</td>
<td>3/24</td>
<td>6/6</td>
</tr>
<tr>
<td>Average initial white count/cu mm</td>
<td>40,600</td>
<td>195,000</td>
</tr>
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</table>
Case 20 (Table 1) illustrates the value of the B-cell antiserum in detecting small numbers of leukemic cells in a patient with pancytopenia. With a total white blood cell count of only 3200, 10% of cells appeared to be atypical blasts by virtue of their large nuclei and one or more prominent nucleoli (Fig. 1). These cells showed bright membrane fluorescence with the B-cell antiserum but no fluorescence with the polyvalent Ig antiserum; they also bore no receptors for E or complement. Thus, despite the patient's age of 30 yr, his few leukemic blasts reacted as did those from the majority of children with ALL. This case may be contrasted to the three other adults with ALL in this series (Table 2) whose blasts were nonreactive with the B-cell antiserum but expressed receptors for either complement or E. The latter patients presented with high blast counts and a mediastinal mass, and they have died or relapsed within 1 yr following diagnosis. Patient 20 had a low blast count and no mediastinal mass. He has not been followed for sufficient time to determine his prognosis.

With respect to clinical features, there were differences noted in the numbers of patients with mediastinal involvement, high white cell counts, and numbers of relapses. All 6 of the negative cases examined, but only 3 of 26 positive cases, showed mediastinal involvement (Table 3). This difference was highly significant \( p < 0.001 \). The peripheral white blood cell counts of the negative group were much higher (195,000/cu mm) than those of the positive group (40,600/cu mm).

Data on the number and duration of remissions were limited by the relatively short periods the patients had been on treatment. The data available, however, showed that 16 ALL patients in the positive group were in remission for periods of 1–19 mo without any relapses. Two patients have relapsed and one has died. Of the six ALL patients in the negative group, one has died and three others have relapsed.

The ratios of males to females in both groups were not significantly different. However, there was a difference in median age; that of the positive group was 5 yr, whereas that of the negative group was 10 yr.

Included in the data are three patients with DLL, two of whom developed ALL (patient 25, Table 1; 7, Table 2). Blast cells from one of these patients (25, Table 1) reacted with the B-cell antiserum. In this case lymphoma blast cells obtained from the liver, bone marrow, and pleural fluid all expressed SmIg in addition to the B-cell antigen. These lymphoblasts also had prominent cytoplasmic lipid vacuoles and morphologically resembled Burkitt lymphoma cells. Blasts from the two DLL (patients 7 and 8, Table 2) which did not react with B-cell antiserum formed E rosettes and the patients had prominent mediastinal involvement. Blasts from DLL patient 8 (Table 2) also had receptors for complement (see also ALL patients 4 and 6, Table 2).

In contrast to the reactivity of ALL blasts, those taken from the bone marrow of a patient with disseminated neuroblastoma did not react with the B-cell antisera and did not express any of the lymphocyte markers examined. Absorption studies (Table 4) showed that the null-cell ALL cells absorbed out the anti-B-cell activity when tested against several other null-cell ALL cells, but the same number of absorbing T-cell ALL cells had no effect on the anti-B-cell activity.
DISCUSSION

A main finding in this study is that null-cell ALL patients could be positively identified by the presence of B-lymphocyte antigens on their blast cells. The B antigen detected here by rabbit antisera is found on B lymphocytes, B-cell lines, and leukemia cells from approximately 70% of acute myelocytic leukemia, ALL, and chronic myelocytic leukemia patients and more than 90% of CLL patients. From absorption data it appears that the same B antigen is present on all these cell types.

The absorption data shown in Table 4 indicate that the positive ALL cells express a common antigen detected by the anti-B-cell antiserum and the negative ALL cells do not express this antigen by the methods of detection used. From the present study it is clear that the ALL cells which express the B-cell antigen are those which have previously been considered to be "null"-cell ALL cells, i.e., they did not appear to express currently recognized T- or B-cell markers. From a clinical point of view it appears that this group of patients with null-cell ALL has a better prognosis than patients with T-cell ALL. Our present study also shows that those ALL cells which do not express the B-cell antigen do express either T-cell markers or complement receptors. Therefore, the B-lymphocyte antigen represents a positive marker for the recognition of null-cell ALL cells which previously have been distinguished only by the lack of T- and B-cell surface markers.

There is a considerable amount of evidence that the B antigen detected here by rabbit heteroantisera represents part of the common or nonvariable region of the recently discovered B alloantigens. Several B-alloantigenic specificities have been detected by human multiparous pregnancy sera. There is some evidence that the B alloantigens represent the lymphocyte-defined determinants which are responsible for stimulation in the mixed lymphocyte culture reaction. Because of comparable tissue distribution and genetic loci within the major histocompatibility region, human B alloantigens have also been considered to be the human analogue of the murine Ia antigens (immune response gene-associated antigens).

<table>
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<tr>
<th>Patient</th>
<th>Type</th>
<th>No.</th>
<th>27, 11</th>
<th>4, 1</th>
<th>5, 1</th>
<th>8, 1</th>
<th>17, 1</th>
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<tr>
<td>5, 2</td>
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<td>+</td>
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</tbody>
</table>

* All null-cell ALL cells.
† Patient number.
‡ Table number.
§ ND, not done.
The evidence that the rabbit anti-B-cell antisera are reacting with the non-variable part of the human B-alloantigen molecule is as follows. (1) Both the rabbit and human anti-B-cell antisera react with the same populations of target cells. These include B lymphocytes, B lymphoblastoid cell lines, and approximately 70% of leukemia cells. (2) F(ab')2 fragments of the rabbit antisera specifically block the cytotoxicity of the human B-cell alloantisera. (3) The molecular weight of the antigen detected by the rabbit anti-B-cell sera and the human anti-B-cell sera is the same i.e., 65,000 daltons.

When tested against normal peripheral blood B lymphocytes, cultured B-cell lines, and leukemia cells, the rabbit antisera react with all cells detected by the entire range of human B-lymphocyte alloantisera, whereas the latter show variable specificities from which five main groups of reactivity emerge. Thus, it appears that the alloantisera may detect the variable region and the rabbit antisera the common region of the B-lymphocyte antigen molecule.

In a certain number of the positive cases (Table I) the percentage blast cells was a little different from the percentage B-cell antigen-positive cells determined by immunofluorescence. Part of this difference may be due to technical problems or to the fact that the percentage of blast cells was determined from blood smears, whereas the percentage of immunofluorescence-positive cells was determined from Ficoll-Hypaque prepared cells. There is also the possibility, however, that there were some blast cells present which did not react with anti-B-cell antisera. For example, this phenomenon might be due to a particular stage of the cell cycle that the blast cell is in. In addition, there are some normal nonblast cells, such as monocytes and B lymphocytes, which would appear positive by immunofluorescence. In normal marrow a subpopulation of erythroid normoblasts and myeloblasts also appeared to be positive. In total, the percentage of positive cells in normal bone marrow ranged from 0 to 2%.

There have been various reports in the literature of heteroantisera which react with the majority of leukemia cells but not the majority of normal lymphocytes (i.e., T cells). Although no direct comparisons have been made between antisera, in the cases where the antisera were raised in rabbits to papain digests of B-cell lines it is probable that the antisera are of similar specificity to those described here. The antisera raised to papain-digested B-cell-specific glycoproteins described by Schlossman et al., appear to be similar in many respects to our anti-B-cell antisera. It is known that anti-Raji antiserum has anti-B-cell reactivity. The serum raised to antilymphocyte serum-coated ALL cells described by Brown et al. appears to be different in that it reacts mainly with ALL cells and not with normal B- or T-cell antigens. Nonhuman primate antisera described by Mohanakumar et al. appear to detect morphologic subclasses of leukemia.

Case 20 (Table I) illustrates an advantage of immunofluorescence over cytotoxicity in the evaluation of certain patients. Whereas this case may have been uninterpretable or interpreted as negative by cytotoxicity with the B-cell antiserum due to the low percentage of peripheral leukocytes expressing B alloantigens, immunofluorescence combined with phase microscopy allowed specific detection of the B alloantigens on the membrane of the leukemic blasts (Fig. 1). Immunofluorescence done with the polyvalent Ig antiserum and
cytocentrifuge preparations of the peripheral leukocytes incubated with E, EAC, and in this case EA confirmed that these blasts lacked other currently used lymphocyte markers and would therefore have been designated as “null” cells by these criteria.

In this case (20) and others (cases 9, 12, 13, 19, 20, and 25, Table 1) where the percentage of blast cells was less than 50% of the total cells, it was the blast cells that were immunofluorescent-positive. The small T lymphocytes were negative (Fig. 1). The blast cells which were larger in size, having larger nuclei with conspicuous nucleoli, were easily distinguished from the normal T lymphocytes which formed E rosettes in these mixed populations.

It appears from our results that those leukemic blasts which do not express any of the currently used lymphocyte markers (i.e., null-cell type blasts) can be identified by the presence of B alloantigens. In a limited study of six patients, Fu et al. showed that blasts from four patients appeared to be of the null-cell type and expressed B alloantigens, whereas the remaining two which did not appear to express B antigens were of T-cell origin. However, we have found that lymphoblasts which fail to express B antigens may also lack T-cell markers. Only three of six of our non-B-antigen group formed E rosettes. Five of these six, however, possessed complement receptors and all six expressed at least one of these markers.

From this and other studies of ALL and DLL, the complement receptor appears to be an additional independent marker which may occur in the absence of SmIg and the receptor for E. Moreover, we have shown that its presence may be exclusive of B alloantigens in these disorders. This finding differs from CLL in which leukemic blasts bear B alloantigens. SmIg, and complement receptors. In ALL, detection of the complement receptor may have similar prognostic significance to detection of the receptor for E since both seem to be associated with a high white cell count, a mediastinal mass, and a possible poor prognosis. In our study three patients who had ALL cells with complement receptors but no T markers were part of the B-antigen-negative group. Three of these patients have relapsed and one has died. Thus, it is possible that, besides T-cell markers, complement receptors also define a high-risk group. Large numbers of patients with longer periods of follow-up will be needed to confirm this trend.

Note added in proof. Since submitting this manuscript, we studied lymphoblasts of a 9-yr-old girl with a mediastinal mass and diffuse poorly differentiated lymphocytic lymphoma and leukemia. Her lymphoblasts were null with respect to rosetting and SIg. They also did not react with our rabbit anti-B-cell antiserum. However, they showed immunofluorescence with a specific anti-T-cell antiserum made by immunizing rabbits with MOLT-4 cells. These results are in agreement with observations made by Kersey et al. and Kaplan et al., who also found ALL null cells expressing serologically defined T antigens.

ACKNOWLEDGMENT

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and making available all clinical records. We also thank Eileen Garratty and JaNahn Scalapino for their excellent technical assistance and Dr. Louis K. Diamond for his continuing support and advice.

REFERENCES


23. Mann DL, Rogentine GN, Halterman


Immunofluorescent method for positive identification of null-cell type acute lymphocytic leukemias: use of heterologous antiserum

M Kadin and RJ Billing