

## Acute Myeloblastic Leukemia-Associated Antigens: Detection and Clinical Importance\*

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### A. Abstract

Antigenic compounds from the surface of leukemic myeloblasts are shed *in vitro* on short-term culture. Blast cells radiolabeled by lactoperoxidase iodination release soluble compounds that react immunologically with alloantisera to leukemia-associated antigens. Partially characterized soluble antigens were used to raise heteroantisera in monkeys that are selectively reactive with leukemic myeloblasts and unreactive with nonleukemic cells. Monkey heteroantisera were used to further characterize soluble leukemia antigens. Sera from patients with acute myeloblastic leukemia inhibit the reactivity of the heteroantisera, suggesting that soluble leukemic antigen is released *in vivo* as well.

### B. Introduction

The description of antigens on leukemic blast cells has increased our understanding of human leukemia (Greaves 1979). Heteroantisera raised in mice, rabbits, or monkeys to leukemic antigens have been useful in defining tissues of origin of leukemic cells and improving diagnostic accuracy (Baker et al. 1974, 1976, 1978, 1979). Solubilization of leukemic antigens and biochemical characterization of antigenic compounds may yield further insight into the nature of the leukemic process (Taub

et al. 1978). Biochemical studies involving extraction of leukemic cell membrane-associated antigens with proteolytic enzymes (Billing and Terasaki 1974; Metzgar et al. 1974) or hypertonic potassium chloride (Gutterman et al. 1972) have generally yielded inhomogeneous or incompletely characterized products. Assays for antigenicity have been semiquantitative and depended either on inhibition of agglutination or cytotoxicity or on the reactions to intracutaneous skin testing (Mavligit et al. 1973).

Because our preliminary observations suggested that certain membrane components may be "shed" in soluble form from blast cell surfaces (Taub et al. 1976) just as from certain normal cells (Cone et al. 1971), we have analyzed material released into the supernatant medium of cultured myeloblasts.

Partially characterized compounds from the leukemic myeloblast cell surface have been used to raise heteroantisera in monkeys that are selectively reactive with leukemic myeloblasts but are unreactive with nonleukemic cells. Sera from patients with acute leukemia inhibit reactivity of the antisera, suggesting that similar compounds are shed *in vivo*.

### C. Methods

#### I. Preparation of Radiolabeled Soluble Antigen

Leukemic cells were obtained from the peripheral blood of patients with acute myeloblastic leukemia on initial presentation, with white blood cell counts greater than  $50.0 \times 10^9/l$ , and with greater than 99% myeloblasts on differential white count. To

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$1 \times 10^8$  cells in 2 ml phosphate-buffered saline (PBS; pH 7.0) were added 1.0 mC sodium iodide  $^{125}\text{I}$ , 200  $\mu\text{l}$  of lactoperoxidase (Sigma, 0.25 mg/ml), and 25  $\mu\text{l}$  of 0.03% hydrogen peroxide. The cells were incubated at room temperature for 10 min; during this period 25  $\mu\text{l}$  of the peroxide solution was added twice. The reaction was terminated by adding 8 ml 0.01 M cysteine and 0.01 M potassium iodide in PBS. The cells were washed thrice in Hanks balanced salt solution and placed in culture at 37°C in 5 ml minimal Eagle's medium. After 4 h the medium was discarded and the cells were washed and reincubated. The supernatant was harvested at 24 h. Cell suspensions showing less than 80% viability were not used.

## II. Immunoprecipitation

To microtiter U-plate wells (Cooke Engineering Co., Alexandria, Virginia) prewashed with bovine serum albumin were added 20  $\mu\text{l}$  of alloserum and 20  $\mu\text{l}$  of the  $^{125}\text{I}$ -labeled supernatant. All tests were done in triplicate. The plates were shaken and allowed to stand for 1 h at 4°C. Coprecipitation was carried out by adding 100  $\mu\text{l}$  of Staphylococcal protein A (Enzyme Centre Incorporated, Boston, Mass.) to each well. The plates were shaken again, kept 15 min at 22°C, and then spun at 1800 r/min for 10 min. The supernatant liquid was gently sucked out of the wells, and the precipitates were washed three times in PBS (pH=7.2) and transferred to cuvettes. Radioactivity was counted in a Beckmann Biogamma II gamma spectrometer.

## III. Gel Filtration Chromatography

Gel filtration chromatography was conducted by applying 0.5 ml aliquots of culture supernatant on to 0.9 $\times$ 90 cm columns (Glenco Scientific, Inc.) of Bio-Gel A-1.5m (approximately 8% agarose, 200–400 mesh) equilibrated and eluted at 4°C with 0.01 M ammonium acetate at a hydrostatic pressure of 20 cm water.

## IV. Isoelectric Focusing

Solutions for isoelectric focusing were added to 0.75 ml of 40% ampholine (pH 3.5 to 10) and applied to an LKB 8100 column at 4°C to a total volume of 110 ml with sucrose gradient solution (Abraham and Bakerman 1977).

## V. LDS-Polyacrylamide Disc Gel Electrophoresis

The pooled fractions or immunoprecipitates from these fractions were boiled with 1% (weight/volume) lithium dodecyl sulfate (LDS)/0.01 M lithium phosphate buffer, pH 7.0, and then incubated

further with this detergent at 37°C for 30 min. The resulting polypeptide subunits were resolved by LDS-polyacrylamide disc gel electrophoresis carried out according to Laemmli (1970) except for the substitution of LDS for sodium dodecyl sulfate in order to conduct the experiments at 4°C (Delepelairre and Chua 1979).

## VI. Cells

Enriched T- or B-lymphocyte preparations were obtained either by selective rosetting of T-lymphocytes using sheep erythrocytes or by removing adherent B-lymphocytes by absorption on a flask coated with affinity-purified goat antihuman Fab. The details of T- and B-cell enrichment procedures have been published earlier (Mohanakumar et al. 1979).

## VII. Preparation of Antiserum

Heterologous anti-AMLSGA serum was prepared by injecting a monkey (*M. spesiota*) intravenously and intradermally (50  $\mu\text{g}$ ) three times, with a period of 14 days between immunizations (Mohanakumar et al. 1974). For intradermal injections the antigen suspended in PBS pH 7.2 was mixed with an equal volume of Freund's complete adjuvant (H37Ra). The antiserum described in this report was obtained 7 days after the third inoculation with antigen.

## VIII. Absorption of Antisera

Immune and the preimmune sera were heat inactivated at 56°C for 30 min and then absorbed twice for 20 min at 4°C with an equal volume of cells (pooled normal human platelets, leukocytes, bone marrow cells, or myeloblasts).

## IX. Microcytotoxicity Assay

A standard Amos modification of the microtechnique described by Mittal et al. (1968) was used for unfractionated cell preparations, and the incubation time with complement was extended to 2 h when enriched B- and T-lymphocytes were used as targets. Selected nontoxic rabbit complement was used throughout.

## D. Results

### I. Partial Characterization of Surface Compounds

Leukemic myeloblasts radiolabeled with the lactoperoxidase iodination technique released labeled soluble compounds into supernatant

media. Following 24-h incubation, cells remained >90% viable by trypan blue exclusion. Gel filtration chromatography in 8% agarose of the labeled supernatant yielded two distinct peaks, coincident in radioactivity and protein concentration. The second peak was selectively reactive by coprecipitation with antileukemic antisera obtained from patients receiving immunotherapy with leukemic myeloblasts (Taub et al. 1978).

Supernatant material derived from radiolabeled blasts and eluted in the second peak from the agarose column was applied to a DEAE cellulose column and yielded a single peak, coincident in radioactivity and protein levels. Carbohydrate content of the eluted material was estimated at 10% by weight. This material retained immunologic activity with antileukemic alloantisera as measured by coprecipitation.

## II. Development of Heteroantisera

Antisera raised in monkeys to the compounds eluted from DEAE cellulose columns was tested for cytotoxic reactivity against leukemic and nonleukemic cells (Table 1). In complement-dependent cytotoxicity testing the monkey anti-AMLPGA was reactive with cells from leukemic patients but was unreactive with nonleukemic peripheral blood or bone marrow cells. Absorption of anti-AMLPGA with leukemic myeloblasts from patients with acute myeloblastic or chronic myelocytic leukemia removed all antileukemic activity. Ab-

sorption with leukemic lymphoblasts or leukemic lymphocytes removed activity against ALL or CLL cells but did not remove antimyeloblast activity. Anti-AMLPGA was unreactive with B-lymphocytes, including those of an identical twin to a patient with AML, and absorption with B-lymphocytes did not reduce antimyeloblast activity. In contrast, the rabbit anti-Ia was reactive with all nonleukemic B-lymphocytes. Antileukemic activity was not reduced by absorption of anti-AMLPGA with the enriched mononuclear fractions of nonleukemic marrow or with neutrophils from nonleukemic patients.

## III. Further Characterization of Surface Compounds

The antileukemic heteroantisera were applied to the further isolation and characterization of compounds from the myeloblast cell surface. Compounds eluted from gel columns were applied to isoelectric focusing columns and fractions obtained were analyzed for reactivity by coprecipitation with the heteroantisera. A major reactive peak was obtained at pI 7.8. Analysis of the immune precipitate by LDS-PAGE yielded a homogeneous peak with molecular weight estimated between 70,000 and 80,000 daltons.

## IV. Testing of Patients' Sera

Serum samples were tested for their ability to interfere with coprecipitation of soluble radio-

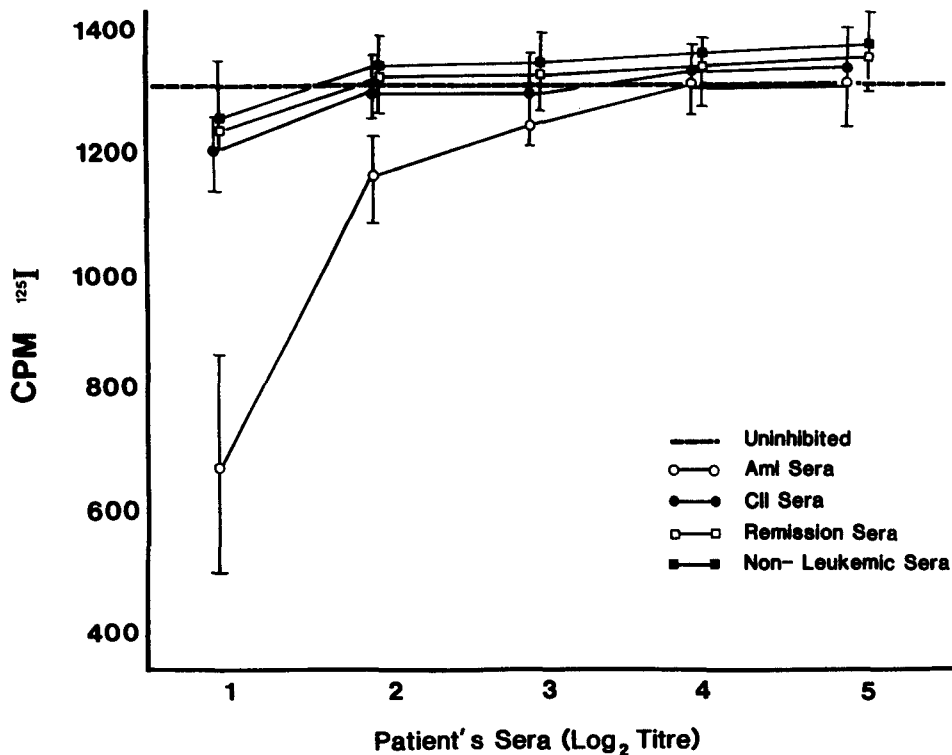
Serum absorbed with	Target cells					
	AML (10) <sup>a</sup>	CML (5)	ALL (5)	CLL (5)	T-cells (10)	B-cells (5)
Unabsorbed	+ <sup>b</sup>	+	±	±	-	-
AML <sup>c</sup>	-	-	-	-	-	-
CML	-	-	-	-	-	-
ALL	+	+	-	-	-	-
CLL	+	+	-	-	-	-
B-cells	+	+	±	+	-	-

**Table 1.** Reactivity of monkey anti-AML antiserum

<sup>a</sup> Number of samples tested

<sup>b</sup> Plus (+) indicates all samples were reactive. Minus (-) indicates all samples were negative. Both signs (±) indicate weak positive reactions in some samples

<sup>c</sup> Abbreviations: AML, acute myeloblastic leukemia; CML, chronic myelocytic leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia



**Fig. 1.** Inhibition of coprecipitation by patient's sera. Sera from AML patients with high blast counts inhibit the precipitation of leukemic antigen by monkey antimyeloblast sera. Significant inhibition is obtained at the first dilution of patient's sera ( $P < 0.05$ )

labelled leukemia antigen by antileukemic heteroantisera (Fig. 1). Sera were tested from seven patients with AML in relapse (peripheral blood blast count  $> 50,000/\text{mm}^3$ ), five patients with chronic lymphocytic leukemia (lymphocyte count  $> 50,000/\text{mm}^3$ ), five patients with AML in remission, and five patients with nonleukemic disease. Significant inhibition was seen with low dilutions of sera from patients with high myeloblast counts ( $P < 0.05$ ).

## E. Discussion

The development of techniques for lactoperoxidase-catalyzed surface radioactive labeling of viable cells has provided an important tool for analysis of the composition and turnover of cell membrane components (Hubbard and Cohen 1972; Humphreys et al. 1976). An important advantage of this surface-labeling technique is that of topological as well as chemical specificity; only antigens sufficiently exposed on the membrane surface to be accessible to the action of peroxidase (Vidal et al. 1974) and consisting of substantial amounts of histidine- or tyrosine-containing protein will be heavily labeled (Hubbard and Cohen

1972). We chose to analyze material shed into the supernatant from membranes of viable cultured myeloblasts in order to circumvent the problem of contamination with HLA antigens encountered in some attempts to solubilize leukemic cell membrane antigens directly. Metzgar et al. (1974) used trypsin digestion, 3 M potassium chloride extraction, or autolytic treatment to prepare soluble LAAs. Only trypsin treatment proved effective, but large amounts of HLA activity were solubilized along with LAA.

The antiserum that we have raised to this antigen is highly reactive with leukemic myeloblasts from patients with AML or CML (Ramachandar et al. 1975) and following appropriate absorption is unreactive with histocompatibility antigens, including Ia antigens, normal marrow fractions, leukemic lymphoblasts, and leukemic lymphocytes. Absorptions with normal peripheral blood cells, bone marrow cells, lymphoblasts, or CLL cells does not remove activity against myeloblasts.

Although the antiserum is selectively reactive with leukemic myeloblasts, it is not necessarily leukemia specific. The antigens recognized may be present in a cell population occurring infrequently in normal blood or marrow or may be well masked on the cell surface of

nonleukemic cells (Greaves 1979). Leukemic blast cells carry oncofetal antigens (Granatek et al. 1976) or antigens characteristic of specific phases of the cell cycle (Pasternak et al. 1974), and these may be recognized by the antiserum. Selective reactivity against myeloblasts may be explained by cell surface glycoprotein changes characteristic of leukemic cells (Andersson et al. 1979; Khilanani et al. 1977; van Beek et al. 1978). Van Beek et al. (1978) have documented structural differences in fucose-containing glycopeptides in leukemic myeloblasts compared to nonleukemic leukocytes, and Andersson et al. (1979) have demonstrated surface glycoprotein patterns diagnostic of acute myeloblastic leukemia. Studies of serum and urine of patients with AML have yielded characteristic compounds resulting from alteration of normal glycoproteins (Rudman et al. 1976). Since the compounds obtained in the present study were not prepared by adding proteolytic or dissociating agents but were rather shed into the supernatant during short-term incubation, we have characterized material that is associated with the cell surface but is not likely to be an integral membrane glycoprotein; furthermore, it may represent intact native compounds rather than fragments.

We have previously described the detection of leukemic antigen in the bone marrow of patients with AML in remission (Baker et al. 1979). The increase in reactivity which occurs in marrow before relapse may allow the early use of reinduction chemotherapy. The technical difficulties inherent in studying fresh bone marrow specimens have led us to study antigen levels in serum. Further refinement of immunologic assays for detection of antigen in serum may allow trials of therapeutic intervention upon early detection of increase in tumor burden.

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