C.1.

ISOLATION OF A CELL SURFACE ANTIGEN SPECIFIC FOR HUMAN ACUTE MYELOGENOUS LEUKEMIA CELLS

by

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ABSTRACT

Methods are described whereby three protein bands (#1, #2 and #3), apparently unique to cells of patients with acute myelogenous leukemia (AML), were isolated on non-reducing polyacrylamide gels. Antisera raised against all three proteins demonstrated absolute specificity, in the ELISA, for absorbed cell membrane preparations from cells of patients with AML and did not react with comparable cell membrane preparations from cells of normal individuals or those of patients with lymphoproliferative disorders. Inhibition studies showed that AML band#1 (68,000 dalton molecular weight) material was capable of totally inhibiting the reaction, in the ELISA, of all three antisera (anti-AML#1, #2 and #3) with absorbed AML cell membrane preparations. When AML cell membranes were examined in SDS-PAGE, only material identified as AML band#1 was detectable, indicating that AML bands#2 and #3 represented aggregates of AML band#1 or artifacts of non-reducing PAGE and the cell membrane preparative procedures. AML band#1 material was shown to be homogenous by two-dimensional gel electrophoresis with a molecular weight of 68,000 daltons and a mean pI of 7.16. AML band#1 material was also shown to be capable of inhibiting the reaction of anti-AML#1 antiserum with the promyelocytic cell line HL-60, as demonstrated by FACS IV analysis, whereas material from normal cell membranes could not demonstrate this inhibition.

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LIST OF ABBREVIATIONS

1. ALL: Acute Lymphocytic Leukemia.
2. AML: Acute Myelogenous Leukemia.
3. AMML: Acute Monomyelocytic Leukemia.
4. APML: Acute Promyelocytic Leukemia.
5. CLL: Chronic Lymphocytic Leukemia.
6. CML: Chronic Myelogenous Leukemia.
7. DNA: Deoxyribonucleic Acid.
8. ELISA: Enzyme-linked Immunosorbent Assay.
9. FACS(IV): Fluorescence-activated Cell Sorter.
10. FITC: Fluorescein Isothiocyanate.
11. G-6-PD: Glucose-6-phoshate Dehydrogenase.
12. IEF: Isoelectric Focussing.
13. LAA: Leukemia-associated Antigen.
14. 2-ME: 2-mercaptoethanol.
15. PAGE: Polyacrylamide Gel Electrophoresis.
16. 2D-PAGE: 2-dimensional Polyacrylamide Gel Electrophoresis.
17. SDS-PAGE: Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis.
18. PBS: Phosphate Buffered Saline.
19. PBL: Peripheral Blood Leucocyte.
20. PCV: Polycythemia Vera.
21. RNA: Ribonucleic Acid.

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"....For afterwards a man finds pleasure in his pains, when he has suffered long and wandered long. So I will tell you what you ask and seek to know."

- Homer.

INTRODUCTION

In the fields of tumour immunology and tumour virology there has been a great deal of speculation on the relatedness of carcinogenesis and viral oncogenesis. There has also been a concommitant increase in the amount of research directed toward determining the manner by which oncogenic viruses bring about neoplastic transformation of normal cells. Numerous papers in the literature have dealt with the isolation and cloning of DNA sequences from RNA tumour viruses which cause neoplastic transformation when used to transfect normal recipient cells (1-5). Many investigators have reported, using cloned transforming sequences as probes, the presence of normal cellular genes homologous to the cloned viral oncogenes in the DNA of normal, non-neoplastic cells (6). Subsequent studies have shown that these normal cellular genes are actively transcribed in neoplastic cells and transcriptionally silent in normal, non-neoplastic cells (7). Other research has suggested that the targets for transformation, induced by virus or chemical carcinogens, comprise a small but discrete set of cellular oncogenes which are present, but inactive, in normal, non-neoplastic cells (8). Experiments using sheared DNA from human neoplasms have demonstrated that the transforming potential and ultimate neoplastic phenotype are encoded in the DNA of these neoplastic cells (9). Additional findings indicate that different neoplasms carry different transforming sequences

suggesting that more than one cellular oncogene is responsible for the variety of known human neoplasms (9). There is, at present, some research being directed toward the isolation of the protein products of these cellular oncogenes and also toward determination of their role(s) in the transformation process (10). There has been a certain amount of speculation as to whether or not these proteins represent gene products of the viruses themselves or products of normally "silent" cellular genes transcriptionally activated, directly or indirectly, by viral integration into the host cell DNA (11). Other researchers have attempted to isolate transformation-specific or induced proteins from virally transformed cells. At least two laboratories have had some success but the question of function still remains unanswered (10). The accepted view at the present time is that these proteins represent normal growth regulatory proteins. The inappropriate expression of these proteins, precipitated by viral promoter insertion or an analogous activation event, in the cell causes an imbalance in the host cell feedback growth regulatory system, the consequences of this imbalance representing the first step(s) in the transformation process (6).

In regard to human AML (Acute Myelogenous Leukemia), the involvement of oncogenic viruses or chemical carcinogens as causative agents in leukemiogenesis is unclear. Little is known about the disease itself, apart from its clinical manifestations and physiological consequences. The majority of human leukemia research has been conducted at the clinical

level and has dealt with the chronic leukemias rather than the acute leukemias. Some of the research with human CML (Chronic Myelogenous Leukemia) has provided some useful starting points for AML research. CML has been shown to be a clonally-derived disease, in that cells characteristic of CML are derived from a single stem cell in the bone marrow of the "pre-leukemic" individual, which has become leukemic due to the action of an extrinsic factor(s). The clonal origin of CML and other hematopoetic stem cell disorders correlates very well with the current theories on the origins of human cancers, since it is thought that clonal diseases would originate from rare oncogenic events, such as somatic mutation or viral promoter insertion next to a normal cellular oncogene, acting on a single stem cell (12). In CML this appears to be the case since only specific cell lineages are "leukemic", shown by the presence of a characteristic chromosomal anomaly of chromosome 22 known as the Philadelphia chromosome (Ph^1) as well as the presence of a single type of glucose-6-phosphate dehydrogenase (Gd^a or Gd^b) in the cells of G-6-PD heterozygotes with CML (13). All cells derived from the leukemic stem cell possess the Ph^1 and the factors contributing to the appearance of this anomaly are most likely involved in the cause of the disease as well. These factors, their origins and their targets are still unknown (13). The alteration in CML occurs in the myeloid stem cell and it is supposed that the replacemant of the normal stem cells by the leukemic stem cells ultimately gives rise to a myeloid stem cell population exempt from normal growth control

mechanisms in the bone marrow of leukemic individuals (14,Figure 1A).

In AML the overall picture is less clear. There have been several reports in the literature concerning leukemia-associated antigens (LAAs) and AML in which the AML-LAA has been described as a glycoprotein of 75,000-80,000 dalton molecular weight (15), a glycoprotein of 400,000 dalton molecular weight (16) or cell stage dependent proteins of 86,000 and 88,000 dalton molecular weights (17). The antisera raised to these various proteins show high specific reactivity with the antigen itself and cell lines bearing the antigen on their cell surface. Significant cross-reactivity has also been observed between these antisera and cells from related (AML, AMML and CML) and non-related (ALL, CLL, myelomas and lymphomas) stem cell disorders. This spectrum of reactivity probably reflects the presence of common stem cell antigens, differentiation antigens of common LAAs present on the cell surface of these malignant cells. Although genetic studies have not revealed characteristic chromosomal anomalies in AML analogous to the Ph^1 in CML, these studies have implied that defined genetic events are intimately involved in creating these anomalies and probably represent causative agents for the disease as well. In DNA transfection experiments, using DNA from the promyelocytic leukemia cell line HL-60, it has been demonstrated that this DNA is capable of transforming recipient mouse NIH 3T3 cells (18). This experiment strongly supports the contention that within the

Figure 1A: Ontogeny of the cell lineages involved in human acute myelogenous leukemia (AML) and other hematopoetic stem cell disorders.

AML: Acute Myelogenous Leukemia.

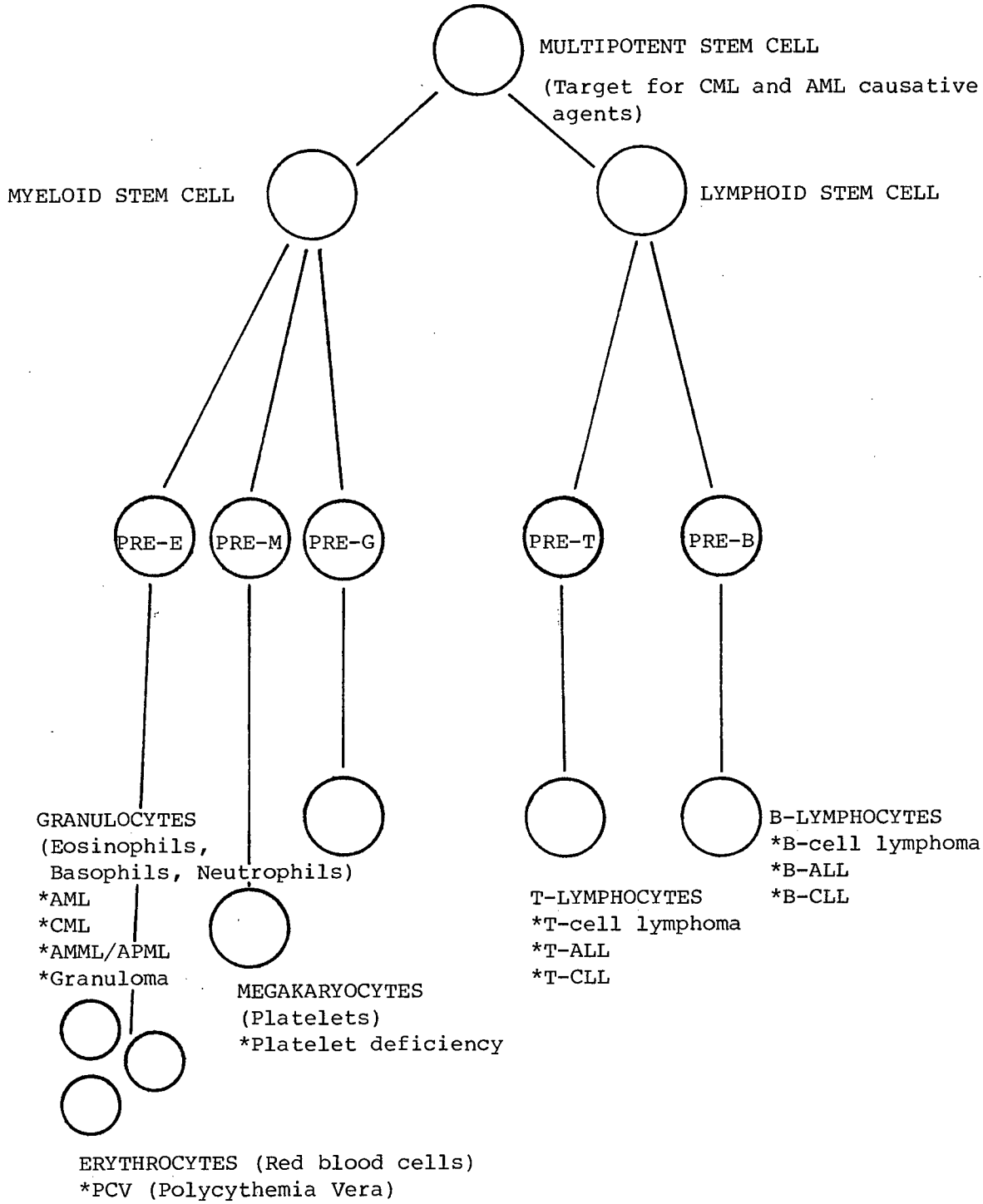
CML: Chronic Myelogenous Leukemia.

ALL: Acute Lymphocytic Leukemia.

CLL: Chronic Lymphocytic Leukemia.

PCV: Polycythemia Vera.

AMML/APML: Acute Mono-/Promyelocytic Leukemia.



Disease states denoted by an asterisk (*).

DNA of leukemic cells resides a gene(s) responsible for the observed transforming ability and final neoplastic phenotype of these cells. At the present time, we have isolated and partially characterized what appears to be an AML-specific cell surface antigen (19,20) and have also produced a high titre antiserum to this marker in rabbits (20). When this antiserum is tested in the ELISA of FACS IV, it shows absolute specificity for AML cells or cell membrane extracts whereas no reactivity is observed using comparable cells or cell membrane extracts from normal individuals (20,21). When tested in the FACS IV, using a broader panel of cell samples, this same antiserum shows specificity for the bone marrow cells and peripheral blood leucocytes of both AML and CML individuals. Again, no reactivity is seen with unrelated stem cell disorders or normal cell samples (22). An interesting and significant finding is that this antiserum reacts with the peripheral blood leucocytes of AML individuals in clinical remission (22). This result is significant in demonstrating the presence of this "malignancy marker" on the surface of putatively normal cells. The observation that the presence of this "marker" does not appear to interfere with the normal differentiation of these cells is also interesting. Both of these results imply that the event causing the expression of the AML-LAA on the cell surface of leukemic and remission cells does not represent the oncogenic event responsible for the onset of leukemiogenesis. Apart from showing that the gene(s) responsible for production of the

AML-LAA is expressed in AML, CML and remission cells which appear to be differentiating normally, these studies have shown AML to be a disease of clonal origin in which the malignant clone is derived from a pluripotent stem cell (22). With our isolation of an AML-LAA and data from tumour virology suggesting the relatedness between viral transformation and the induction of transformation-specific proteins, it is tempting to regard the AML-LAA as a product of a virally-activated normal cellular oncogene, a product of a recombinant oncogene (a gene composed of recombined normal cellular and viral oncogenes) or an aberrantly expressed growth factor or receptor. There is, at present, no evidence to support any of the above suppositions. Since so little is known about the precise cellular mechanisms involved in transformation and ultimately what effect is exerted by the oncogene product to bring about transformation, questions regarding the nature and function of the AML-LAA abound. Is the AML-LAA a normal growth regulatory gene(s) product? Is this gene(s) product overproduced or inappropriately expressed in leukemic cells? Does expression of the AML-LAA ultimately result in leukemic transformation? Answers to the above questions are not immediately available and are the subjects of ongoing research.

MATERIALS & METHODS

Preparation of isolated cell membranes;

$1x10^7$-10^9 AML cells or normal PBLs were used to prepare cell membranes by sonication of the cell sample for 2-3 min, in 30 sec bursts, on ice at a setting of 30% probe intensity on a Fisher Scientific Sonic Dismembrator(Model 300) following freezing and thawing. Cell debris was removed by centrifugation of the cell sample at 400xg for 10 min. Cell membranes were pelleted from the supernatents by centrifugation at 50,000xg for 180 min. Membrane pellets were resuspended in minimal volumes of PBS (phosphate buffered saline, pH 7.2, 0.01M phosphate) and sonicated as described above or extracted in 3.0M KCl overnight at $4^{o}C$. The cell membrane sample was then centrifuged at 50,000xg for 180 min following sonication or KCl extraction and dialysis against PBS. Pellets were resuspended to a final volume of 5.0 ml in PBS and the total protein concentration of both the pellet and supernatent were determined by the method of Lowry et al. (23). Both the pellet and supernatent were also tested for reactive material using specific antiserum and the ELISA (24).

Feedback antiserum;

Antiserum was prepared against a pool of normal PBL cell membrane extracts. Rabbits were immunized with this antiserum, as antigen, in a 50% emulsion of Freund's complete adjuvant

in four distal intramuscular sites in 250 ul volumes. Rabbits were immunized monthly and, after a 6-8 week immunization schedule, bled weekly. Activity of this antiserum was estimated in the ELISA (24). This antiserum was used in the feedback procedure to raise an anti-AML antiserum. This anti-AML antiserum was produced utilizing the feedback method previously used to raise antiserum specific for a human bronchiogenic carcinoma antigen (25) and antiserum to tumour-associated antigens in the murine mastocytoma cell line P815 (19). The procedure was carried out as follows:

Anti-normal human antiserum was reacted with a 3.0M KCl extract of AML cell membranes in order to determine optimal proportions for immune precipitation. This ratio of antiserum and AML cell membrane extract were mixed together and allowed to stand at 4°C for 24 h, following which the precipitate was removed by centrifugation, and more anti-normal human antiserum was added to the soluble mixture to bring it to a final concentration of 10-fold excess of anti-normal human antiserum. The mixture was allowed to stand for a further 24 h and then centrifuged. The supernatent, containing anti-normal human antibodies, soluble complexes of anti-normal antibody and normal antigenic components of the AML cell membrane extract and possibly the tumour-associated antigen(s) of AML cells, was used to immunize rabbits. The soluble material was precipitated from the supernatent with alum and administered in 1.0 ml volumes (0.5 ml intramuscularly and 0.5 ml intraperitoneally) to young adult female albino

rabbits. Rabbits so immunized were bled after a 4-6 week interval, following which they were immunized and bled weekly. The antiserum recovered from these rabbits was absorbed twice on immunoadsorbent columns, prepared from normal PBL membrane extracts, prior to testing in the ELISA.

Anti-AML antiserum (anti-AML#1,#2,#3):

Fractions containing eluted protein, from preparative polyacrylamide gels, shown to have antigenic activity in the ELISA were used to immunize young adult female albino rabbits. The eluted material was mixed with Freund's complete adjuvant in a 50% emulsion to a total volume of 1.0 ml and injected intramuscularly into four distal sites. Rabbits were given another immunization 4 weeks later and bled 1 week after the second immunization. This antiserum, following testing in the ELISA, was found to have insignificant levels of anti-normal activity and therefore was not adsorbed on immunoadsorbent columns prepared with normal cell membrane extracts. An anti-normal antiserum was also prepared by hyperimmunizing rabbits with a normal human cell membrane preparation and bleeding these rabbits weekly, following several monthly immunizations.

Immunoadsorbents;

Immunoadsorbents containing anti-normal human antiserum or normal cell membrane extracts were prepared as follows: Antiserum raised in rabbits to a pool of normal PBL cell membranes or antigen extracted from these same normal PBL

membranes was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) (26,27). Prior to use, the immunoadsorbent columns were equilibrated with borate buffered saline, pH 8.5. Samples were layered onto the columns and cycled over twice, during a 90 min period, using borate buffered saline. All cell membrane samples were treated in this manner prior to testing. This treatment was found to remove approximately 90% of the protein present in the cell membrane preparations. The "column pass" fractions, presumably enriched for tumour-associated cell membrane components, were used for subsequent studies and immunizations to produce additional antiserum. Some anti-AML antiserum preparations were cycled over immunoadsorbent columns containing immobilized normal cell membrane components. This treatment was found to remove the majority of the anti-normal human activity present in these anti-AML antisera, as detected by the ELISA. The absorbed anti-AML antisera were used for further studies in the ELISA and FACS IV. Immunoadsorbent columns were purged after each adsorption by washing with 0.1N HCl followed by equilibration with PBS, pH 7.2.

Enzyme-linked immunosorbent assay (ELISA);

Antisera were assayed using the enzyme-linked immunosorbent assay (ELISA) following a previously described protocol (2). Briefly, eluted reactive material from native poyacrylamide gels or antigen in the form of absorbed AML or normal cell membrane extracts was coated onto ELISA microtitre

plates (Cooke Engineering, Alexandria, Va.) at 300 ng/ml in 0.05M carbonate buffer, pH 9.6, in 100 ul volumes for 18 h at 4°C. Plates were washed three times with PBS-Tween 20 buffer and reacted with the test antiserum for 60 min at room tempeature and washed an additional three times with PBS-Tween 20 buffer. Sheep anti-rabbit Ig, which had been glutaraldehyde coupled to alkaline phosphatase (Sigma Type VII) (28), was then added to the ELISA plates in 100 ul volumes at a 1/1500 dilution. Reaction was allowed to proceed a further 90 min at room temperature following which the plates were washed again with PBS-Tween 20 buffer and developed for alkaline phosphatase activity with p-nitrophenyl phosphate-Na (Sigma 104) for 45 min at room temperature. ELISA plates were read for absorbance at 405 nm in a Titertek Multiskan (Flow Laboratories, Inglewood, Ca.). Plates were blanked against a row of wells containing antigen alone and all tests were run at least twice and in triplicate on each occassion.

The ELISA was also used to caary out inhibition studies with AML band#1 material. All antisera (anti-AML#1,#2,#3) were normalized for reactivity with the absorbed AML cell membrane preparation and dilutions of each antisera were selected so that all yielded equivalent absorbances at 405 nm in the ELISA on this antigen after 45 min incubation at room temperature. Each antiserum was pre-incubated with various concentrations of AML band#1 material (from 0.0 to 3.0 ug/ml) for 18 h at 4°C, after which all preparations were tested for reactivity on ELISA plates which had been pre-coated with absorbed AML cell

membrane extract at 300 ng/ml.

Polyacrylamide gel electrophoresis (PAGE);

Electrophoresis was carried out according to the procedure of Laemmli (29), except that in some instances the sodium dodecyl sulphate (SDS) and 2-mercaptoethanol (2-ME) were omitted. Samples of absorbed cell membrane extracts were applied to 7.5% polyacrylamide gels, for both analytical and preparative procedures, and electrophoresed at 70V for 5-6 h. Preparative gels were run using a one-tooth comb (1.5 mm x 10.0 cm) and loading 250-500 ug of protein onto the gel. Molecular weight determinations were accomplished by co-electrophoresing samples with protein standards, containing bovine serum albumin (67,000 dalton), ovalbumin (43,000 dalton), carbonic anhydrase (30,000 dalton), trypsin inhibitor (20,100 dalton) and lysozyme (14,400 dalton). Gels were stained with either Coomassie Blue, according to the procedure of Fairbanks et al. (30), or silver stained according to Wray et al.(31). For preparative gels a section of the gel was removed and stained, the remainder being stored at -70°C until it was required for slicing and elution of protein.

Elution of protein;

Stained and unstained portions of preparative gels were lined up and illuminated from below using a light box. Bands were cut from regions of the unstained gel corresponding to locations on the stained section of the gel shown to contain

detectable protein. Gel slices were placed in small, capped glass tubes and crushed with a spatula. 2.0 ml of PBS were added to each tube and elution was effected by end-over-end mixing in a Labquake rotator (Labindustries, Berkeley, Ca.) at 4°C for 48 h. Eluted protein was collected by vacuum filtration and protein concentrations of all fractions were determined using the method of Lowry et al. (23). Testing of the eluted material for antigenic activity was accomplished by using a rabbit anti-AML antiserum (19,20) and the ELISA.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE);

Gel purified AML band#1 material was analysed on 2D-PAGE according to the procedure of O'Farrell et al. (35). In brief, a 100 ul sample containing 36 ul of AML band#1 (40.0 ug/ml), 2.25 ul of a 1.0M Tris-HCl solution, pH 6.8 (0.05M final conc.), 2.25 ul 2-ME (5% final conc.), 4.5 ul of a 20% SDS solution (2% final conc.), 5 ul of 40% LKB Ampholines (2% final conc.), 8 ul of a 10% Triton X-100 solution (8% final conc.) and 55 mg solid urea (8M final conc.) was added to a single isoelectric focussing tube gel (4% polyacrylamide, 2 mm x 11.5 cm) and focussed at 400V until the "volt-hours" (V-h) totalled between 7,500 and 10,000V-h. After isoelectric focussing (IEF), the tube gel was extruded from the coated tube by attaching a short segment of Tygon tubing to the top of the tube and using a 1.0 ml syringe filled with distilled water at the other end of the tubing to push the gel onto a clean glass plate. The tube gel was then prepared for running in the second dimension

by equilibrating it with SDS-PAGE running buffer (384mM glycine, 50mM Tris-HCl and 0.1% SDS), pH 6.8, and 5% 2-ME for at least 2 h with gentle agitation on a Junior Orbit Shaker at room temperature. After equilibration, the tube gel was set on top of the stacking gel bed (3% polyacrylamide, pH 6.8) and secured into position by allowing a 1% agarose solution in running buffer to solidify over the tube gel-stacking gel contact area. The second dimension was then run under previously described conditions (cf. above) and when the run was completed, stained according to the procedure of Wray et al. (31). Molecular weight standards were included in the second dimension by forming a well in the 1% agarose overlay using a plastic well-former (5 mm x 1.5 mm x 3.0 cm). The well-former was removed when the agarose overlay had solidified and molecular weight protein standards were added before the running of the second dimansion commenced.

Cell line;

The human promyelocytic cell line HL-60 was obtained from Dr. R.C. Gallo, N.C.I., Bethesda, Md. It was maintained in RPMI 1640 supplemented with 10% fetal calf serum in a humidified 37°C incubator in 10% CO_2. Cells were harvested when the cell density reached 1 x 10^6/ml, washed in serum-free medium prior to cell membrane preparation or washed in serum-containing medium and labelled for FACS IV analysis, as described below.

Fluorescent antibodies;

Fluorescein-labelled DEAE-purified goat anti-rabbit IgG was prepared according to a standard procedure (32). Briefly, the goat antibody (10 mg/ml) was dialyzed against 0.15M NaCl, after which it was dialyzed for 5 h against 0.5M bicarbonate buffered saline, pH 8.5, and finally against 0.05M bicarbonate buffered saline, pH 9.2. The reaction was terminated by dialysis against 0.02M PBS, pH 7.2, at $4^{o}C$ for 4 h. Unbound fluorescein isothiocyanate (FITC) was removed from FITC-IgG conjugates by passage of the material over Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). A ratio of fluorochrome to protein (F:P) of 4 was determined by spectrophotometric analysis (33).

Cell labelling;

HL-60 cells were harvested and washed twice in PBS containing 5% fetal calf serum, following which 1×10^{6} cells were incubated for 1.5 h on ice with 0.2 ml of antiserum (normal rabbit serum, anti-AML or anti-normal human antiserum) diluted to 1/10 in PBS, pH 7.2. All antisera were centrifuged before use to eliminate aggregates. Cells were washed three times in PBS and 0.2 ml of fluoresceinated goat anti-rabbit IgG at 1/20 was added to each cell pellet. Cells were incubated a further 1.5 h on ice, washed once in PBS, and then centrifuged through 100% fetal calf serum. The cells were finally suspended in 1.0 ml PBS containing 5% fetal calf serum for FACS IV analysis.

Fluorescence-activated cell sorter (FACS) IV analysis;

Twenty-five thousand cells from each cell sample were analyzed on a Becton Dickinson FACS IV using the 488 nm wavelength of the Spectra Physics Model 164-05 argon laser at a power setting of 400 mW. The standard filter for FITC analysis was used (520-long pass filter). The FACS IV was standardized using glutaraldehyde-fixed chicken red blood cells (34) and fluorescent monodispersed carboxymethylated microspheres (d= 1.75 um 0.02 SD; Polysciences Inc., Warrington, Pa.).

All anti-AML antisera were titrated against the HL-60 cell line in the FACS IV and compared with the reactivity of the positive control (anti-normal human) antiserum. All anti-AML antisera showed approximately the same reactivity as the positive control antiserum on the HL-60 cells and as a result, in inhibition studies, only anti-AML#1 antiserum was used. Both test antiserum (anti-AML#1) and control antisera (normal rabbit serum and anti-normal human) were absorbed with AML band#1 material for 18 h at 4°C with the antisera diluted 1/10 and the inhibiting antigen at 40.0 ug/ml. Antisera were subsequently tested on HL-60 in the FACS IV. These inhibition assays, using the same antisera, were repeated using absorbed normal human cell membrane extracts as well.

RESULTS

Antiserum was prepared against AML, using the antibody feedback procedure, and tested in the ELISA against both AML and normal human cell membrane extracts. Representative results are shown in Figure 1, in which KCl extracts of 16 AML and 12 normal cell samples were assayed in the ELISA using absorbed anti-AML antiserum. These results show that the anti-AML antiserum reacted with all 16 AML cell extracts whereas no equivalent reactivity was observed with any of the 12 normal cell extracts. These results are also indicative of the fact that this antiserum is recognizing an antigen common to AML cells which is not detectable in normal cell extracts, at least in this assay procedure. The possibility that this antiserum was detecting a blast cell antigen or a differentiation antigen not found on lymphoid cells was investigated by examining extracts of bone marrow cells, on the assumption that this cell population would contain blast cells of the myeloid cell lineage. The results shown in Figure 2 indicate that the antigen is not present in detectable levels in either cell population, regardless of whether the bone marrow sample came from clinincally normal individuals or those with various hematopoetic stem cell disorders.

In subsequent studies, sonicated cell membrane preparations of a pool of normal human white blood cell membranes and an equivalent preparation of AML blast cells were used. After

Figure 1: ELISA of individual extracts of either AML or normal peripheral blood leucocytes (PBL) with absorbed anti-AML antiserum.

AML cell extracts: (O).

Normal cell extracts: (▲).

Anti-AML antiserum at 1:100 dilution.

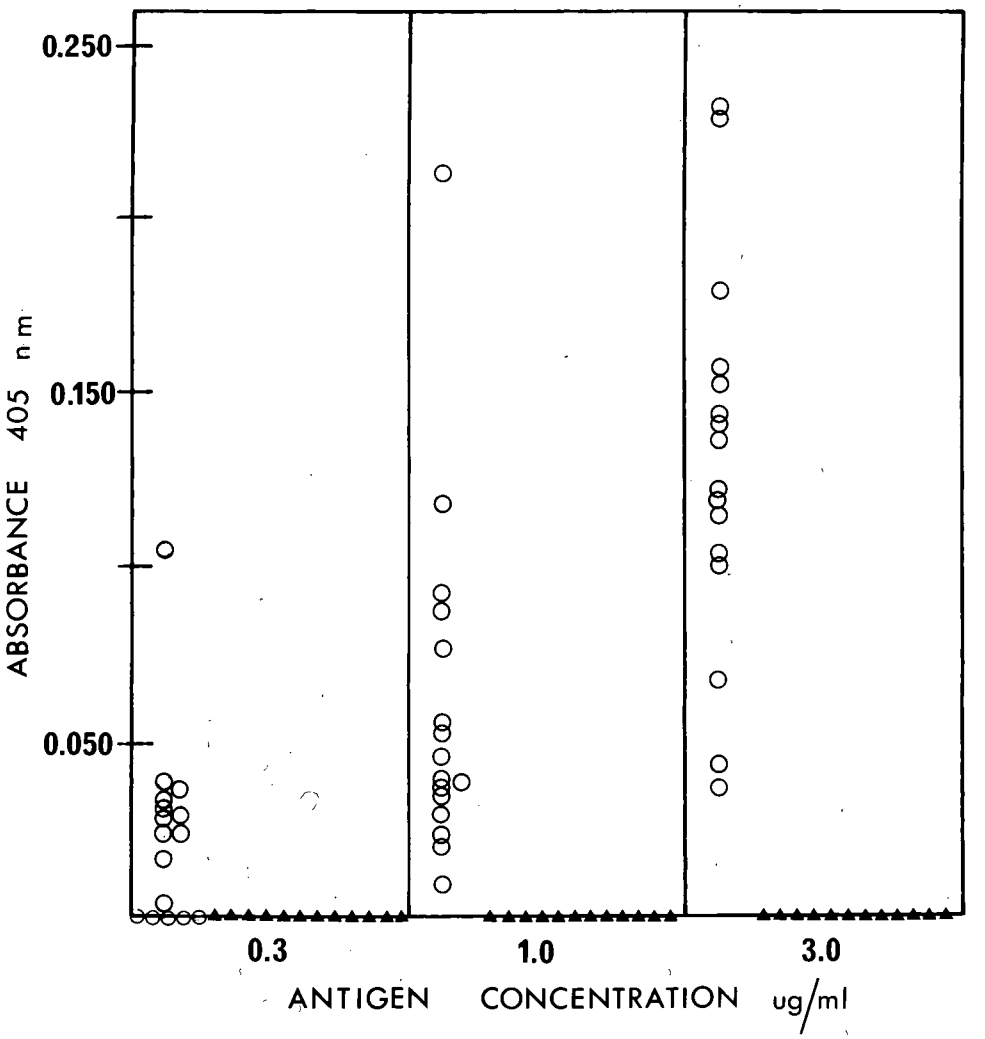

0.250
0.150
0.050
ABSORBANCE 405 nm
0.3
1.0
3.0
ANTIGEN CONCENTRATION ug/ml

Figure 2: ELISA of extracts from AML and various bone marrow cell samples with either anti-AML or anti-normal human antiserum.

a) Antigen preparations from cell extracts of two patients with AML.

b) Individual bone marrow cell extracts from patients with a variety of hematopoetic stem cell disorders as follows:

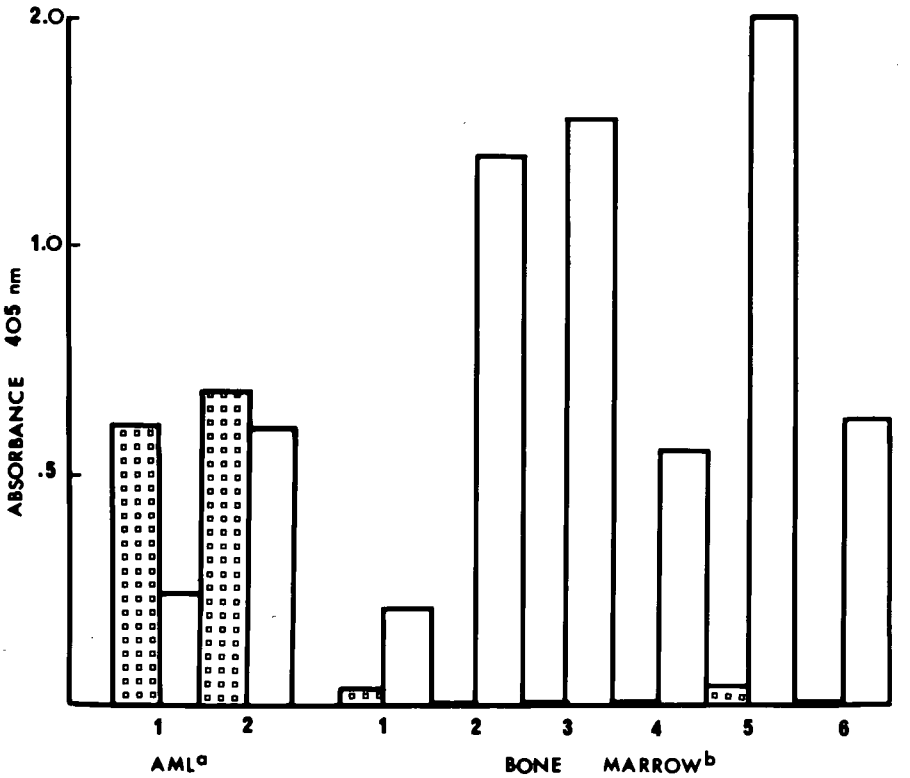
1. Normal bone marrow.
2. Lymphoma patient with malignant cell infiltration in the bone marrow.
3. Multiple myeloma patient with malignant cell infiltration in the bone marrow.
4. Lymphoma patient with minimal malignant cell infiltration in the bone marrow.
5. Acute lymphocytic leukemia patient with malignant cell infiltration in the bone marrow.
6. Normal bone marrow.

All cell extracts were used at 3.0 ug/ml.

Anti-AML and anti-normal human antisera were used at 1:100 dilution;

Anti-AML antiserum: (▦).

Anti-normal human antiserum: (□).


2.0
1.0
.5
ABSORBANCE 405 nm
1
2
1
2
3
4
5
6
AML[a]
BONE MARROW[b]

absorption of these cell membrane extracts on an immunoadsorbent column, containing anti-normal human antiserum, and running on a 7.5% polyacrylamide gel, under non-reducing conditions, a band pattern emerged in which the normal cell membrane preparation displayed a single protein band and the AML cell membrane preparation showed the presence of 4 unique protein bands (Fig. 3). Preparative gels were run on the AML cell membrane sample and each of the four protein bands were eluted and examined for reactivity with the feedback anti-AML antiserum in the ELISA (19). This examination showed AML bands#1,#2 and #3 to be reactive whereas AML band#4 showed no significant reactivity above background. Consequently, AML band#3 material was used for preliminary studies and material eluted from preparative gels was used to raise specific antiserum in rabbits. This antiserum (anti-AML#3) showed essentially absolute specificity in the ELISA for cell extracts from patients with myeloproliferative disease and did not react with equivalent cell extracts from individuals with no apparent disease or with lymphoproliferative disorders. Further work, using this anti-AML antiserum with bone marrow cells from a variety of patients in the FACS IV showed, similarly, that only cells of patients with myeloproliferative disease fluoresced in the presence of this antiserum (21).

The anti-AML#3 antiserum was also found to react strongly with AML bands#1 and#2, eluted from the same preparative gel (20). This indicated that AML bands#1,#2 and #3, at least, shared common antigenic sites and that this antigenic identity

Figure 3: Analytical non-reducing gel pattern from membrane extracts of normal human PBL and blast cells from an AML patient.

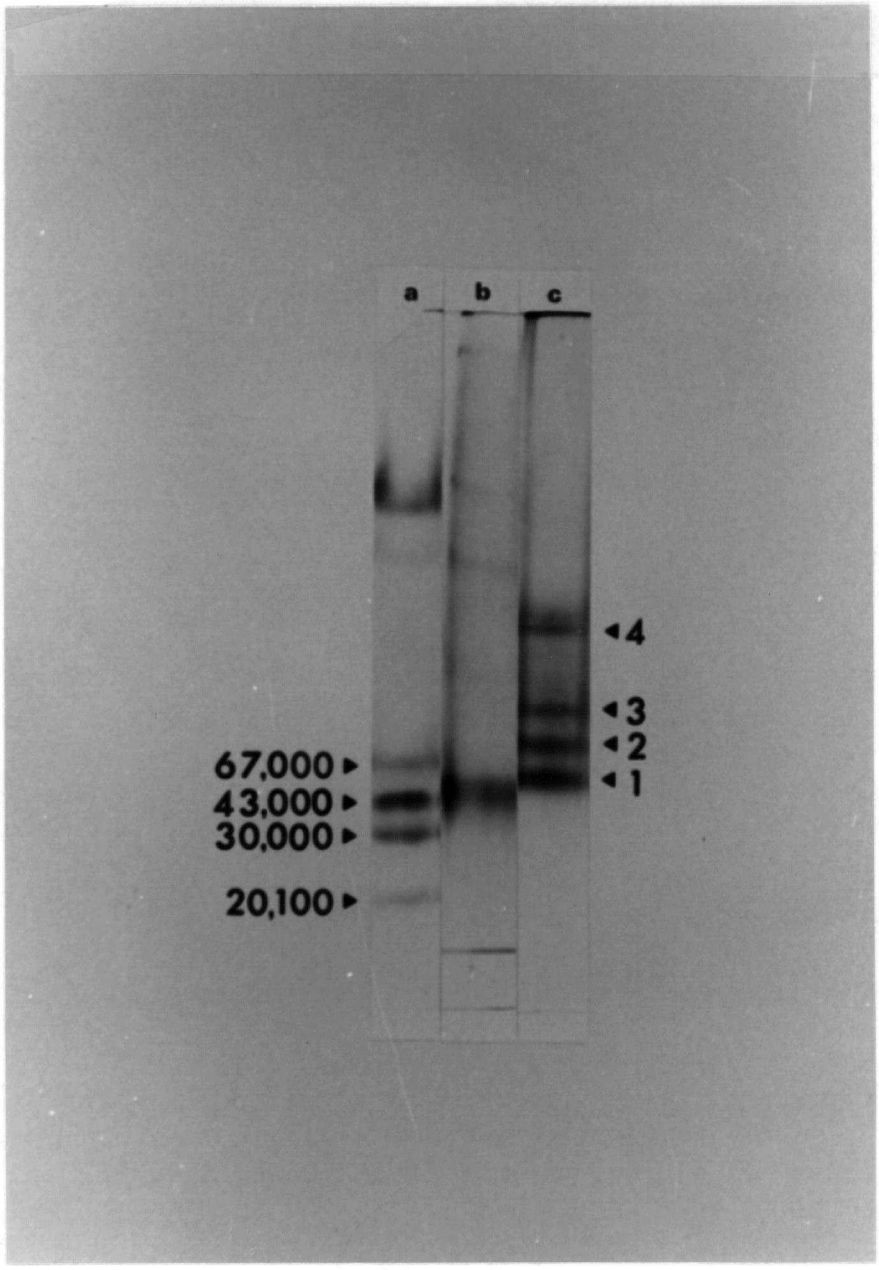
lane a: Molecular weight standards.

lane b: Normal human PBL membrane extract.

lane c: AML blast cell membrane extract.

Both cell membrane preparations had been absorbed twice on an immunoadsorbent column containing anti-normal human antibody, prior to PAGE.

Gel stained in Coomassie Blue according to Fairbanks et al. (1971).


a
b
c
4
3
2
1
67,000
43,000
30,000
20,100

might be reflected in structural similiarities as well. Because of the considerable cross-reactivity between AML bands#1,#2 and #3, the possibility that AML bands#2 and#3 might represent aggregated forms of the lowest molecular weight constituent (AML band#1) was considered.

In order to determine whether similar gel band patterns would develop when extracts from other AML cell membrane preparations were run, or if analogous protein bands would be seen in cell membrane preparations from patients with lymphoproliferative disorders, a panel of cell membrane samples were prepared, absorbed and run on 7.5% SDS-PAGE (Fig.4). These gels appear to confirm that AML bands#2 and #3, observed originally, were probably artifacts of the preparative procedures and represented aggregates of AML band#1 material, since the common feature between extracts of cells from patients with AML is the presence of AML band#1. Membrane preparations from normal cells or those from individuals with hematopoetic stem cell disorders, other than AML, do not possess this protein band. It is interesting to note that the APML/AMML (acute pro-/ monomyelocytic leukemia) cell membrane sample contains a band that is slightly lower in molecular weight than AML band#1. This may represent an unmodified form of AML band#1 in that APML/AMML cell membrane preparations react, in the ELISA, with the anti-AML#1 antiserum, which may be indicative of structural similarities.

In order to test further the contention that AML bands#2 and #3 represent aggregates of AML band#1, antisera to all three

Figure 4: SDS-PAGE of a number of absorbed membrane extracts from a variety of cell sources.

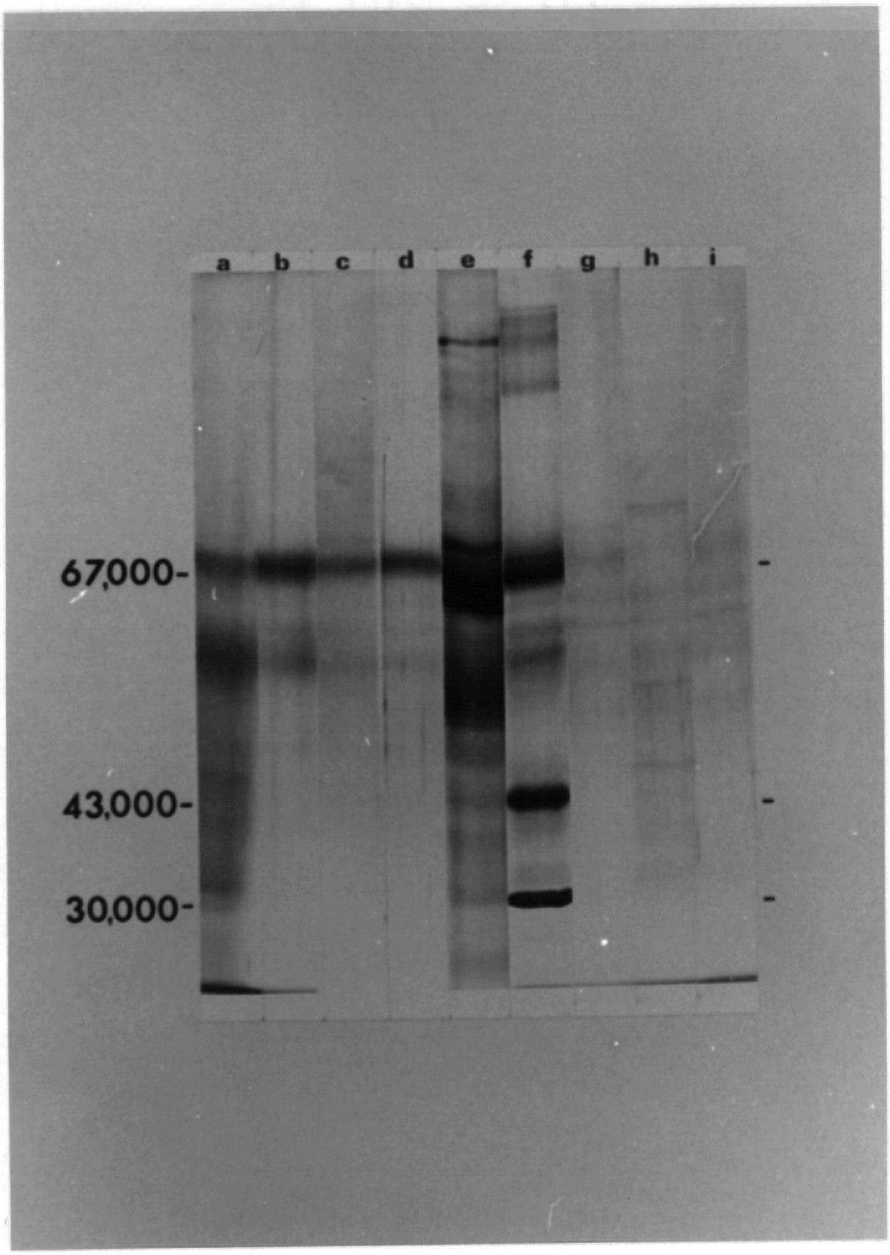
lanes a-d: Absorbed membrane extracts of four individual AML cell samples.

lane e : Absorbed membrane extract of an AMML cell sample.

lane f : Molecular weight standards.

lanes g-i: Absorbed membrane extracts of a pre-CLL, an ALL bone marrow and a normal human buffy coat cell samples, respectively.

Gel stained using the silver staining method of Wray et al. (1981).


a b c d e f g h i
67,000-
43,000-
30,000-

protein bands were raised in rabbits. The specificity of each antiserum was tested in the ELISA using absorbed AML cell membrane samples and an equivalent preparation of normal human white blood cell membranes. Results (Fig.5) show that all the antisera react specifically with the AML cell membrane sample and do not react with the normal human preparation. In order to investigate the possibility that each antiserum was directed to similar, if not identical, antigens, all present in AML band #1, inhibition assays were performed using AML band#1 material as the inhibitor. As shown in Figure 6, all the antisera were 100% inhibited by the same concentration of AML band#1 (about 1.0 ug/ml). This result supports the contention that all three antisera are directed to the same antigenic determinants and that the three protein bands in the AML samples share antigenic and possibly structural identity. Since AML band#1 is found to be uniquely present in all preparations tested from individuals with myeloproliferative disease, the character and structure of AML band#1 and its origin become more important. In order to establish that AML band#1 constituted a single component, 2D-PAGE was performed on a number of purified AML band#1 preparations from a variety of AML cell membrane samples. A representative gel pattern is shown in Figure 7 in which it can be seen that AML band#1 is composed of a single protein component of approximately 68,000 dalton molecular weight with a mean pI of 7.16. The "tailing" or streaking seen in Figure 7 is probably due to differential glycosylation of the AML band #1 material.

Figure 5: Specificity of various anti-AML antisera for absorbed AML extracts in the ELISA.

The antisera were titrated on ELISA wells containing 300 ng/ml of either absorbed AML antigen or absorbed normal human antigen;

anti-AML#1 antiserum	(on AML antigen)	(▲).
"	(on normal antigen)	(△).
anti-AML#2 antiserum	(on AML antigen)	(●).
"	(on normal antigen)	(○).
anti-AML#3 antiserum	(on AML antigen)	(■).
"	(on normal antigen)	(□).

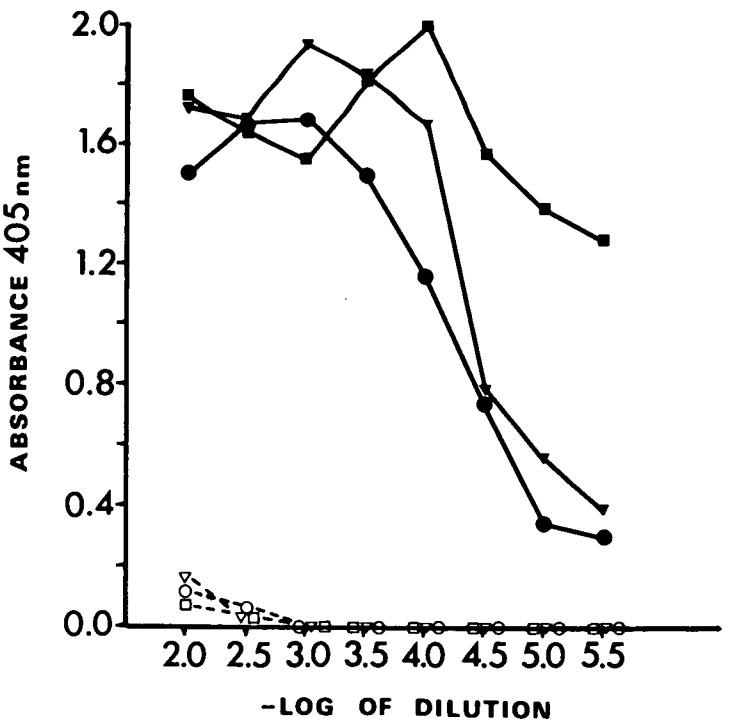

2.0
1.6
1.2
0.8
0.4
0.0
ABSORBANCE 405nm
2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5
-LOG OF DILUTION

Figure 6: Inhibition of anti-AML antisera in the ELISA with various concentrations of AML band#1.

Anti-AML antisera which had been pre-incubated with various concentrations of AML band#1 were tested for reactivity on absorbed AML antigen in the ELISA. ELISA wells contained 300 ng/ml of the absorbed AML antigen. The antisera are as follows;

anti-AML#1 antiserum (▲).

anti-AML#2 antiserum (■).

anti-AML#3 antiserum (●).

Data are presented as percent inhibition.

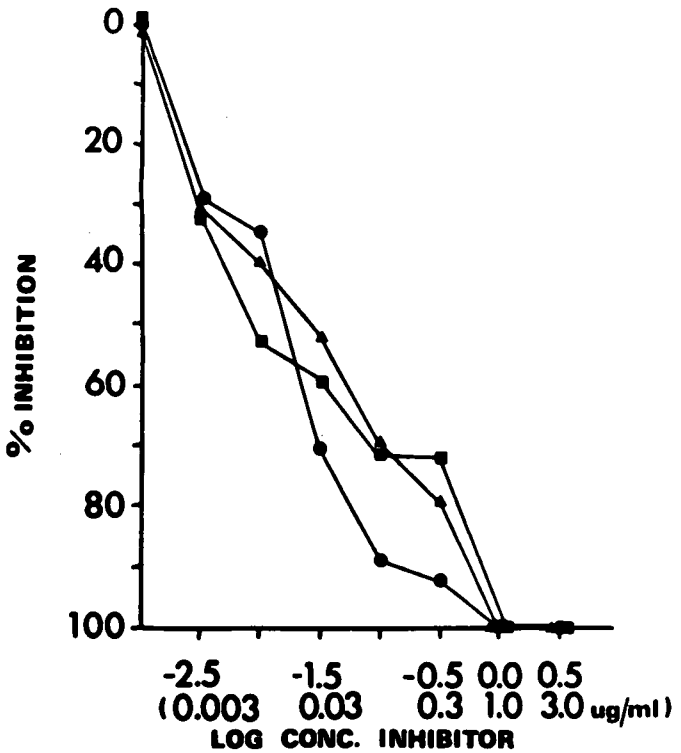
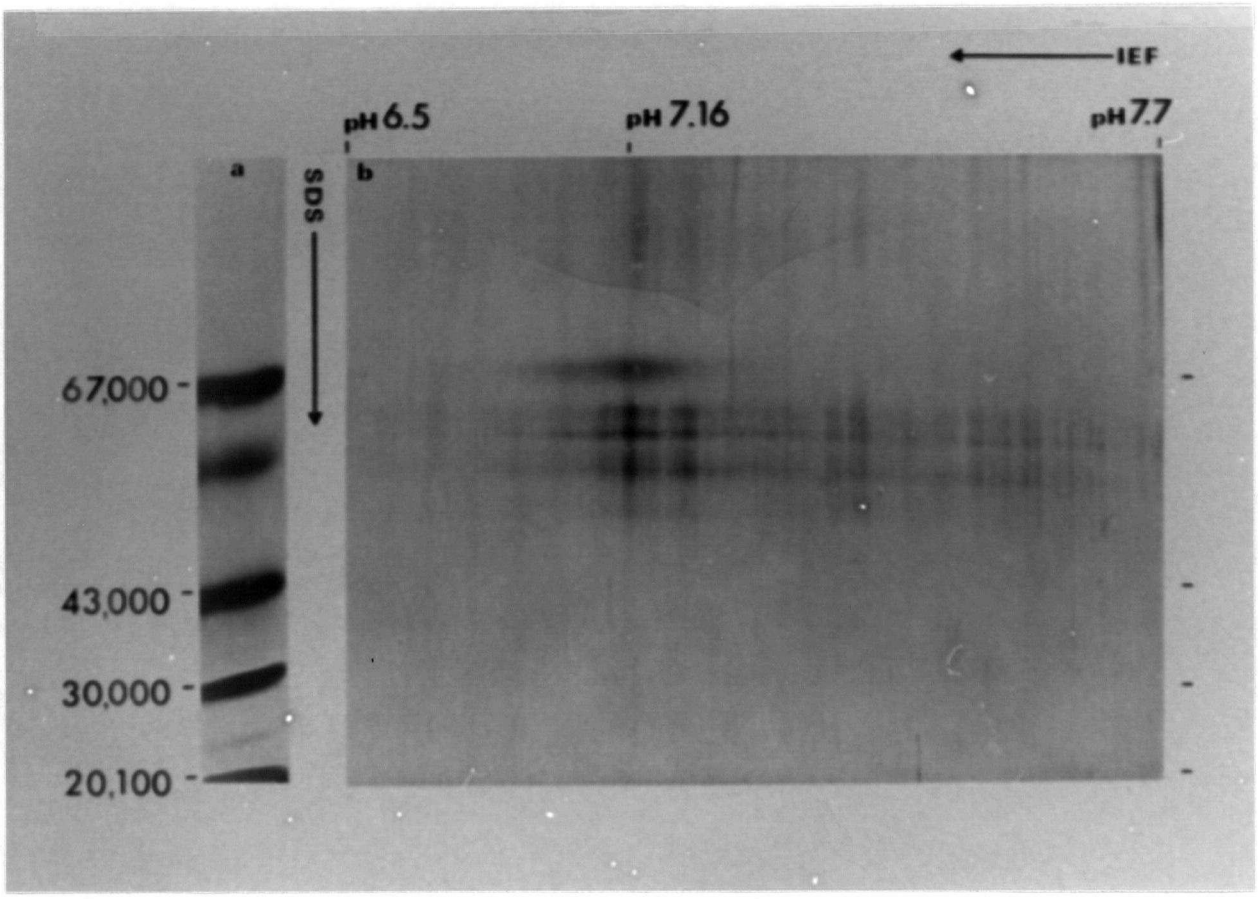

0
20
40
60
80
100
% INHIBITION
-2.5
(0.003
-1.5
0.03
-0.5
0.3
0.0
1.0
0.5
3.0 ug/ml)
LOG CONC. INHIBITOR

Figure 7: Two-dimensional polyacrylamide gel profile of AML band#1.

lane a: Molecular weight standards.

lane b: 5 ug of AML band#1 material was loaded onto an IEF tube gel containing 2% LKB Ampholines. The sample was focussed at 400V for 24h, after which it was extruded and equilibrated with SDS-PAGE running buffer. The IEF tube gel was placed onto a 7.5% PA gel and electrophoresed at 70V for 5h. The finished gel was then silver stained according to the method of Wray et al. (1981).

The lines seen on the gel between 67,000 and 55,000 dalton molecular weight are artifacts of the SDS-PAGE procedure. These same lines appear on 'blank gels' (gels run without any protein present) and therefore represent artifacts of either the SDS-PAGE running conditions or the silver-staining procedure.


IEF
pH 6.5
pH 7.16
pH 7.7
a
b
SDS
67,000
43,000
30,000
20,100

Earlier studies using the anti-AML#3 antiserum on patients' cells in the FACS IV have shown that this antiserum detected a cell surface component present only on cells of patients with myeloproliferative disorders (20). This antiserum also reacted with the promyelocytic leukemia cell line HL-60 in the FACS IV. Studies with anti-AML#1 antiserum showed that it also reacted with HL-60 and appeared to react with patients' cells in a manner identical to the anti-AML#3 antiserum. To establish that the material with which anti-AML#1 was reacting on HL-60 was indeed AML band#1, an inhibition assay in the FACS IV was carried out. It can be seen that AML band#1 material successfully blocks the reaction of anti-AML#1 antiserum with HL-60 cells (Fig.8a and Table I). Conversely, normal human cell membrane preparations successfully block the reactivity of the anti-normal human antiserum with HL-60 (Fig.9b and Table I). The partial inhibition of both the anti-normal human antiserum by AML band#1 (Fig.8b and Table I) and the anti-AML#1 antiserum by the normal human cell membrane preparation (Fig.9a and Table I) can be accounted for by the possibility that AML band #1 is comprised of both leukemia-associated and normal cellular components. The presence of both 'leukemic' and 'normal' cellular moieties together on the same molecule most probably arises as a result of 'leukemic-transformation' of normal hematopoetic stem cell(s).

Table 1. Results of FACS IV analysis of HL-60 cells with antisera pre-incubated with AML band#1, absorbed normal human cell membrane antigen or PBS prior to labelling and analysis.

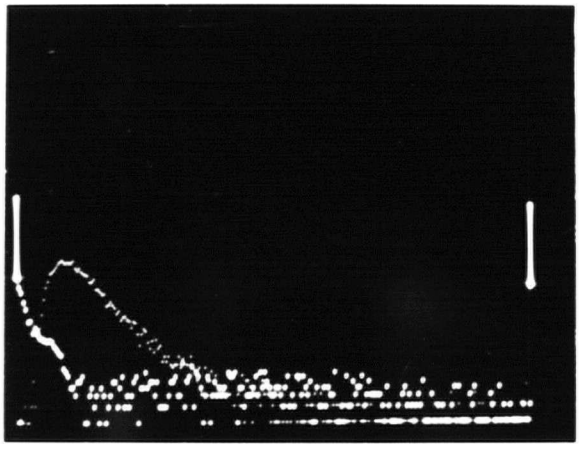
Antiserum	Inhibitor	Number of Cells Fluorescing
Normal rabbit serum	-	3787
"	normal antigen	4127
"	AML band#1	3485
Anti-normal human	-	22,392
"	normal antigen	3560
"	AML band#1	20,927
Anti-AML#1	-	13,175
"	normal antigen	13,370
"	AML band#1	4547

Figure 8: Inhibition analysis of anti-AML#1 and anti-normal human antisera with AML band#1 in the FACS.

panel a: Inhibition of anti-AML#1 by AML band#1 material in the FACS IV using HL-60 cells. The upper distribution pattern shows the fluorescence of HL-60 cells with anti-AML#1 antiserum. The lower distribution pattern shows HL-60 cells with anti-AML#1 that has been pre-incubated with AML band#1.

panel b: Failure of AML band#1 to inhibit the fluorescence of HL-60 cells reacted with anti-normal human antiserum. The upper distribution pattern shows the fluoresence of HL-60 cells with the anti-normal human antiserum. The lower distribution pattern shows HL-60 cells with anti-normal human antiserum that has been pre-incubated with AML band#1.

a



b

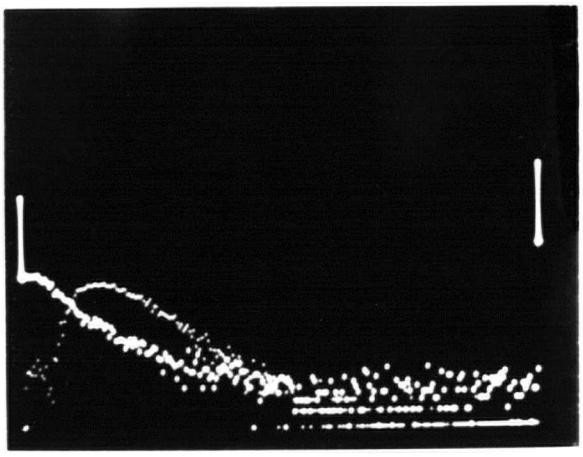
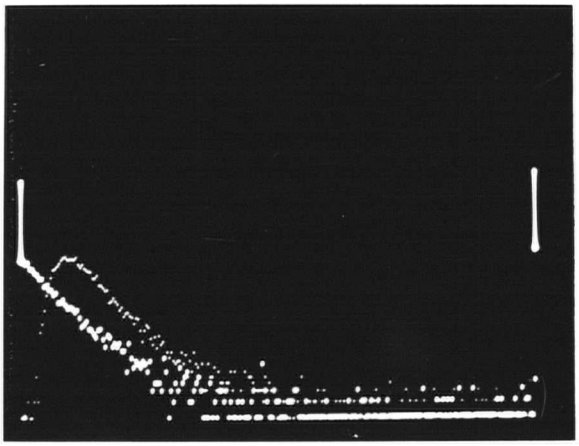


Figure 9: Inhibition analysis of anti-AML#1 and anti-normal human antisera with absorbed normal human cell membrane antigen in the FACS.

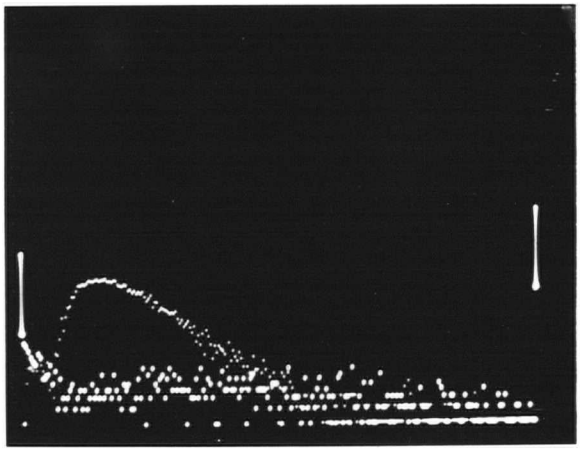
panel a: Failure of absorbed normal human cell membrane antigen to inhibit the fluorescence of HL-60 cells reacted with anti-AML#1 antiserum. The upper fluorescence pattern indicates reactivity of the anti-AML#1 antiserum in the absence of normal human antigen. The lower fluorescence pattern shows the reactivity of anti-AML#1 antiserum with HL-60 in the presence of normal human antigen.

panel b: Inhibition of anti-normal human antiserum by normal human cell membrane antigen in the FACS IV using HL-60 cells. The upper line indicates fluoresence in the absence of normal human antigen, whereas the lower line demonstrates the inhibition seen in the presence of the normal human cell membrane antigen.

a



b



DISCUSSION

The data presented here, and that published previously (19, 20), show that the antisera prepared in rabbits by injection of gel purified protein from absorbed membrane extracts of AML cells react exclusively, in the ELISA, with AML cell membrane preparations and not with equivalent preparations of cell membranes from either clinically normal individuals or those with lymphoproliferative disorders. The specificity of this anti-AML antisera was confirmed in further studies, in which bone marrow cells and PBL from patients with myeloproliferative disorders reacted with the antisera in the FACS IV whereas comparable cells from normal individuals or those with a variety of lymphoproliferative disorders did not (21), indicating that the antigen in question was present on the cell surface. In a previous publication (20), we showed that absorbed membrane preparations of AML cells, when run on non-reducing PAGE, demonstrated the presence of 3 apparently unique, immunologically cross-reactive protein bands. At this time, we have clarified, to some extent, the nature of these proteins in various ways. When absorbed AML cell membranes are run on SDS-PAGE, only the lowest molecular weight band (AML band#1) is consistently present. This band has been present in all cell membrane preparations examined from cells of patients with myeloproliferative disorders and has not yet been detected in identical cell membrane preparations of cells from normal

individuals or those from patients with lymphoproliferative disorders. Antisera raised to each of the 3 protein bands present in the absorbed AML cell membrane sample are inhibitable, in the ELISA, with gel purified AML band#1 material. These data confirm the contention that the higher molecular weight protein bands seen in some AML cell membrane preparations represent aggregates of AML band#1 in association with some other cellular moieties. The molecular weights of the 4 protein bands seen in Figure 3 are 56,000, 80,000, 110,000 and 240,000 daltons molecular weight respectively. The molecular weight of AML band#1, seen in Figure 4, is approximately 68,000 daltons and this is further confirmed since after 2D-PAGE, the protein migrates with an apparent molecular weight of 68,000 daltons and a pI of 7.16. The apparent discrepancy in the molecular weight of AML band#1 is most likely due to the different PAGE conditions used to run the absorbed AML cell membrane sample in Figures 3 and 4.

At present, the precise nature and origin of the AML band #1 antigen is not known. It has recently been shown to be present on a high percentage of both bone marrow cells and PBL of patients with AML or CML (21). Most interesting, has been the finding that the AML antigen is not only present on the blast cells of patients with the active disease but also on a high percentage of noramally differentiating lymphoid and myeloid cells of patients in clinical remission. These observations imply that the expression of this antigen on the surface of a pleuripotent stem cell is an indication of a

potentially malignant change in that cell. At this time, we have not been able to demonstrate the presence of this antigen on cells from normal individuals or patients with lymphocytic leukemia or lymphomas. There are a number of possibilities regarding the origin of this antigen. It may represent a viral gene product since these have been shown to be present at the cell surface and are usually highly glycosylated. It is also possible that the antigen represents a normal differentiation antigen which is present at such low concentrations and on such a low number of cells that it has not been detected in normal preparations, by the tests used here. These possibilities are presently under investigation. Ongoing studies are also addressing the number and distribution of the AML antigen on various cell populations (blast cells, remission cells, etc.), the copy number per cell, its possible function and whether or not the antigen is a recombinant gene product. At present, the intention is to clone the gene(s) encoding this putative AML antigen and address the question of how expression of this gene(s) product is related to the onset of leukemia or to the dominance of a potentially malignant stem cell clone in the bone marrow of AML or remission patients. Regardless of the outcome of these studies, the importance of this AML antigen appears to be largely clinical due to its apparent specific expression in AML and CML cells.

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"....and so there ain't nothing more to write about, and I am rotten glad of it, because if I'd knowed what a trouble it was to make a book I wouldn't a tackled it and I ain't agoing to no more. But I reckon I got to light out for the Territory ahead of the rest, because Aunt Sally she's going to adopt me and sivilize me and I can't stand it. I been there before."

- Mark Twain from 'Huckleberry Finn'.