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Michael A. Baker , M.D.

Judith A. Falk , B.Sc.

Walter H. Carter , Ph.D.

Robert N. Taub , M.D., Ph.D.
*Virginia Commonwealth University, Medical College of Virginia*

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# EARLY DIAGNOSIS OF RELAPSE IN ACUTE MYELOBLASTIC LEUKEMIA

## Serologic Detection of Leukemia-Associated Antigens in Human Marrow

MICHAEL A. BAKER, M.D., JUDITH A. FALK, B.SC., WALTER H. CARTER, PH.D., ROBERT N. TAUB, M.D., PH.D., AND THE TORONTO LEUKEMIA STUDY GROUP

**Abstract** We tested serial bone-marrow samples from 47 adults with acute myeloblastic leukemia in remission for reactivity with heteroantiserums to leukemia-associated antigens, to determine whether imminent relapse could be detected in patients with acute leukemia. Of 26 patients who relapsed by standard morphologic criteria, 21 had increased immunoreactivity of bone marrow for one to six months (mean, 3.7 months) before relapse. High concordance was observed between a positive test and relapse during the period of study (chi-square = 27.53, $P<0.001$). The median time to relapse after a positive test was four months, as compared with the median remission duration of 19 months for the whole group ($P<0.02$, Peto's log-rank analysis). Serologic detection of leukemia-associated antigens in marrow may be a reliable indicator of imminent relapse in acute myeloblastic leukemia. (N Engl J Med 301:1353-1357, 1979)

THE survival of patients with acute myeloblastic leukemia correlates closely with the duration of first complete remission.[1] When patients have evidence of reappearance of myeloblasts in the marrow, the current practice is to attempt to reinduce remission with chemotherapy; however, reinduction is seldom successful when this point is reached.[1,2] Detection of relapse before the appearance of morphologically detectable myeloblasts in the bone marrow would allow reinduction chemotherapy to be introduced at an earlier time and could result in prolonged duration of first remission.[3]

From the departments of Medicine, Pathology, and Surgery, Toronto Western Hospital, University of Toronto, and the Division of Oncology, Department of Medicine, and the Department of Biostatistics, Medical College of Virginia, Richmond, VA (address reprint requests to Dr. Baker at Toronto Western Hospital, 399 Bathurst St., Toronto, Ontario, Canada M5T 2S8).

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The members of the Steering Committee were Michael Baker, Kenneth Shumak, and Bernadette Garvey; Robert N. Taub, Medical College of Virginia, served as a consultant.

Patients were also referred by the Division of Oncology, Department of Medicine, Medical College of Virginia, Richmond, VA.

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Antigens associated with human acute myeloblastic leukemia have been identified by means of in vivo or in vitro assays of cell-mediated immunity,[4-6] as well as serologic[7-9] and biochemical[10] techniques. We have used heteroantiserums to human myeloblastic leukemia-associated antigens[8,11] to detect imminent relapse in bone marrow from patients with acute myeloblastic leukemia in remission.

## METHODS

### Patient Selection and Clinical Follow-up

Adults with acute myeloblastic leukemia were given cytarabine (cytosine arabinoside), 100 mg per square meter of body-surface area intravenously over 24 hours, by means of continuous infusion for seven days, along with daunorubicin, 45 mg per square meter on Days 1, 2, and 3, to induce remission.[12] If necessary, a second course was given, consisting of cytarabine for five days and daunorubicin for two days at the same daily dosage. Remission-maintenance chemotherapy was given as follows: 10 doses of cytarabine, 100 mg per square meter every 12 hours, and 10 doses of 6-thioguanine, 100 mg per square meter by mouth every 12 hours, with both drugs given for the first five days of each month. Bone-marrow samples for testing were drawn at each monthly visit, immediately before chemotherapy.

Remission was considered complete if less than 5 per cent of the bone-marrow cells were blast cells. Patients were tested simultaneously for reactivity of marrow cells with heteroantiserums and were considered to have a positive test if more than 30 per cent of mononuclear cells were reactive (an increase of more than two standard deviations above the mean for remission marrows of $13\pm7$ per cent).

### Cell Preparation and Storage

Remission leukocytes and normal peripheral blood leukocytes were separated from heparinized samples by means of layering on Ficoll-Isopaque. Bone-marrow cells were similarly prepared to obtain a population enriched in mononuclear cells. Phagocytic cells

were removed by incubation for 10 minutes at 37°C in the presence of iron filings. Cells were used fresh or after storage in RPMI 1640 (Grand Island Biological Company, Grand Island, NY), autologous plasma, and 10 per cent dimethyl sulfoxide at −80°C or −196°C.[13] Viability of frozen stored cells was greater than 90 per cent. Phytohemagglutinin-stimulated lymphocytes were prepared with the large-tube method of Waithe and Hirschhorn.[14] Granulocytes were prepared by layering samples on Ficoll–Isopaque with an excess of diluted fresh blood and recovering cells from the suspension above the red-cell button (Bryant N: personal communication).

### Preparation of Serums

Techniques for production of antiserums have been previously reported.[8,11] In brief, three-month-old male mice of the CBA/J strain (Jackson Laboratories, Bar Harbor, ME) were injected intraperitoneally with cyclophosphamide, 200 mg per kilogram of body weight, and four hours later were injected intravenously with $10^8$ frozen, stored remission leukocytes. Seven, 14, and 21 days later, the mice were injected subcutaneously and intraperitoneally with $10^8$ frozen, stored leukemic blast cells from the same patient in relapse, together with complete Freund's adjuvant (on Days 14 and 21). Mice were bled for antiserums on the 28th and 35th days.

### Direct Testing for B-Cell Reactivity

Serum samples were screened for B-cell reactivity with the standard complement microcytotoxicity test on two test panels: a panel of 20 chronic-lymphocytic-leukemia cells of B-cell type, and a panel of B-cell and T-cell-enriched populations from the peripheral blood leukocytes of 10 disease-free unrelated persons.[15,16]

### Immunofluorescence Testing

Serum samples were stored at −80°C and centrifuged on the day of use to eliminate aggregates. Antileukemia serum was incubated with cells at 4°C for 30 minutes. Cells were washed thrice, resuspended in heparinized medium with 0.01 per cent sodium azide, and incubated for 30 minutes at 4°C with fluorescein-conjugated anti-mouse IgG, IgM, or IgA, diluted to a concentration of 1:5 in medium.[17] Cells were identified with interference contrast optics and then observed for the presence of bright surface fluorescence (rings or dots) with a Ploem vertical illuminator. Background counts determined with fresh or frozen, stored cells were less than 5 per cent. Nonspecific binding of antibody to Fc receptors was minimized by means of the removal of phagocytic cells with iron filings, the use of sodium azide, incubation at 4°C, precentrifugation of serum, and the use of goat antiserums.[17]

### Absorptions

Antiserums were absorbed with leukocyte, bone-marrow, tonsil-cell or myeloblast suspensions; cells were stored at −80°C or in liquid nitrogen and were thawed as described above. Platelets used for absorption were obtained as washed, fresh, pooled human-platelet concentrate on the day of absorption. Phytohemagglutinin-stimulated lymphocytes were used fresh on the day of harvesting. Antiserum, 50 μl, was absorbed with $2\times10^8$ cells or $1\times10^{11}$ platelets in a 0.1-ml Beckman microcentrifuge tube at 4°C for 45 minutes, then centrifuged at $3000\times g$ for three minutes and transferred to additional tubes containing $2\times10^8$ cells or $1\times10^{11}$ platelets.

## Results

After absorption with pooled human platelets, indirect immunofluorescence showed that serum samples were reactive with a majority of cells in samples of peripheral blood from 25 patients and of bone marrow from 73 patients with acute myeloblastic leukemia (Table 1). The level of immunofluorescence detected in peripheral blood and bone marrow from patients with acute myeloblastic leukemia in remission was significantly lower than that in patients in relapse ($P<0.001$ by one-sided t-test). Values obtained from patients in remission were significantly higher than values in patients without leukemia ($P<0.01$).

Reactivity of antiserum with blood or bone-marrow cells (enriched in the mononuclear fraction) from patients with acute lymphoblastic leukemia, with chronic myelocytic leukemia in blast crisis, or without leukemia, and with peripheral blood lymphocytes rendered blast-like by stimulation with phytohemagglutinin was close to background values. Serum samples were nonreactive with peripheral blood cells from patients with chronic lymphocytic leukemia, and quantitative absorption with leukemic lymphocytes did not reduce antimyeloblast activity (Table 2). Serum samples were unreactive on cytotoxicity testing with peripheral blood mononuclear cells obtained from patients without leukemia and enriched in B cells. Quantitative absorption of antiserum with leukemic lymphoblasts, phytohemagglutinin-stimulated lymphocytes, cells obtained from normal marrow, or

Table 1. Specificity of Heteroantiserums.

| Target-Cell Population* | Source of Target Cell | | | |
|---|---|---|---|---|
| | Peripheral Blood | | Bone Marrow | |
| | *no. of samples* | *% fluorescence* | *no. of samples* | *% fluorescence* |
| AML blasts | 25 | 65±15† | 73 | 72±15 |
| AML remission | 22 | 7±4 | 193 | 13±7 |
| CML blast crisis | 3 | 71±13 | ND‡ | ND |
| ALL blasts | 6 | 9±6 | 5 | 9±9 |
| CLL lymphocytes | 10 | 2±2 | ND | ND |
| Nonleukemic | 10 | 3±2 | 40 | 3±2 |
| PHA-stimulated | 3 | 2±1 | ND | ND |

*AML denotes acute myeloblastic leukemia, CML chronic myelocytic leukemic, ALL acute lymphoblastic leukemia, CLL chronic lymphocytic leukemia, nonleukemic those patients with benign disease, and PHA-stimulated phytohemagglutinin-stimulated peripheral blood leukocytes from patients with benign disease.

†Expressed as mean ±SD of cells fluorescent above background.

‡ND denotes not done.

Table 2. Retention by Heteroantiserums of Activity against Leukemic Myeloblasts after Absorption.*

| Cells Used for Absorption | Source of Myeloblasts | | | |
|---|---|---|---|---|
| | Peripheral Blood | | Bone Marrow | |
| | *no. of samples* | *% fluorescence* | *no. of samples* | *% fluorescence* |
| Unabsorbed | 25 | 65±15‡ | 73 | 72±15 |
| ALL blasts | 3 | 54±6 | 3 | 61±8 |
| CLL lymphocytes | 10 | 57±10 | 3 | 66±14 |
| Normal marrow | 10 | 58±12 | 3 | 61±6 |
| Normal granulocytes | 5 | 60±8 | 3 | 66±4 |
| PHA-stimulated | 3 | 54±12 | 3 | 68±8 |
| AML blasts | 10 | 3±2 | 3 | 4±2 |

*Quantitative absorption × 3 at 4°C.

†ALL denotes acute lymphoblastic leukemia, CLL chronic lymphocytic leukemia, PHA-stimulated phytohemagglutinin-stimulated normal peripheral blood leukocytes, and AML acute myeloblastic leukemia.

‡Expressed as mean ±SD of cells fluorescent above background.

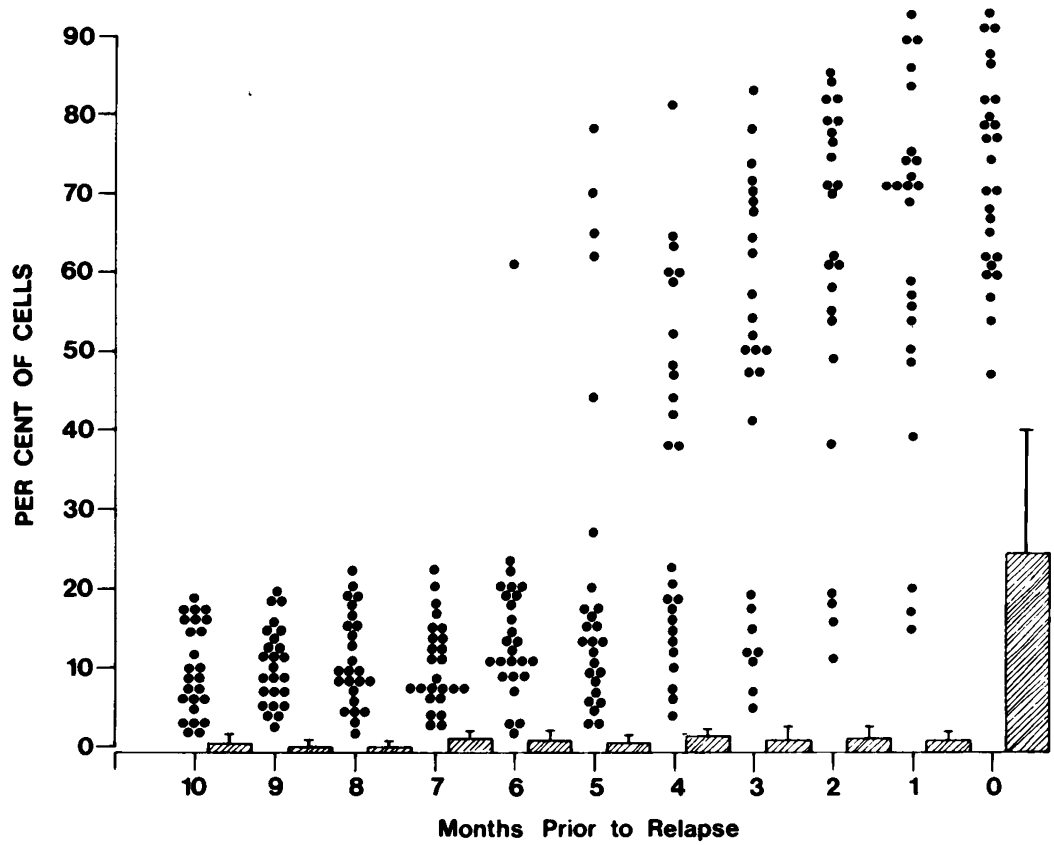


Figure 1. Bone-Marrow Specimens Examined for 10 Months before Relapse in 26 Patients with Acute Myeloblastic Leukemia in Remission.

The number of myeloblasts in the marrow (mean per cent ±S.D.) in all patients tested each month is shown in the columns. The percentage of mononuclear cells immunoreactive in each marrow specimen tested is represented by a solid circle. A test was considered positive if more than 30 per cent of cells were immunofluorescent (>2 S.D. above the mean for remission marrows of 13±7 per cent).

A positive test occurs one to six months (mean, 3.7 months) before relapse as defined by morphologic criteria.

granulocytes from normal peripheral blood did not reduce reactivity against leukemic myeloblasts, but reactivity was removed by absorption with myeloblasts (Table 2). The few reactive cells seen in the control preparations resembled small lymphocytes on phase microscopy. Comparable results were obtained with fresh or frozen cells.

Serial bone-marrow samples from adults with acute leukemia in remission were tested for reactivity with heteroantiserums. At least three marrow samples were obtained at four- to eight-week intervals in 47 patients. Follow-up testing of samples was done for a period of four months to 3.5 years, with a mean follow-up of 14±8 months. During this follow-up period 26 of 47 patients relapsed according to morphologic criteria. A positive test for increased immunoreactivity of bone marrow (greater than two standard deviations above the mean value for remission marrows of 13±7 per cent, i.e., >30 per cent) was found before morphologically detectable relapse in 21 of 26 patients. A positive test preceded the appearance of blast cells by a mean of 3.7 months (range one to six months) (Fig. 1). In five of the patients under study, relapse was seen morphologically before an increase in immunoreactivity was detected. In two cases the marrows drawn eight weeks and four weeks before relapse were technically inadequate for study (samples clotted or too few cells were obtained). In three patients, no increased immunofluorescence was noted in the sample drawn one month before relapse. One patient, who has been in continuous complete remission for over four years, has shown a high degree of immunologic reactivity on three separate occasions several months apart but has remained in complete remission for two years since the first positive test.

Two patients who were tested at the time of diagnosis of leukemia had a high level of reactivity with serum that declined during the first two months of remission. One patient in apparent remission for less than three months maintained a high level of marrow reactivity with serum during the brief remission period.

The time in remission before a positive test correlated with the time before relapse after the positive

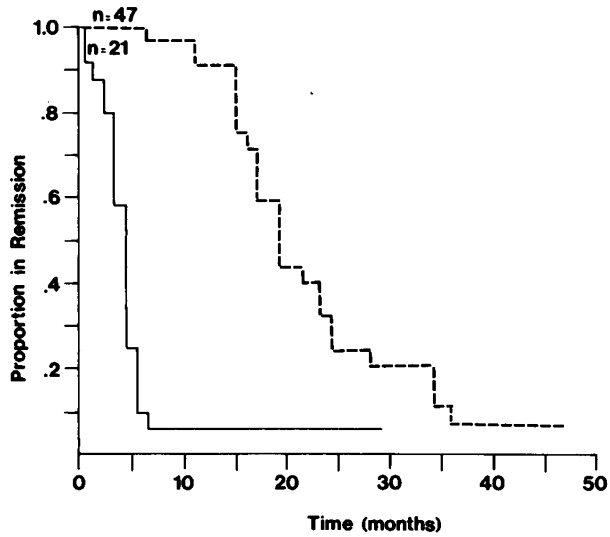


Figure 2. Duration of Remission in All 47 Patients (Broken Lines) and Duration of Remission after a Positive Test in the Subgroup of 21 Patients (Solid Lines) with Increased Immunoreactivity in the Bone Marrow.

The median time to relapse after a positive test was four months, as compared with a remission duration of 19 months in the entire group ($P<0.02$ by Peto's log-rank analysis).

test ($R = 0.53$, $P<0.02$). High concordance between a positive test and relapse during the period of the study was reflected in a chi-square test of independence for a 2-by-2 contingency table (chi-square = 27.53, $P<0.001$). The median time to relapse after a positive test was four months, as compared with a median remission duration of 19 months in the whole group (Fig. 2) ($P<0.02$ by Peto's log-rank analysis).

## Discussion

Antigens associated with human leukemic myeloblasts have been identified with serologic techniques employing either antiserums derived from patients receiving immunotherapy with leukemia-cell vaccines[15,18] or heteroantiserums raised in mice,[8,11] rabbits,[19-21] or nonhuman primates.[7] We have previously shown that heteroantiserums raised in mice rendered tolerant to remission cells with cyclophosphamide and then challenged with blast cells from the same patient could be used to detect leukemia-associated antigens on human myeloblasts in tests of complement-dependent cytotoxicity.[8,11] The use of the indirect-immunofluorescence technique permits identification of a minor population of cells in a heterogeneous cell population when the background activity is reduced to a low level.[9]

Our antiserums are directed toward antigens represented on myeloblasts rather than more mature granulocyte forms, lymphocytes, or other leukemic cells. This finding does not prove, however, that the antigens detected are leukemia-specific. Histocompatibility antigens,[22,23] differentiation antigens,[24,25] and fetal antigens[26,27] have all been detected on leukemic myeloblasts, and our antiserums may be directed against one or a combination of these substances. Of importance to this study, however, is the ability of the antiserums to identify cell-surface antigens associated with the leukemic state in a background of nonleukemic cells.[26]

The serums did not have reactivity with histocompatibility antigens, including the Ia (HLA-DR) antigens expressed on normal B cells and cells from patients with chronic lymphocytic leukemia. Although serum samples were screened against B-cell-enriched nonleukemic cells by means of cytotoxicity testing alone, serum samples were also unreactive with cells from patients with chronic lymphocytic leukemia when tested with both cytotoxic and immunofluorescence techniques. This observation is particularly important since recent studies have shown cross-reactivity between mouse immune serum and human histocompatibility antigens including Ia antigens.[16]

Serial monitoring of patients in remission with use of these antiserums appears to allow detection of imminent relapse an average of 3.7 months before morphologically detectable relapse occurs in the bone marrow. The correlation observed between the length of remission before positive immunoreactivity and the time until relapse after the test makes it unlikely that a positive test was simply a random event during the course of treatment.

Residual leukemic activity in marrow obtained during remission has been demonstrated with stimulation of autologous lymphocytes in vitro,[28] detection of reverse transcriptase,[29] and observations of abnormal in vitro growth characteristics.[30] In this study, we have found that immunologically detectable activity is present on small mononuclear cells in the bone marrow before the appearance of morphologically identifiable blast cells. The immunofluorescence-positive cells resemble small lymphocytes but could represent a myeloid precursor. Before leukemic relapse occurs, precursors or stem cells in the marrow may pass through stages of differentiation that express leukemia-associated antigens but do not yet resemble blast cells morphologically.[26] The antigens expressed may represent differentiation markers and may result from the variations in cell products that occur with changes in biologic state.[31]

Alternatively, reactivation of leukemia may effect changes that cause nonleukemic cells to display new determinants before leukemic blast cells become evident. Increases in sialotransferases or fucosyl transferases in serum may disturb glycoprotein synthesis and change cell-surface carbohydrate moieties[32] — an effect similar to that responsible for changes in blood-group substances in patients with leukemia.[33] Still another alternative is that the reactive cells may not

synthesize antigen but, rather, adsorb antigen from the plasma. We have demonstrated a rapid shedding of leukemia-associated antigens from blast cells in vitro,[10] and it is possible that when such molecules are shed by reactivated leukemic cells in vivo, they adsorb to nonleukemic cells in the marrow.

Over half the adults with acute myeloblastic leukemia will have complete remissions if current chemotherapeutic regimens are used.[1-3] Most patients will relapse within two years in spite of remission-maintenance regimens, but the time elapsing before relapse may vary from three months to five years.[1-3] Present strategy calls for the use of reinduction chemotherapy when morphologically identifiable blast cells are detected in the bone marrow during monthly screening examinations; however, reinduction chemotherapy has generally been unsuccessful. Intermittent use of prophylactic reinduction therapy during remission has been precluded by the toxicity of the regimens used and the wide variation of remission duration. Our results show that serologic detection of leukemia-associated antigenic activity in marrow is a reliable indicator of imminent relapse. Use of reinduction chemotherapy at this point may prolong remission duration and survival. Treatment trials are needed to test this hypothesis.

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