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Characterization of Compounds Shed From the Surface of Human Leukemic Myeloblasts In Vitro

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Human leukemic myeloblasts shed glycoproteins from the cell surface during short-term in vitro culture. Shed surface glycoproteins yield a characteristic profile when studied by gel chromatography, isoelectric focusing, immune precipitation, and polyacrylamide gel electrophoresis. Isolation of immunologically active material yields a compound of approximately 75,000–80,000 daltons, with an isoelectric point of pI 7.6 to 7.9. Various morpholgical subtypes of acute myelogenous leukemia shed these compounds, but they are most easily obtained from the more differentiated M2 and M4 types as compared to the undifferentiated M1 type. The shed compounds appear to be quantitatively and qualitatively different from compounds shed by other leukemic cells or nonleukemic cells.

CONTINUOUS SHEDDING of cell-surface constituents has been described in malignant cells from both animal and human tumors.[1-3] Compounds shed into the host circulation may confer survival advantage to the malignant cells. The released compounds may be antigenic to the host and play a role in the escape of malignant cells from immunologic destruction.[1]

Human leukemic myeloblasts continuously shed surface compounds into supernatant medium in vitro.[4] In the present study we have examined the shed surface glycoproteins by gel chromatography, isoelectric focusing, immune precipitation, and polyacrylamide gel electrophoresis, and a characteristic profile has been demonstrated that differs quantitatively and qualitatively from compounds shed by other leukemic and nonleukemic cells.

MATERIALS AND METHODS

Cell Surface Labeling and Collection of Shed Material

Leukemic cells were obtained from the peripheral blood of patients at the time of initial presentation of acute myelogenous leukemia who showed white blood cell counts greater than 50.0 × 10^9/liter with greater than 99% myeloblasts. Cells were used either fresh or after freeze storage in 10% dimethyl sulfoxide in liquid nitrogen followed by thawing in a 37°C water bath before use. Cell suspensions with less than 90% viability at the start of the procedure were discarded. The HLA-A, B, and Ia-like antigens (DR, MT, and MB) were determined using a standard microcytotoxicity assay.[5]

Human leukemic cell lines HL-60[6] and KG-1[7] were maintained at 37°C in 5% CO in RPMI (GIBCO, Grand Island, N.Y.) supplemented with 1% sodium pyruvate, nonessential amino acids, and 10% heat-inactivated fetal calf serum (GIBCO). Penicillin and streptomycin were added to the medium, and periodic assays were conducted for mycoplasma contamination. Cultures were divided twice weekly.

All cells were labeled and incubated under the same culture conditions. To 10^8 cells in 2 ml RPMI were added 1.0 mCi sodium iodide ^{125}I, 200 μl of lactoperoxidase (Sigma, St. Louis, Mo. 0.25 mg/ml), and 25 μl of 0.03% hydrogen peroxide. The cells were incubated at 22°C for 10 min; during this period, 25 μl of hydrogen peroxide solution was added twice. The reaction was terminated by adding 8 ml of 0.01 *M* cysteine and 0.01 *M* potassium iodide in RPMI. The cells were washed thrice in RPMI and placed in culture at 37°C in 5 ml minimal Eagle's medium. After 4 hr the medium was discarded, the cells washed, and reincubated. The supernatant was harvested at 24 hr. Cell suspensions showing less than 80% viability at the end of the incubation were not used.

Immunoprecipitation

Twenty microliters of alloantiserum or heteroantiserum and 20 μl of the ^{125}I-labeled supernatant were added in triplicate to microtiter U-plate wells (Cooke Engineering Co., Alexandria, Va.) prewashed with bovine serum albumin. The plates were shaken and allowed to stand for 1 hr at 37°C. Antigen–antibody complexes were precipitated with 100 μl of staphylococcal protein A (Enzyme Center Incorporated, Boston, Mass.). The plates were shaken again, kept 15 min at 22°C, and then spun at 1800 rpm for 10 min. The supernatant liquid was gently sucked out of the wells, and the precipitates were washed three times in phosphate-buffered saline (pH 7.2) and the wells were transferred to counting vials. Radioactivity was counted in a Beckmann Biogamma II gamma spectrometer.

Gel Filtration Chromatography

Half milliliter aliquots of concentrated culture supernatant were applied to 0.9 × 90 cm columns (Glenco Scientific, Inc., Toronto, Ont.) of Bio-GelA-1.5 m (approximately 8% agarose, 200–400 mesh), equilibrated, and then eluted at 4°C with 0.01 *M* ammonium acetate at a hydrostatic pressure of 20 cm water. Fractions of 1–2 ml were collected, and the radioactivity of each was counted in a gamma 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 using crystalline bovine serum albumin as standard.[8]

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Samples of culture supernatant were also applied on to 0.9 × 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 were standardized in separate experiments by applying proteins of known molecular weight (horse apo-ferritin, human gamma-globulins, human transferrin and ovalbumin, from Schwarz/Mann, Toronto, Ont.). In addition, homogeneous human liver fatty acid synthetase, which was assayed by spectrophotometric method (Gilford spectrophotometer 2400-S, Mississauga, Ont.), was used as a standard.[9]

Isoelectric Focusing

Samples for isoelectric focusing were added to 0.75 ml of 40% ampholine (pH 3.5–10) and applied to an LKB 8100 column at 4°C to a total volume of 110 ml with sucrose gradient solution.[10] On elution, protein levels within the pH gradient were monitored by measuring the optical density at 280 nm and the ^{125}I radioactivity of each fraction was determined in a gamma counter. Pooled fractions for immunologic and biochemical studies were concentrated by Minicon concentrators with removal of sucrose and ampholytes.

LDS–Polyacrylamide Disc Gel Electrophoresis

The pooled fractions and immunoprecipitates from these fractions were boiled with 1% (w/v) 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,[11] except for the substitution of LDS for sodium dodecyl sulfate in order to conduct the experiments at 4°C.[12] Gels were in 0.6 × 10 cm tubes. In other experiments, in addition to dissociation with LDS, samples were incubated with 0.1 *M* 2-mercaptoethanol in order to cleave any disulfide bonds prior to disc gel electrophoresis. Radioactivity was determined in serial 0.5-cm gel slices in the gamma counter.

Antisera

Anti-HLA-A and B and anti-Ia antisera of defined specificity have been previously described.[13] A.TH anti-A.TL congenic murine serum is reactive to a high titer (1:1280) with Ia-positive human cells in binding, cytotoxic, and immunoprecipitation assays.[14,15] Analyses of the reactivity by one- and two-dimensional gel electrophoresis and by sequential precipitation have shown that the anti-Ia mouse sera is cross-reacting with monomorphic, nonallelic determinants common to human Ia antigens.[14,15] These sera were reactive by cytotoxic and binding assays with leukemic myeloblasts used in this study. Both the alloantisera and the heteroantisera were used in immunoprecipitation studies to rule out the presence of specific histocompatibility antigens in the soluble leukemic compounds.

Monkey antiserum (Dys) raised against myeloblast-associated antigens has been previously described in detail.[16,17] Briefly, antisera were raised in *Macaca speciosa* by injection of compounds shed from myeloblast cell membranes. Antisera were absorbed with platelets, B lymphocytes, lymphoblasts, and nonleukemic marrow cells and retained reactivity by complement-mediated cytotoxicity against myeloblasts from patients with acute myeloblastic leukemia or chronic myelogenous leukemia in blast crisis. Absorbed sera were unreactive with B or T lymphocytes, lymphoblasts, nonleukemic marrow, or peripheral blood neutrophils.[16,17]

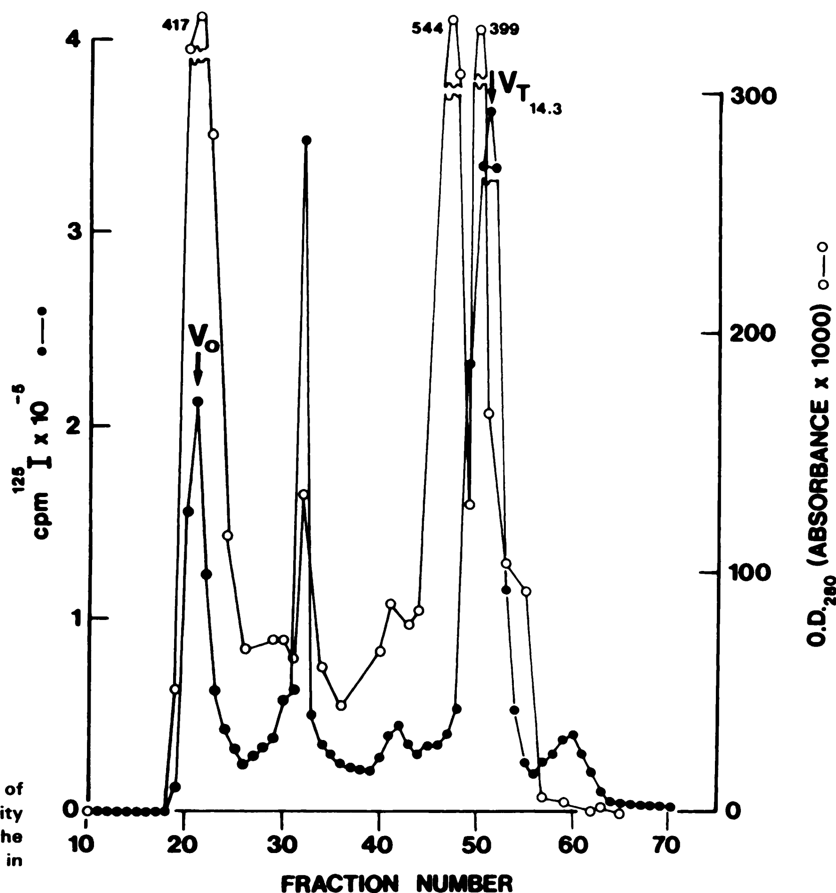

Fig. 1. Gel filtration chromatography of radiolabeled myeloblast supernatant. Reactivity with antimyeloblast serum was confined to the radiolabeled peak of high specific activity in fractions 31–34.

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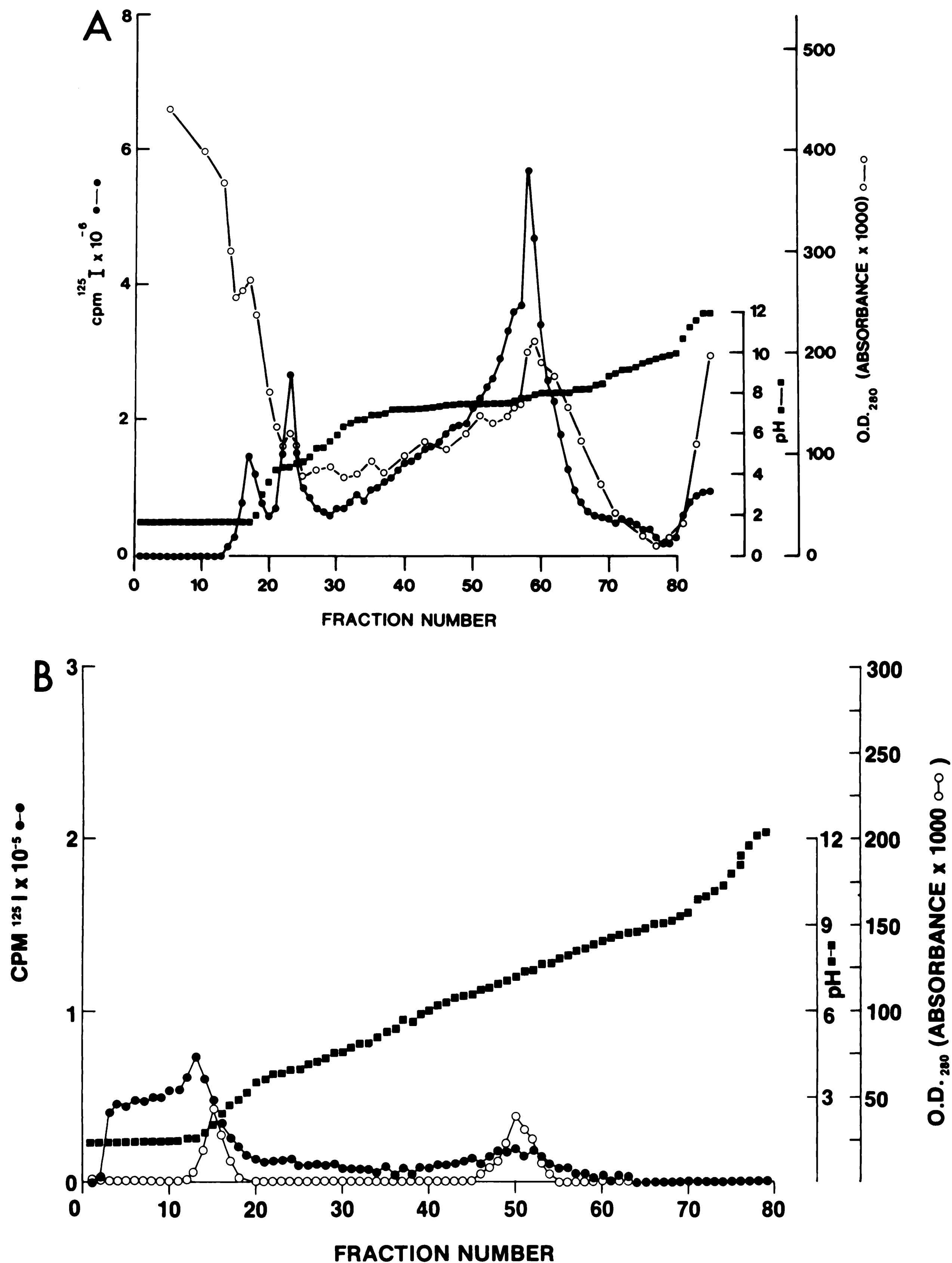

Fig. 2. (A) Isoelectric focusing of active fractions derived from gel filtration chromatography of material shed from leukemic myeloblasts. Reactivity with antimyeloblast serum was confined to the radiolabeled peak of high specific activity at isoelectric point 7.8, fractions 57–60. (B) Isoelectric focusing of compounds shed by nonleukemic peripheral blood granulocytes. (C) Isoelectric focusing of compounds shed by leukemic lymphoblasts (acute lymphoblastic leukemia). (D) Isoelectric focusing of compounds shed by leukemic lymphocytes (chronic lymphocytic leukemia).

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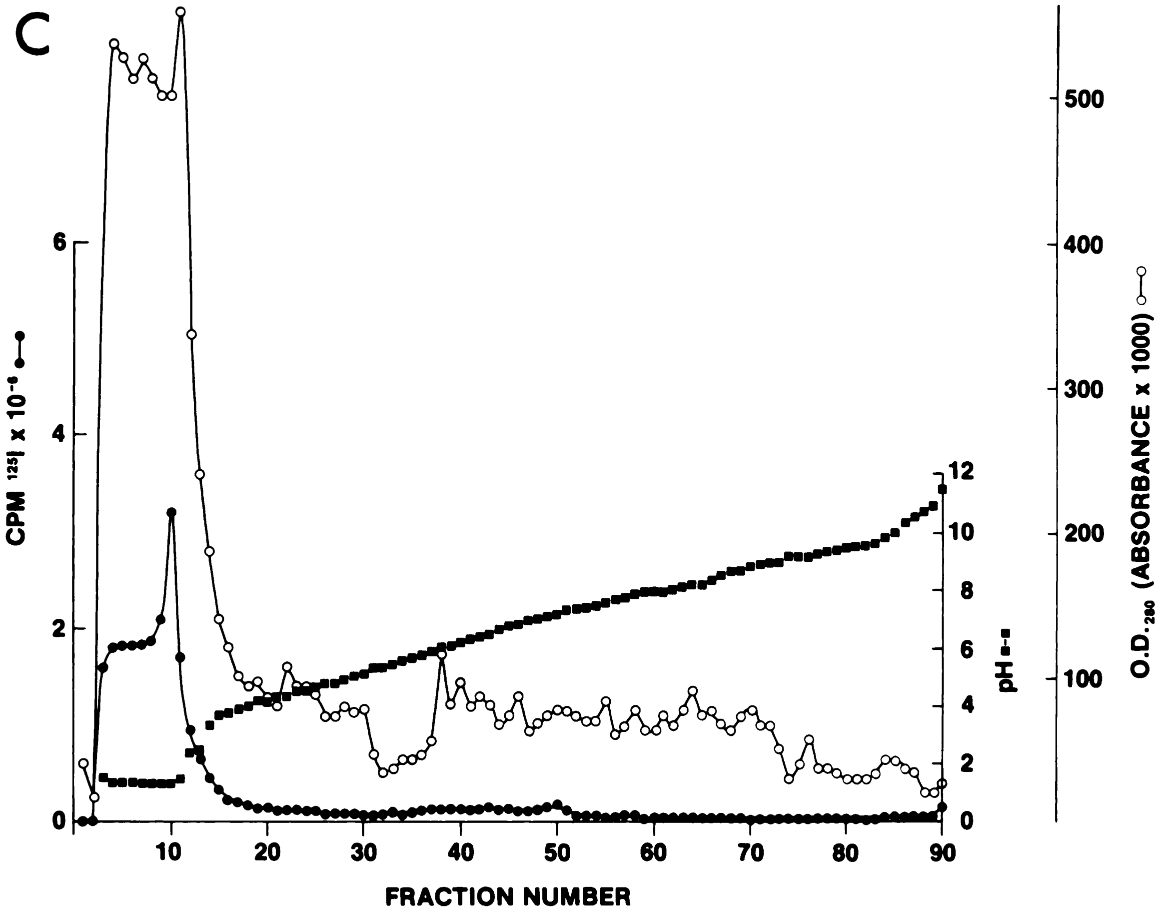

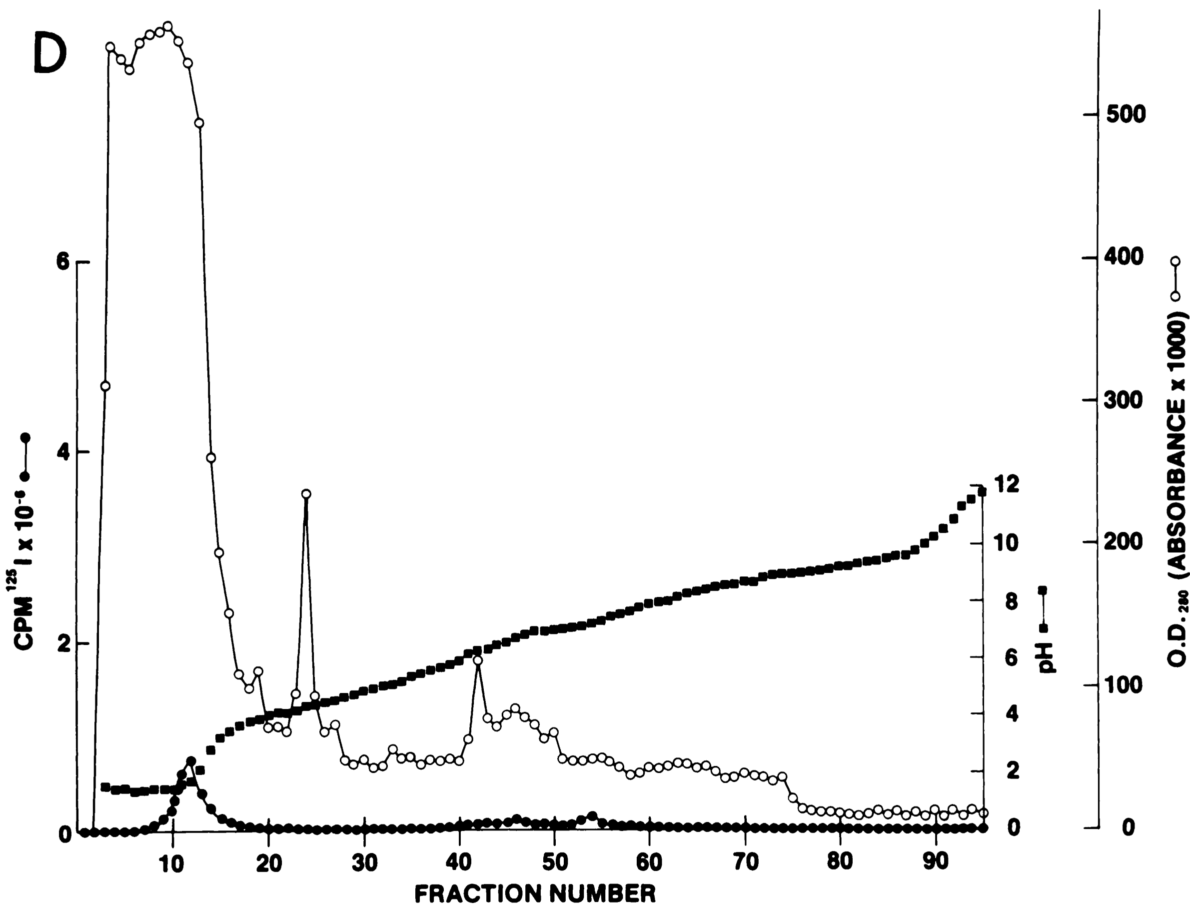

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Table 1. Shedding of Radiolabeled Compounds According to Morphological Diagnosis

Morphological Diagnosis	BioGelA-1.5*M* Included Peak	Isoelectric Focusing Peak at pI 7.8
Acute myelogenous leukemia	22/25*	5/9*
Undifferentiated-M1	2/3	0/3
Acute myelocytic-M2	6/8	2/3
Promyelocytic-M3	2/2	ND
Acute myelomonocytic-M4	12/12	3/3
Acute lymphoblastic leukemia	3/3	0/3
Chronic lymphocytic leukemia	0/3	0/3
Chronic myelocytic leukemia†	1/1	1/1
Cell lines		
HL-60	1/1	1/1
KG-1	1/1	1/1
Nonleukemic lymphocytes	0/3	0/3
Nonleukemic granulocytes	0/3‡	0/3‡

*Number of patients whose cells yielded radiolabeled compounds over the number tested.

†Peripheral blood mononuclear cell fraction (granulocyte precursors including blasts).

‡Small peaks of low specific activity seen (see Fig. 2B).

RESULTS

Myeloblasts from 25 patients with acute myelogenous leukemia were radiolabeled and incubated in short-term culture. Cells from 22/25 patients shed radiolabeled compounds into the supernatant medium over a 24-hr period. The radiolabeled compounds released gave a consistent pattern when analyzed by gel filtration chromatography (Fig. 1). For 9 patients, the major peak from gel filtration chromatography was further analyzed by application to isoelectric focusing columns. A major radiolabeled peak was obtained in 5 of the cases at isoelectric point pH 7.8 (Fig. 2A).

A morphological analysis of cells examined is presented in Table 1. Of three patients with undifferentiated (M1) leukemia, two patients shed compounds that were included in the BioGelA column, but none showed a peak at pI 7.8 on isoelectric focusing. Six of eight patients with acute myelocytic (M2) morphology shed compounds isolated on gel chromatography and two of three showed a peak at pI 7.8. The two patients with promyelocytic leukemia (M3) shed material that could be isolated on gel chromatography, but insufficient cell numbers were available for testing on isoelectric focusing. Twelve patients with acute myelomonocytic leukemia (M4) were studied, and all shed radiolabeled compounds included in the BioGelA column and 3/3 tested gave peaks at pI 7.8 on isoelectric focusing.

Radiolabeling procedures, gel chromatography, and isoelectric focusing of shed compounds were also carried out under the same experimental conditions on granulocytes and lymphocytes from peripheral blood of 3 nonleukemic patients, lymphoblasts from 3 patients with non-B, non-T acute lymphoblastic leukemia, granulocyte precursors (including blasts) from the peripheral blood of a patient with chronic myelocytic leukemia (CML), cells from promyelocytic cell line HL-60, and cells from myeloblastic cell line KG-1 (Table 1). Peripheral blood granulocytes showed considerably less shedding of surface compounds in culture. A small protein peak of low specific activity was obtained at pI 8.2 (Fig. 2B). Leukemic lymphoblasts demonstrated active shedding of radiolabeled compounds in culture and a major included peak was obtained on gel chromatography. No radiolabeled peak occurred in the alkaline range on isoelectric focusing (Fig. 2C). Leukemic lymphocytes similarly demonstrated active shedding in vitro, but peaks of low specific activity were seen only at lower pI (Fig. 2D). HL-60 and KG-1 cells and granulocyte precursors in CML actively shed compounds that have gel filtration and isoelectric focusing patterns indistinguishable from those of myeloblasts studied. Monkey anti-human myeloblast serum[16,17] was reactive in coprecipitation testing with the major included peak obtained from BioGelA-1.5 *M* chromatography of radiolabeled compounds released from AML cells from 10 patients and that of HL-60 and KG-1 cells (Table 2). This peak was unreactive with antisera to HLA-A, B or DR specificities that were reactive with the intact parent cells. Antiserum Dys was unreactive with the excluded peak derived from AML cells or with peaks obtained from AML, CLL cells, or normal leukocytes.

Table 2. Specific Immune Precipitation of Radiolabeled Compounds From Leukemic Myeloblasts Following Fractionation on a BioGelA-1.5*M* Column

	Cells					
Antiserum	AML (10 Patients)	ALL	CLL	Normal	HL-60	KG-1
Monkey serum Dys	22.9 ± 12.4*	0.7 ± 0.2	0.9 ± 0.3	0.5 ± 0.2	29.4 ± 11.5	26.0 ± 9.5
Normal monkey serum	0.3 ± 0.3	0.8 ± 0.4	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	0.2 ± 0.2
anti-HLA-A, B	0.5 ± 0.3					
Anti-HLA-DR	0.7 ± 0.3					
Normal human serum	0.4 ± 0.3					

*Calculated as cpm ^{125}I precipitated/cpm ^{125}I total in well × 100 and expressed as mean and standard deviation.

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Table 3. Specific Immune Precipitation of Fractions Obtained From Isoelectric Focusing of Leukemic Myeloblasts

Antiserum	Percent Precipitation						
	Fractions 0–20 (Peak 1)	Fractions 20–30 (Peak 2)	Fractions 30–50	Fractions 50–57	Fractions 57–60 (Peak 3)	Fractions 61–70	Fractions 71–80
Monkey serum Dys	0.8 ± 0.8*	2.6 ± 2.2	3.6 ± 1.3	3.3 ± 1.7	31.9 ± 12.0†	3.8 ± 1.3	0.9 ± 0.2
Normal monkey serum	ND	1.4 ± 1.5	2.2 ± 0.8	2.7 ± 0.8	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.8

*Calculated as cpm ^{125}I precipitated/cpm ^{125}I total in well × 100 and expressed as mean and standard deviation, fractions obtained from 3 cells. Protein concentration was adjusted to 35 μg/100 μl.

†$p < 0.01$.

Consecutive fractions obtained from isoelectric focusing of active fractions from gel chromatography were pooled and tested for reactivity with antimyeloblast serum. Significant precipitation was seen only with the major radioactive protein peak at pI 7.8 in material obtained from leukemic blast cells or cell lines (Table 3). The immunoreactive fractions did not precipitate with antisera active against the HLA-A, B, and Ia antigens of the parent myeloblasts (Table 4). Sequential precipitation showed that 90% of antigen was cleared in a single reaction with monkey serum Dys and that reaction with anti-HLA-A, B, and anti-Ia sera did not interfere with subsequent precipitation by monkey serum Dys (Table 4). Radiolabeled compounds shed from lymphoblasts, leukemic lymphocytes, and normal peripheral blood luekocytes did not precipitate with monkey antimyeloblast serum, and absorption of this antiserum with lymphoblasts, leukemic lymphocytes, nonleukemic leukocytes, or bone marrow fractions enriched for mononuclear cells did not reduce the precipitation of radiolabeled compounds from myeloblasts.

The active fractions were reversibly retained on concanavalin-A or lentil lectin agarose beads but not peanut lectin or soybean lectin beads, indicating significant carbohydrate content with mannose exposure but not galactose. No change in isoelectric point was seen after treatment of cells or supernatant with neuraminidase.

The immune precipitate formed by antiserum Dys and the active peak from isoelectric focusing was analyzed by LDS-PAGE. A major radioactive peak was obtained (Fig. 3) that did not change position following the addition of 2-mercaptoethanol, indicat-

Table 4. Sequential Precipitation of Active Fractions Obtained From Isoelectric Focusing of Leukemic Myeloblasts

Initial Antiserum	Precipitation* With Initial Antiserum	Sequential Precipitation* With Monkey Serum Dys	
	1	2	3
Monkey serum Dys	24.7 ± 7.9*	3.2 ± 1.2	0.9 ± 0.3
Normal monkey serum	1.6 ± 0.9	ND	ND
Anti-HLA-A, B serum†	0.6 ± 0.2	21.6 ± 8.2	4.8 ± 1.8
Anti-HLA-DR serum†	0.5 ± 0.2	25.1 ± 9.3	5.7 ± 3.0
Normal human serum	0.7 ± 0.4	ND	ND
Mouse anti-Ia‡	1.8 ± 1.0	ND	ND

*Calculated as cpm ^{125}I precipitated/cpm ^{125}I total in well × 100 and expressed as mean and standard deviation. Protein concentration 80 μg/100 μl.

†Allosera active against intact myeloblasts.

‡ATH anti-ATL serum active in immune precipitation of human Ia antigens.

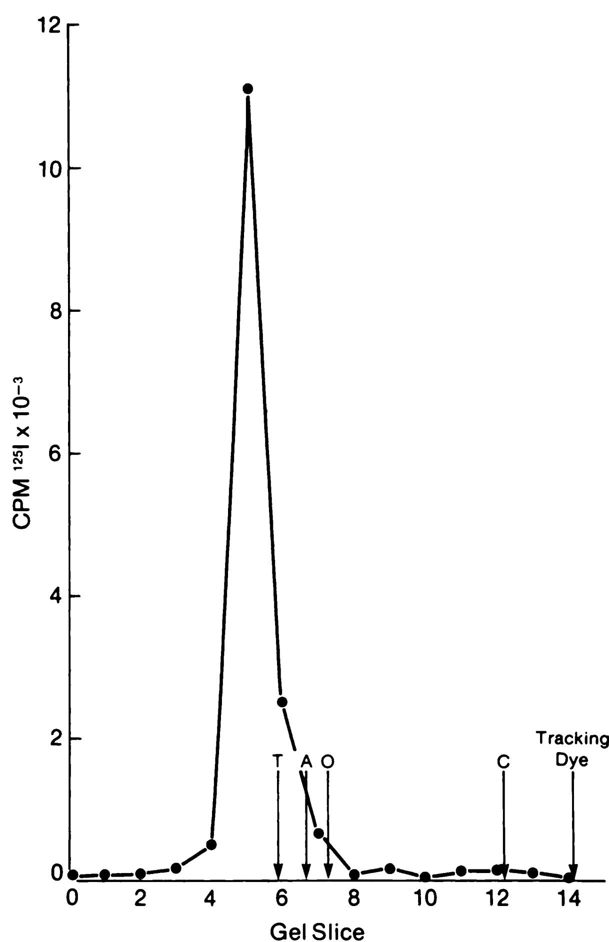

Fig. 3. Lithium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitate formed by antimyeloblast serum and active peak from isoelectric focusing. A single radioactive band was obtained. T, human transferrin; A, bovine serum albumin; O, ovalbumin; C, horse heart cytochrome-C.

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ing that interchain disulfide bonds were not present in the compound. The estimated molecular weight is 75,000–80,000 daltons. This peak is in the same position as the major peak seen on LDS-PAGE of the compound obtained directly from the active fractions of isoelectric focusing.

DISCUSSION

In the present study, we have shown that human leukemic myeloblasts shed glycoproteins from the cell surface during short-term in vitro culture. Shed surface glycoproteins have yielded a characteristic profile when studied by gel chromatography, isoelectric focusing, immune precipitation, and polyacrylamide gel electrophoresis. Various morphological subtypes of acute myelogenous leukemia shed these compounds, but they are most easily obtained from the more differentiated M2 and M4 types as compared to the undifferentiated M1 type. The shed compounds are quantitatively and qualitatively different from compounds shed by other leukemic cells or nonleukemic cells.

Isolation of immunologically active material yields a compound of approximately 75,000–80,000 daltons with an isoelectric point of pI 7.6–7.9. Since compounds were shed without the addition of proteolytic or dissociating agents, they may be peripheral rather than integral membrane structures[18] or may have been cleaved by proteolysis from intracellular enzymes.[1]

Shedding of compounds from the glycocalyx has been described as a general property of malignant cells.[1] It is possible that leukemic myeloblasts release compounds normally present on the leukocyte cell surface at a rate high enough to detect by the methods employed in this study. A small amount of shedding was observed from nonleukemic granulocytes and lymphocytes, with some granulocytic compounds showing isoelectric points at pI 7.8. More shedding from nonleukemic cells might have been seen under different culture conditions. Immune precipitation with an antileukemic heteroantisera[16,17] was confined to compounds shed from myeloblasts or leukemic cell lines, suggesting that compounds released are antigenically different from those released by the nonleukemic cells. Leukemic transformation may be associated with alterations in carbohydrate of normal cell surface glycoproteins. Increases in sialyl- or fucosyl-transferases in plasma may result in disturbances of glycoprotein synthesis and changes in cell surface carbohydrate moieties similar to those responsible for changes in blood group substances in leukemic patients.[19-21] Van Beek et al.[22] have documented structural differences in fucose-containing glycopeptides in leukemic myeloblasts compared to nonleukemic leukocytes, and Andersson et al.[23] have demonstrated surface glycoprotein patterns diagnostic of acute myeloblastic leukemia. Studies of plasma and urine of patients with AML have yielded characteristic compounds resulting from alteration of normal glycoproteins.[24]

Immune precipitation with an antimyeloblast serum does not necessarily indicate the presence of leukemia-specific antigens. Histocompatibility antigens,[25,26] differentiation antigens,[27,28] and fetal antigens[29,30] have all been detected on leukemic myeloblasts. Antisera to histocompatibility antigens did not precipitate with the compounds obtained on isoelectric focusing, and high titer antisera to Ia antigens did not clear the solution of immunoreactive compounds so that activity against HLA-A, B, or DR specificities is unlikely. Lack of reactivity with nonleukemic bone marrow cells and failure to suppress colony-forming units in culture of nonleukemic marrow suggest that the antigen is not heavily represented in nonleukemic presursors,[16,17] although differences in antigen density may explain these findings. Nevertheless, the antisera may be directed towards one or a combination of these antigens in characteristic configuration on the leukemia cell surface. Shedding of surface compounds from leukemic myeloblasts may confer survival advantage on these highly malignant cells and may contribute to coagulation abnormalities seen in this disease.[1]

We have previously shown that detection of antigens associated with leukemic myeloblasts on the surface of cells in bone marrow may be helpful in predicting relapse in patients with acute myeloblastic leukemia in remission.[13] Purification and characterization of these antigens should allow the development of immunoassays of material in serum as well as on cells and should lead to a more precise definition of the relationship between the antigenic materials and the nature of leukemic transformation.

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