Isolation and Partial Characterization of Radioiodinated Myeloblastic Leukemia-associated Cell Surface Glycoprotein Antigen

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ABSTRACT

Peripheral blood myeloblasts from five patients with acute myeloblastic leukemia and peripheral remission leukocytes from two of these patients were radiolabeled by the lactoperoxidase-catalyzed surface radioiodination technique and incubated in a nutrient medium at 37°. Radioactive materials shed from viable cells into the supernatant at 24 hr were purified by gel filtration and by DEAE-cellulose chromatography. The radiolabeled leukemic cells shed relatively few molecular species into the culture medium. The DEAE-cellulose eluate usually contained one major peak in which radioactivity and protein levels were coincident; the molecular weight of this compound was 350,000 to 400,000, and it contained carbohydrate as well as protein. Glycoprotein shed from leukemic cells was specifically reactive in a coprecipitation assay with defined antmyeloblast alloantisera obtained from leukemic patients receiving immunotherapy. No reaction was seen with antisera directed against HLA or B-cell antigens. Material shed from remission cells did not coprecipitate with antileukemic antisera. The isolation of radioactively labeled antigen derived from myeloblasts may ultimately allow the monitoring of human antigen levels in leukemic blood by radioimmunoassay.

INTRODUCTION

Serological and biochemical analyses of human leukemia cell surface antigens can aid in our understanding of the pathogenesis of leukemia and could foster the development of immunodiagnostic techniques. Heteroantisera to human LAA's have been raised in rabbits (15, 17), mice (3, 4), and nonhuman primates (15, 25) and have been shown to discriminate between leukemic and nonleukemic cells in both peripheral blood and bone marrow. We have reported that patients with leukemia receiving immunotherapy will produce alloantisera against leukemia-associated determinants that are distinguishable from normal WBC antigens, including histocompatibility and differentiation antigens (1, 30).

Biochemical studies involving extraction of leukemic cell membrane-associated antigens with proteolytic enzymes (7, 25) or hypertonic potassium chloride (16) 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 (23).

Because our preliminary observations suggested that certain membrane components may be "shed" in soluble form from blast cell surfaces (36), just as from certain normal cells (10), we have analyzed material released into the supernatant medium of cultured myeloblasts. In these studies our alloantisera raised against human LAA were used in a coprecipitation assay to aid in the isolation and partial characterization of myeloblastic LAA following lactoperoxidase-catalyzed cell surface radioiodination.

MATERIALS AND METHODS

Antisera. Human alloantisera against LAA were obtained from patients with acute myeloblastic leukemia who had received immunotherapy for 12 to 24 months with allogeneic leukemia cells from a single donor and Bacillus Calmette-Guérin vaccine (1). Antisera were tested as outlined previously either by the fluorochrome cytotoxicity assay described by Tagasugi (34) or by an indirect immunofluorescence assay with the use of fluoresceinated rabbit anti-IgG (Behringwerke, Marburg, West Germany) (3). With the use of anti-HLA reagents of defined specificity provided by J. Falk, tabulations were made of the HLA antigens of the patients' remission and relapse cells, the immunizing alloantigens, and cells of test panels. Reactivity of these alloantisera could be demonstrated against leukemic myeloblasts and not against leukemic lymphoblasts, remission cells, or nonleukemic cells; serological activity was sometimes present against HLA components and B-cell antigens as well as LAA, but these activities could be absorbed with peripheral blood leukocytes without loss of anti-leukemic cell activity. The characterization of our antisera to LAA is detailed elsewhere (1).

Lactoperoxidase-catalyzed Radiiodination. We studied peripheral blood myeloblasts from 7 patients with acute myeloblastic leukemia in relapse (PIV, OST, COA, MIC, VEI, WAC, and SY5) and peripheral leukocytes from 3 of these patients (OST, COA, and VEI) during complete hematological remission, as diagnosed by the usual criteria (1). Cells were frozen, stored in 10% dimethyl sulfoxide at −70°C in a Revco freezer or at −196°C in liquid nitrogen, and thawed rapidly before use in a 37°C water bath. Cell suspensions with less than 90% viability at the start of the procedure...
were discarded.

To 5 x 10^7 cells in 2 ml PBS (pH 7.0) were added 1.0 mCi [125]IU labeled sodium iode, 200 μl lactoperoxidase (0.25 mg/ml; Sigma Chemical Co., St. Louis, Mo.), and 25 μl 0.03% hydrogen peroxide. The cells were incubated at room temperature for 10 min; during this period 25 μl of the peroxide solution were added twice. The reaction was terminated by adding 8 ml of 0.01 M cysteine and 0.01 M potassium iodide in PBS. The cells were washed twice in Hanks' balanced salt solution and once in Eagle's minimal essential medium containing 5% fetal calf serum. The cells were then placed in culture at 37° in 5 ml Eagle's minimal essential medium containing 10% fetal calf serum. The medium was harvested and changed after 1, 4, and 24 hr. Cell suspensions showing less than 80% viability were not used.

Coprecipitation Assay. Twenty μl of alloserum in 0.9% NaCl solution and 20 μl of the [125I]-labeled supernatant were added to microtitr U-plate wells (Cooke Engineering Co., Alexandria, Va.). The plates were shaken and allowed to stand for 15 to 20 hr at 4°. Coprecipitation was carried out by adding 20 μl of polyvalent rabbit anti-human immunglobulin antiserum or rabbit anti-mouse IgG (Behring Diagnostics, Somerville, N. J.) to each well. The plates were shaken again, kept 15 to 20 hr at 4°, and then spun at 1800 rpm for 10 min. The supernatant liquid was gently sucked out of the wells, and the precipitates were washed 3 times in PBS (pH 7.2). Wells containing precipitate were then counted for radioactivity in a γ scintillation spectrometer.

Gel Filtration and Ion-Exchange Chromatography. Gel filtration chromatography was conducted by applying 0.5-ml aliquots of culture supernatant onto 0.9- x 90-cm columns (Glenco Scientific, Inc., Houston, Texas) of 8% agarose (Bio-Gel A, 1.5 m, 200 to 400 mesh) equilibrated and eluted at 4° with 0.01 m ammonium acetate at a hydrostatic pressure of 20 cm water. Fractions of 1 to 2 ml were collected, and the radioactivity was counted in a γ spectrometer. The protein profile was determined by measuring the absorbance at 280 nm. Protein concentrations in specific pools of radioactive material were determined by a microbiuret procedure with crystalline bovine serum albumin as standard (26). Samples of culture supernatant were also applied onto 0.9- x 60-cm Sephadex G-100 (fine) columns that were developed and monitored as described for agarose, except for a hydrostatic pressure of 50 cm. The Sephadex and agarose columns had been standardized in separate experiments by applying proteins of known molecular weight (horse apoferritin, human γ-globulins, and human transferrin and ovalbumin; Schwarz/Mann, Orangeburg, N. Y.). In addition homogeneous human liver fatty acid synthetase, which was assayed by a spectrophotometric method (Gilford spectrophotometer 2400-S), was used as a standard (31).

DEAE-cellulose (Whatman DE52) chromatography was conducted by applying aliquots of concentrated 8% agarose eluates at 10 mg protein per ml of packed resin. After the column was washed with 3 volumes of equilibrating solution (ammonium acetate, pH 7.2, at a conductivity of 3 mmho), it was developed with a linear ammonium acetate gradient (3 to 17 mmho, pH 7.2) pumped at about 0.3 ml/min. Eluate fractions were monitored for radioactivity and protein. In some experiments total carbohydrate was quantified by the phenol-sulfuric acid procedure (12).

RESULTS

Coprecipitation of Radioactivity from Unfractionated Supernatants of Radioiodinated Blast Cells and Remission Cells (Table 1). Myeloblasts and remission cells from Patient OST were adjusted to 5 x 10^7 cells in 1 ml of medium and subjected to lactoperoxidase-catalyzed iodination. The supernatant was harvested at 24 hr from cultured labeled cells and incubated with a panel of antisera from 3 patients (DIX, RIC, and LOG) receiving immunotherapy with third-party allogeneic myeloblasts (1) and with serum from a normal AB Rh-positive donor. Somewhat more trichloroacetic acid-precipitable [125I] radioactivity was incorporated into the blast cell than remission cell supernatant. A greater proportion of radioactivity was precipitated by allogeneic anti-LAA antisera from the supernatant of blast cells than of remission cells; in the former instance much more was precipitated than could be accounted for by differences in protein-bound [125I]. Normal serum precipitated equivalent amounts of radioactivity from blast cells and remission cells.

Gel Filtration Chromatography. After application to a column of 8% agarose, the myeloblast supernatants from Patients OST, MIC, and WAC (Chart 1, A, B, and C) and from 4 other patients (PIV, VEI, COA, and SYS, not shown) yielded similar elution profiles. Remission leukocytes from Patient OST, (Chart 1D) and from Patients VEI and COA (not shown) showed an elution profile with much lower levels of activity and a near absence of the characteristic initial radioactive peak.

Three sets of adjacent fractions (35 to 45, 45 to 55, and 55 to 65) were pooled from both the blast cell and the remission leukocyte eluates and were tested in parallel coprecipitation assays against several alloantisera (Table 2). Anti-LAA alloantisier (DIX) diluted 1:1 precipitated 12.4% of the total acid-precipitable radioactivity from the pooled fractions of the major peaks eluted from the blast cell supernatant but did not precipitate fractions of remission leukocyte extracts. Anti-HLA-A2 precipitated a small amount of radioactivity from Fraction "A" of OST blast cells but not OST remission leukocytes (the remission cells of

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>OST blast cells</th>
<th>OST remission cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-AML(^b) (RIC)</td>
<td>8251 ± 1391(^c)</td>
<td>1731 ± 388</td>
</tr>
<tr>
<td>Anti-AML (DIX)</td>
<td>7829 ± 1350</td>
<td>1680 ± 400</td>
</tr>
<tr>
<td>Anti-AML (LOG)</td>
<td>4495 ± 890</td>
<td>985 ± 295</td>
</tr>
<tr>
<td>Normal</td>
<td>548 ± 60</td>
<td>457 ± 48</td>
</tr>
<tr>
<td>10% TCA(^d)</td>
<td>34630</td>
<td>25330</td>
</tr>
</tbody>
</table>

\(^a\) Net cpm in washed precipitate from 20 μl of supernatant.  
\(^b\) AML, acute myeloblastic leukemia.  
\(^c\) Mean ± S.D. of 3 replicate determinations.  
\(^d\) Net cpm in trichloroacetic acid precipitate from 20 μl of supernatant.
Table 2
Coprecipitation of radioactivity from fractionated supernatant of short-term cultured, surface-radioiodinated OST blast cells and remission leukocytes

<table>
<thead>
<tr>
<th>Antisera</th>
<th>35–45 (cpm Ab(^{5})·cpm TCA(^{7}))</th>
<th>45–55</th>
<th>55–65</th>
<th>35–45 (cpm Ab(^{5})·cpm TCA(^{7}))</th>
<th>45–55</th>
<th>55–65</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIX 1:1</td>
<td>151:6,565 (2.3)(^{d})</td>
<td>634:5,110 (12.4)</td>
<td>420:20,100 (2.1)</td>
<td>109:4,737 (2.3)</td>
<td>652:824 (2.3)</td>
<td>271:358 (2.0)</td>
</tr>
<tr>
<td>DIX 1:4</td>
<td>145:6,900 (2.1)</td>
<td>345:4,528 (8.1)</td>
<td>272:12,350 (2.2)</td>
<td>87:4,143 (2.1)</td>
<td>72:3,010 (2.4)</td>
<td>281:332 (2.1)</td>
</tr>
<tr>
<td>Anti HLA-A2</td>
<td>140:4,506 (3.1)</td>
<td>277:11,541 (2.4)</td>
<td>253:14,883 (1.7)</td>
<td>146:6,639 (2.2)</td>
<td>75:3,574 (2.1)</td>
<td>27:1,172 (2.3)</td>
</tr>
<tr>
<td>Anti HLA-A1</td>
<td>ND(^{e})</td>
<td>268:11,654 (2.3)</td>
<td>101:7,959 (2.4)</td>
<td>79:3,761 (2.1)</td>
<td>47:2,472 (1.9)</td>
<td>27:1,176 (2.3)</td>
</tr>
<tr>
<td>AB serum</td>
<td>267:15,720 (1.7)</td>
<td>ND</td>
<td>166:7,207 (2.3)</td>
<td>105:4,772 (2.2)</td>
<td>62:3,444 (1.8)</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{a}\) See text.
\(^{b}\) Background precipitated by antisera and rabbit anti-human immunoglobulin from each of two 20-μl aliquots of pooled concentrated fractions. Mean of 2 determinations. See text.
\(^{c}\) Background precipitated by 10% trichloroacetic acid from a third 20-μl aliquot.
\(^{d}\) Numbers in parenthesis, percentage.
\(^{e}\) ND, not done.
OST were known to be HLA-A2 positive). Anti-HLA-A1 and normal human AB Rh-positive serum precipitated minimal quantities of radioactivity.

**DEAE-Cellulose Chromatography.** Supernatant material derived from blast cells of Patient OST (and Patient COA, not shown) that eluted from the agarose column in the major peak was applied to a DEAE-cellulose column and yielded a single peak characterized by congruence of radioactivity and protein levels (Chart 2). The carbohydrate content by weight of the eluted material was estimated at 10%.

**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 component (19, 20). An important advantage of this surface-labeling technique is that it is of topological as well as chemical specificity; only antigens sufficiently exposed on the membrane surface to be accessible to the action of peroxidase (37) and consisting of substantial amounts of histidine- or tyrosine-containing protein will be heavily labeled (19). The use of specific antisera to precipitate solubilized iodinated membrane determinants has aided in identifying and monitoring the isolation of cell surface-associated immunoglobulin, H2, g, thy-1, and histocompatibility antigens (38), as well as human lymphocyte surface antigens (39). The availability in our laboratory of xeno- and alloantisera reactive by cytotoxicity and immunofluorescence with leukemic relapse cells and unreactive with remission leukocytes (1, 3) prompted us to apply radioiodination techniques in the study of membrane antigens of human myeloblasts.

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. (25) used trypsin digestion, 3 m potassium chloride extraction, or autolytic treatment to prepare soluble LAA's. Only trypsin treatment proved effective, but large amounts of HLA activity were solubilized along with LAA (25). In some of our preliminary experiments (D. A. Roncari, unpublished data), digestion of myeloblasts with papain failed to release LAA activity, although HLA activity could be released from either normal or leukemic cells.

Our data suggest that there is a rapid turnover of cell surface glycoproteins of myeloblastic leukemic cells as compared with remission cells and that large amounts of soluble material are liberated into the culture supernatant. The agarose chromatography studies of relapse and remission cells suggest that myeloblasts from different patients with acute leukemia may shed a common leukemia-associated soluble glycoprotein antigen (AMLSGA) (M.W., 350,000 to 450,000) into the culture medium. This molecular weight is considerably greater than that reported for LAA's obtained by papain digestion of leukemic cell membranes (25) or by affinity column purification (M.W., 27,000) (8). It is also larger than soluble HLA antigens (M.W., 60,000), β-2-microglobulin (M.W., 12,000) (35), or B lymphocyte-specific cell-surface antigen (M.W., 23,000 and 30,000) (20).

Certain carcinoembryonic antigens, including carcinoembryonic antigen, are characterized by high-molecular-weight (up to 400,000) glycoproteins that are shed in large amounts from the surfaces of rapidly dividing normal or neoplastic cells and may be found in the serum of cancer patients as oligomers of different sizes (29).

In our studies the antigen isolated from blast cell supernatants may be the result of both a quantitative and a qualitative change in the synthesis of a normal membrane component. Agarose chromatography of the supernatants of cultured relapse cells and of remission cells showed similar radioactivity elution profiles. The quantity of radioactivity, however, was much larger in the preparations from leukemic cells, indicating either a greater affinity of AMLSGA for radioactive iodine, a higher membrane turnover rate of AMLSGA, or both. Qualitatively, only radioactive antigen isolated from the leukemic cells coprecipitated specifically with alloantisera to myeloblasts.

The specificity of the alloantisera used to monitor purification of AMLSGA has been operationally defined by their ability to distinguish between leukemic and normal cells in the same individual. This does not constitute proof that they are detecting an antigen specific for leukemia cells only. The antigen released into the supernatant may be present on normal cells as a cryptic or “buried,” tightly bound antigen. Antibodies can be found in normal human sera that react with some leukemia cells (5) and similarly with determinants on the lymphocyte surface detectable only after neuraminidase treatment (32).

The antigen may be characteristic of mature but relatively rare cells such as peripheral blood B-cells or monocytes. Billing et al. (6) found that myeloblasts may express antigens usually restricted to B-cells. Baker et al. (2) found that monocytic or myelomonocytic leukemia cells possess large amounts of antigen found on normal monocytes. However, repeated testing of our allo- and xenoantisera with panels of monocytes and B-lymphocytes (Baker et al., (1)) has not disclosed specificity for either cell type. It is also possible that the antigen present on leukemic blasts may be integral

**Chart 2. Chromatography of 35S-labeled material**

(Tubes 20 to 23 of Bio-Gel A, 1.5 m, eluate, Chart 1C) on DEAE-cellulose. The sample, which contained 8 mg protein, was applied to a column (2 mg protein per ml packed DEAE-cellulose) developed at 21°C with a linear ammonium acetate gradient (4 to 30 mmh). One-ml fractions were collected.
to normal cells but that its expression is limited to certain phases of the cell cycle (9). Another possibility is that the antigen may be a fetal tissue or blood group determinant expressed on leukemic blast cells because of derepression (14). Harris et al. (18) showed that rabbit antiserum to mouse fetal liver cells reacts more strongly by indirect immunofluorescence with peripheral leukemic cells than with normal donor peripheral lymphocytes.

Blast cells might acquire new antigens by attachment of exogenous immunoglobulin (24) or other serum proteins such as the DJC1 and C2 (33). After lactoperoxidase iodination it is conceivable that such molecules might be labeled and shed into the supernatant along with some membrane components. However, we have not found immunoglobulin by indirect immunofluorescence on the surface of washed, frozen-stored myeloblasts. Also the molecular weight of AMLSGA is different than that of immunoglobulin, nor did any antigenic supernatant radioactivity precipitate with anti-immunoglobulin antisera alone.

It is not yet clear whether the material we isolated is characteristic of myeloblasts or if it might be found on other types of blast cells as well. In addition to be B-cell antigen noted above (6), Chechik and Gelfand (8) identified an antigen (HUTHY-1) in extracts of human thymocytes and leukemic cells, which they found in serum from 4 patients with untreated acute lymphoblastic leukemia; this antigen was also present in the serum of 1 of 8 patients with previously treated acute myeloblastic leukemia.

Our isolation of a radioactively labeled antigen derived from myeloblasts may allow the sensitive monitoring of human antigen levels in leukemic blood by radioimmunoassay. It would be important to learn if the release of soluble tumor cell surface products may interfere with cellular and humoral immunity against the tumor cells themselves. We have observed that the ability of the host to mount a detectable immune response to tumor-associated antigens in chronic myelogenous leukemia is correlated with the clinical course (30). Large numbers of rapidly dividing cells may shed sufficient amounts of soluble antigen in the serum to neutralize any immunotherapeutic effect that might be achieved. The finding of HUTHY-1 in the serum of patients with the high count blast crisis of chronic myelogenous leukemia (8) is consistent with this possibility. More recently, some correlation has been noted between the presence in the blood of soluble leukemia cell membrane components, reduced immune responses, and poor prognosis in patients with acute lymphoblastic leukemia (21).

REFERENCES

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