THE ANALYSIS OF HUMAN MYELOGENOUS LEUKEMIA CELLS IN THE FLUORESCENCE-ACTIVATED CELL SORTER

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ABSTRACT

A cell surface protein from human acute myelogenous leukemia (AML) cells has been purified. (Al-Rammahy et al., Cancer Immunol. Immunother. 9:181, 1980; Malcolm et al., J. Immunol. 128:2599, 1982). This material was used to immunize rabbits. The resulting antiserum (anti-AML) showed myelogenous leukemia specificity in that it reacted with myelogenous leukemia cell extracts and did not react with cell extracts of normal individuals or patients with non-myelogenous leukemia or other malignant disorders in the enzyme-linked immunosorbent assay (ELISA).

Bone marrow and peripheral blood leucocytes (PBL) from either patients with myelogenous leukemia, other disorders or normal individuals were analysed in the fluorescence-activated cell sorter (FACS IV) after labelling with anti-AML, normal rabbit serum (NRS), or antiserum raised to normal human membrane antigens. Of 40 cell samples from patients with AML, 39 reacted strongly with the anti-AML. Similarly, all of 15 specimens from patients with chronic myelogenous leukemia (CML) reacted with the anti-AML. When 42 bone marrow or PBL samples from patients with a variety of lymphoproliferative disorders were examined, only 2 specimens reacted with the antiserum, both from individuals with diagnoses of acute lymphocytic leukemia (ALL). None of the 14 normal bone marrow or PBL

donor specimens tested reacted with the anti-AML. It was also found that essentially all samples from patients in clinical remission from AML had high numbers of cells reactive with the anti-AML. When cells from such individuals were labelled and sorted on the FACS IV, it was found that the cell population fluorescing strongly with the anti-AML contained cells of both myeloid and lymphoid origin.

The AML antigen was used to produce AML specific monoclonal antibody. Spleens from AML-antigen immunized Balb/c mice were fused to NS-1 myeloma parental cells and a myelogenous leukemia specific monoclonal antibody was selected from the hybrid colonies produced. This monoclonal antibody (MAL-1) as well as the rabbit anti-AML has been used to identify myelogenous leukemia patient samples in the FACS IV. In addition, this monoclonal also demonstrates positive fluorescence binding to HL-60 (a promyelocytic leukemia cell line), while there is no binding to lymphocytic leukemia cell lines, CCRF-SB-ALL-B and CCRF-CEM-ALL-T. The MAL-1 monoclonal has been shown to be specific for myelogenous leukemia cell extracts in the ELISA and has been successfully used as an immunoadsorbent for the isolation of the AML antigen from cell extracts. No equivalent antigen was found when cell extracts from normal cells, lymphocytic leukemia cells and lymphoma cells were similarly absorbed.

These findings indicate that both the rabbit anti-AML serum and MAL-1 monoclonal show specificity for an antigen associated with myelogenous leukemia cells.

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CHAPTER I

Introduction

The Stem Cell - Self-Renewal and Differentiation

Haemopoietic stem cells are defined as a small population of marrow cells that are able to give rise to all other haemopoietic cells (pluripotential capacity) as well as being able to regenerate themselves (self-renewal capacity). The prominent importance of the stem cells is exemplified by their unique ability to regenerate a functional haemopoietic system after depletion (i.e. irradiation). Loss of the self-renewal capacity seems to occur concomitantly with the irreversible commitment of the stem cell to a more restricted potential for differentiation. A proposed scheme of bone marrow-derived cell lineages is shown in Figure 1. Since the development of a procedure to quantitate haemopoietic pluripotential cells with the colony forming assay (Till and McCulloch, 1961) many investigators have tried to determine the factors regulating the differentiation of these cells (CFU-S). However, the role of external or internal regulatory factors in determining the decision to produce differentiation progeny remains unknown.

The pluripotent stem cell is naturally of interest when studying disease states such as myelogenous leukemia. Chronic myelogenous leukemia (CML) has been shown to be a clonally-derived disease; the leukemia cells characteristic of CML are derived from a single stem cell in the bone

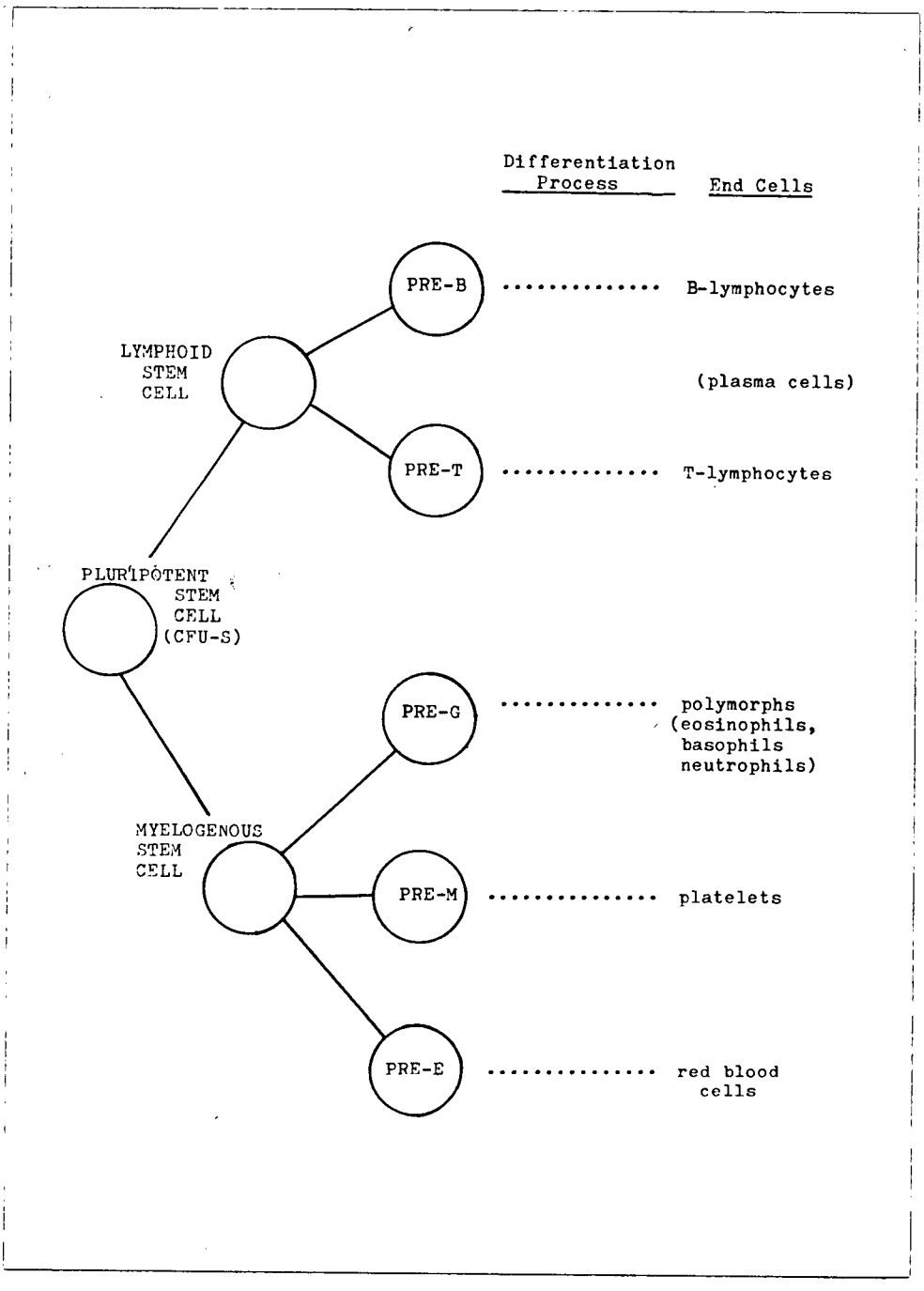
Figure 1. Ontogeny of bone marrow derived cell lineages.

Pre-G = granulocytic progenitors CFU-C as defined by colony assays

Pre-M = megakaryocyte progenitors CFU-M as defined by colony assays

Pre-E = erythroid progenitors primitive BFU-E, mature BFU-E and CFU-E as defined by colony assays

CFU-S = colony forming unit(s) in the spleen


Differentiation Process
End Cells
PRE-B
B-lymphocytes
LYMPHOID STEM CELL
(plasma cells)
PRE-T
T-lymphocytes
PLURIPOTENT STEM CELL (CFU-S)
PRE-G
polymorphs (eosinophils, basophils neutrophils)
MYELOGENOUS STEM CELL
PRE-M
platelets
PRE-E
red blood cells

marrow which has become neoplastic (Barr and Fialkow, 1973; Koeffler and Golde, 1981). CML is characterized by the presence of a chromosomal abnormality called the Philadelphia chromosome, Ph' (which normal arises, 90 per cent of cases, because of a translocation of chromosomal material from the long arm of one of the 22d chromosomes to the long arm of one of the No. 9 chromosomes). The Ph' chromosome has been found in neutrophil, monocyte, erythrocyte, platelet and possibly basophil precursors (Koeffler and Golde, 1981). This alternation occurs in haemopoietic stem cells of patients with CML. The clonal nature of CML is also suggested by the enzyme studies with glucose-6-phosphate dehydrogenase, which is coded by the X chromosome (Fialkow et al., 1978). Women with CML who are heterozygous for this enzyme only have Type A (Gd^A) or B (Gd^B) enzyme in granulocytes, monocytes, eosinophils, basophils, platelets and erythrocytes, suggesting a clonal origin from a common pluripotent stem cell. Fialkow et al. (1978, 1982) also demonstrated that B lymphocytes and B-lymphoblastoid cell lines established from a Gd^A/Gd^B CML patient displayed only one type of G6PD enzyme, indicating that some lymphoid cells arise from the leukemic clone.

It is postulated that leukemic stem cells replace the normal stem cells and ultimately give rise to leukemic blood cells (Wiggans et al., 1978). Current theories of the origins of human cancers are varied, but it is generally thought that clonal diseases, such as CML, may originate from irradiation or a rare oncogenic event, such as somatic mutation or viral integration next to a cellular oncogene acting on a single stem cell (Fialkow et al., 1973; Wiggans et al., 1978). With acute myelogenous

leukemia (AML) however, the picture is less clear. There are no characteristic chromosomal abnormalities unique to AML cells. Although the clonal nature of AML is not clear, Fialkow found a single-enzyme G6PD phenotype in blast cells suggestive of a clonal disorder (Fialkow, 1982). Clearly however, this question remains unresolved.

Cell Surface Antigens on Myelogenous Leukemias

Myelogenous leukemias are at present classified by histochemical reactivity and the morphologic appearance of the malignant cells. Within the last decade, the identification, purification and characterization of cell surface antigens specific for human acute leukemia cells have now been developed and are of potential clinical significance for both the diagnosis and treatment of these diseases. There is considerable evidence in the literature for the presence of tumor-associated antigens which may be common to myelogenous leukemia cells. A number of procedures and animal species have been used to raise xenoantisera to AML cells, all of which have yielded antisera that after appropriate absorptions, demonstrate varying degrees of specificity for AML cells (Baker et al., 1973, 1974, 1981; Durantez et al., 1976; Thränhardt et al., 1978, 1979). Unfortunately, to render these antisera specific, it was necessary to exhaustively absorb them with cells of different cellular lineages, usually resulting in a greatly decreased reactivity to the myeloid leukemia cells. In these studies specificity of the antisera were all assessed by means of complement-mediated cytotoxicity assays. More definitive antisera with marked specificity for AML cell antigens have

been raised in nonhuman primates (Mohanakumar et al., 1978, 1979). In these studies also, cytotoxicity assays were used to define the specificities of the test antisera, and indicated the presence of common AML-associated antigens. With the development of hybridoma derived monoclonal antibodies against cell surface antigens, it seems the difficulties experienced with heteroantisera may be overcome.

Previous work in this laboratory reported on the production of an antisera raised in rabbits to AML membrane extracts which, after absorption, appeared to have specificity for AML cell extracts as assessed by the enzyme-linked immunosorbent assay, ELISA (Al-Rammahy et al., 1980). We tested rabbit antiserum prepared against AML cell extracts by use of a mixture of anti-normal human cell serum and AML KCL-extracted protein (feedback procedure, Al-Rammahy and Levy, 1979) in the ELISA against both the pooled normal antigen and the AML extract. As the unabsorbed antiserum had a considerable amount of reactivity towards normal cell antigens, the antiserum was passed twice over an immunoadsorbent column to which normal cell antigens had been attached. Absorption removed essentially all the reactivity to normal cell antigens, while a considerable reactivity to the AML cell extract was maintained. Indeed, a series of tests were run in which this absorbed antiserum was used in the ELISA with KCL extracts of 16 AML and 12 normal cell extracts. This antiserum reacted with all 16 AML extracts, while it showed no reactivity with extracts of normal cells. As it showed no reactivity to extracts of normal donors' cells the possibility that this reactivity was directed to HLA antigens is exceedingly unlikely.

Furthermore, the antiserum showed no reactivity to PHA blast cell extracts, or to extracts of ALL blast cells indicating that it was not recognizing an antigen common to blast cells. As the antiserum was not reactive with CLL cells (known to be high in Ia) the possibility that it was recognizing a Ia-like component was also unlikely. The possibility that the antiserum was detecting a differentiation antigen not found on lymphoid cells was investigated by testing extracts of bone marrow cells, on the assumption that this population would contain blast cells of the myelocytic lineage. The antigen was not present in detectable levels in these populations either, regardless of whether the marrow specimens came from apparently normal individuals or from those with various malignant conditions; this suggests that the reactivity was not directed toward differentiation antigens found on normal blast cells of lymphocytic or myelogenous lineage (Al-Rammahy et al., 1980).

This feedback procedure resulted in the production, in rabbits, of an antiserum that displayed specific reactivity to extracts of AML patients' cells. The sensitivity of the ELISA allows detection of antigen in nanogram quantities (Kelly et al., 1979); the results obtained with this assay in our laboratory suggest that there may be one unique (or more) but common leukemia-associated antigen on AML cells. The results presented in Chapters II and III of this thesis , in which this antiserum was used to isolate an antigen for the production of a more specific rabbit anti-AML-serum and a mouse monoclonal antibody directed to AML, further support these findings.

CHAPTER II

Analysis of Human Myelogenous Leukemia Cells in the Fluorescence-Activated Cell Sorter Using a Tumour-Specific Antiserum

Introduction

A considerable amount of work has been done on the characterization of cell surface markers which may classify leukemia cells (Maheu et al., 1981; Foon et al., 1982). The majority of those described constitute normal surface markers which may be expressed with greater frequency or density on the surface of leukemia cell populations. Alterations in membrane carbohydrates, such as decreased complex gangliosides (Hildebrand et al., 1972) have been reported with leukemia cells. Some leukemias display unique marker characteristics; for example, glycolipid asiala GM1 is found on cells from patients with acute lymphoblastic leukemia (ALL) but not on cells from patients with other forms of leukemia (Nakahara et al., 1980) and the intracellular enzyme, terminal deoxynucleotidyl transferase (TdT) is detectable in ALL but not AML cells (Foon et al., 1982).

In studies on acute non-lymphocytic leukemias (myelogenous), there has been some evidence that antigens unique for the leukemia cells may also be present. Baker and co-workers (1979) have demonstrated a leukemia associated antigen (LAA) on myelogenous leukemia cells using a specific antiserum raised in mice. They were able to detect imminent relapse of remission patients by demonstrating an increased number of seropositive cells in bone marrow aspirates, detected by immunofluorescence. Similarly, simian heteroantisera have been used to identify what appear to be LAA on myelogenous leukemia cells (Mohanakumar et al., 1980).

As stated in the previous chapter, our laboratory produced a rabbit antiserum with apparently absolute specificity for membrane extracts of human AML cells and no reactivity for equivalent preparations of either peripheral blood or bone marrow cells from either normal individuals or patients with a variety of non-myelogenous disorders. Monitoring for specificity of this antiserum was carried out using the ELISA.

This chapter reports on the use of this specific antiserum as a probe for the isolation of a tumor-specific component from polyacrylamide gels. This isolated material was used to raise a second antiserum in rabbits which subsequently showed apparent specificity for AML cell extracts in the ELISA. Studies using a fluorescence-activated cell sorter (FACS IV) show that this antiserum, when developed with fluorescein-labelled goat anti-rabbit IgG, reacts strongly with cells from bone marrow aspirates and peripheral blood leucocytes of AML patients and of chronic myelogenous leukemia (CML) patients. When cells from patients with a variety of lymphoproliferative disorders (ALL, CLL, lymphomas) were tested, there was no apparent reactivity with the antiserum. Similarly this antiserum did not react with cells from normal individuals. The data reported show that this antiserum is specific for cells of patients with myelogenous leukemias. It is possible that the recognized antigen may constitute a "malignancy marker" for a malignant clone in patients with myelogenous leukemias, since it was found to be present, not only on blast cells but also on other differentiated cell populations both from patients with active disease and in remission.

Materials and Methods

Preparation of Membrane Extracts

AML or normal peripheral blood leucocyte (PBL) membranes were prepared by sonication of cells with four 15 second bursts over ice at a setting of 60 on a Bronwill Biosonik Sonicator (Bronwill Scientific, Rochester, N.Y.). Cell debris was removed by centrifugation of sonicates at 400 x g for 10 min. Membranes were pelleted from the supernatants by ultracentrifugation at 110,000 x g for 90 min. Membrane pellets were resuspended in minimal volumes of PBS (0.01 M pH 7.4) and sonicated again as described. Sonicates were diluted to 5.0 ml in PBS and total protein determined by the method of Bradford (1976).

Immunoadsorbent

Antiserum raised in rabbits to pooled normal PBL membrane extracts from 15 individuals (Al-Rammahy *et al.*, 1980) was coupled to Sepharose 4B (Pharmacia) with cyanogen bromide (Avremeus and Ternynck, 1969; Cuatrecasas, 1970). Prior to use the anti-normal column was equilibrated with borate-saline, pH 8.5. Samples were layered onto the column and cycled over twice using borate saline. Both the AML and normal PBL membrane extracts were treated in this way prior to subsequent testing. This treatment was found to remove approximately 90% of the protein present in the membrane extracts. The fall through fractions were used for further studies.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was performed as previously described (Laemmli, 1970; Gold et al., 1976) except that no SDS or 2-mercaptoethanol was used. Samples of absorbed membrane extracts were applied to 7.5% polyacrylamide gels for both analytical and preparative procedures, and electrophoresed at 50V for 5-6 h. Preparative gels were run using a one-tooth comb and loading approximately 250 µg of protein onto the gel. Molecular weight determinations were carried out by co-electrophoresing samples with low molecular weight protein standards, no SDS (Pharmacia). Gels were stained according to the method of Fairbanks et al. (1971). For preparative gels, a section of the gel was removed and stained, the remainder of the gel being stored at -70°C until it was required for slicing and elution.

Elution of Antigen

Stained and unstained sections of preparative gels were lined up and illuminated from below on a light box. Bands were cut from regions of the unstained gel corresponding to locations shown on the stained section to contain detectable protein. Gel slices were placed in small tubes (Falcon 2003) and crushed with a spatula. Two ml of PBS were added to each tube and elution was effected by end-over-end mixing of the tubes in a Labquake (Labindustries, Berkely, Calif.) at 4°C for 48 h. Eluted protein was collected by filtration over vacuum and protein determinations were carried out on all fractions. Testing of eluted materials for antigenic activity was carried out using a rabbit antiserum previously shown to have specificity for AML cell extracts (Al-Rammahy et al., 1980) in the ELISA.

Antiserum

Fractions of eluted protein shown to have antigenic activity in the ELISA with previously prepared AML-specific antisera were used to immunize young adult female albino rabbits. The eluted material was mixed 1:1 with complete Freund's adjuvant to a total volume of 1.0 ml and injected intramuscularly into four distal sites. Animals were given another immunization 4 weeks later and bled 7 days following the second immunization. We have established that the antigen used to raise the anti-AML antiserum appears to be a homogeneous protein which produces a single spot on two-dimensional gel electrophoresis, has a pI of 7.1 - 7.2 and a molecular weight of 68,000 daltons (Shipman, Malcolm, and Levy, British J. Cancer, In press). Thus, the antiserum used here was prepared against a homogeneous pure protein, according to the standard biochemical criteria.

ELISA

Antisera were assayed by a standard ELISA procedure described previously in detail (Kelly et al., 1979).

Subjects

A total of 40 patients with acute myelogenous leukemia and 15 patients with chronic myelogenous leukemia were studied. Of the AML patients 19 were tested when newly diagnosed, 16 patients were studied when in clinical remission, and 7 as relapsed AML patients. Two other AML patients were analysed post bone marrow transplantation. When attainable,

samples from the same AML patient as newly diagnosed AML patient, as a remission patient, and/or as an AML in relapse were tested. Forty-two patients with leukemias of non-myelogenous origin or with other malignant and non-malignant conditions were used as controls. Fourteen laboratory personnel and bone marrow transplant donors were also used as normal controls.

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 DME supplemented with 10% foetal calf serum in a humidified 37^{o}C incubator in 10% CO_2. Cells were harvested when they reached 1 x 10^6 per ml, washed in medium and labelled for FACS IV analysis as described above.

Fluorescent Antibodies

Fluorescein-labelled DEAE-purified goat anti-rabbit IgG was prepared according to a standard procedure (Wofsy et al., 1980). Briefly, the goat antibody at 10 mg/ml was dialyzed exhaustively against 0.15 M NaCl, after which it was dialyzed for 4 h against 0.5 M bicarbonate-buffered saline, pH 8.5, and finally against 0.05 M bicarbonate-buffered saline, pH 9.2 for 3 h, all at 4^{o}C. The IgG was then dialyzed for 24 h against 100 µg/ml of fluorescein isothiocyanate (FITC, BBL) in a 0.05 M bicarbonate-buffered saline at pH 9.2. The reaction was stopped by dialysis against 0.02 M PBS, pH 7.0, at 4^{o}C for 4 h. Unbound FITC was removed from FITC-Ig conjugates by passage of the material over Sephadex G-25. A ratio of

fluorochrome to protein (F:P) of 4 was determined by spectrophotometric analysis (Wells et al., 1966).

Cells and Cell Labelling

Bone marrow aspirates and peripheral blood samples were obtained from the Division of Haematology, Vancouver, General Hospital or the Cancer Control Agency of British Columbia (Terry Fox Laboratory). Samples obtained were from patients whose diagnoses (AML, CML, or otherwise as referred to in the Tables) were established either prior to or following fluorescence testing. The clinical diagnoses were made by haematologists at, or affiliated with, the Vancouver General Hospital. The tests used included: morphological examination of peripheral blood and bone marrow smears, biochemical tests on bone marrow (Sudan black, PAS, combined esterases, and acid phosphatase tests when necessary), colony growth studies, and chromosomal analyses (in cases of CML). Lymphocyte surface Ig and rosette tests were performed on samples from patients with lymphoid leukemias. Buffy coats were collected from bone marrow samples by sedimentation at 1 g, and from the heparinized peripheral blood samples using Plasmagel (Laboratoire Roger Bellon, Neuilly, France) or by the Ficoll-Hypaque technique (Boyum, 1976; English and Anderson, 1974). In the case of peripheral blood samples, similar results were obtained to those of Boyum (1976) and English and Anderson (1974) regarding the relative purities of mononuclear cell-enriched (96%) and granulocyte-enriched (98%) cell suspensions recovered respectively. The cells were washed in PBS containing 5% FCS and the cell pellet was

suspended in 0.14M ammonium chloride and 0.017 M tris buffer at pH 7.2 to lyse contaminating erythrocytes, followed by a subsequent wash. Then 10^6 cells were incubated for 1.5 h on ice in 0.2 ml of antiserum (anti-normal human, anti-AML or normal rabbit serum (NRS)) diluted to 1/10 in PBS. The normal rabbit serum (NRS) used in these studies was taken from unimmunized rabbits which were used subsequently for immunization with the AML antigen. Consequently, they served as an appropriate negative control in these experiments. All antisera used were centrifuged briefly before use at 20,000 x G 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 another 1.5 h at $0^{o}C$, washed once in PBS, and then centrifuged through 100% FCS. They were finally suspended in 1.0 ml PBS and 5% FCS for FACS IV analysis.

FACS IV Analysis

Twenty-five thousand cells from each samples were analysed 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 by using glutaraldehyde-fixed chicken red blood cells (Herzenberg et al., 1976) and fluorescent monodispersed carboxymethylated microspheres (d = 1.75 m ± 0.02 SD; cat. no. 9847, Polysciences Inc., Warrington, PA, 18976). The results reported here include all those samples which were analysed and which gave acceptable

results in the FACS IV with the positive (anti-normal human) and negative (NRS) controls. Occasionally, it was impossible to obtain samples or book FACS time until the specimens were 48 hours old. Sometimes these samples yielded either extremely high backgrounds with NRS or did not react strongly with the positive control antiserum. Thus, it was impossible in these circumstances to evaluate the reactivity of cells with the anti-AML, and these results were discounted. A certain degree of fluctuation in negative test samples was observed when the cells fluorescing were compared to the NRS control. The anti-AML frequently reacted at a lower level with such cells than did the NRS. It was ascertained that variation in the range of ± 5% in comparison to NRS results was within the margins of error for these studies.

FACS IV Cell Sorting Technique

An APML (promyelocytic) remission sample that had been analysed as significantly positive with anti-AML, was sorted using the FACS IV. Two sort windows were determined, one contained the 25% of the sample showing no or minimal relative fluorescence and one contained the 25% of the sample showing the highest relative fluorescence. The two cell populations were collected in separate tubes containing 1 ml PBS/FCS. The cells between these two windows, representing the remaining 50% of the sample cell population were discarded into the reservoir flask. The head drive frequency was set at 36 KHz, and 2,000 volts were applied across the electrostatic deflection plates. The Eput counters recorded the number of cells collected in the right and left deflection tubes and the FACS was

run at 5 droplets per deflection pulse with the abort on; the droplet delay was set at 14 drops. Cooling water ($2^{o}C$) was circulated around the collection and sample tubes. The cells were then washed once in PBS to remove protein in the FCS and the sample loaded on a cytospin (John's Scientific) at 800 rpm for 8 minutes to obtain slide preparations. The slides were stained routinely with Wright's stain and examined by light microscopy.

Results

Properties of the AML Antigen and Antiserum

Membrane extracts of PBL from patients in AML blast cell crisis and from pooled normal PBL were adsorbed twice on an immunoadsorbent containing rabbit antibodies to normal PBL membrane components. The adsorbed materials (i.e. materials not removed from the preparations by the adsorbent) were run on an analytic nonreducing gel and were stained. Representative results are shown in Figure 2. The AML material contained four distinct bands that did not appear to be present in the equivalent normal preparation. The constituents were isolated by elution of each band from preparative gels. The four preparations were tested in the ELISA for reactivity with the AML-specific rabbit antiserum described previously (Chapter I). Band 1, 2 and 3 showed reactivity in the ELISA, band 3 being the most pronounced (data shown in R. Shipman's M.Sc. Thesis, 1982). The material in band 3 was used to raise an antiserum in rabbits. The resulting antisera, when tested in the ELISA with the absorbed AML extract and the equivalent normal PBL extract, showed marked specificity for the AML preparation and virtually no reactivity with the normal PBL material (Figure 3). To determine whether the three bands represented materials with similar antigenic properties, the antiserum was tested in the ELISA with bands 1 and 2. The results showed that essentially total cross-reactivity existed between the individual bands (R. Shipman, M.Sc. Thesis, 1982). Because these components were isolated from nonreducing

Figure 2. 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. Band 1 = 62,000 daltons M.W.; Band 2 = 89,000 daltons M.W.; Band 3 = 140,000 daltons M.W.

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).

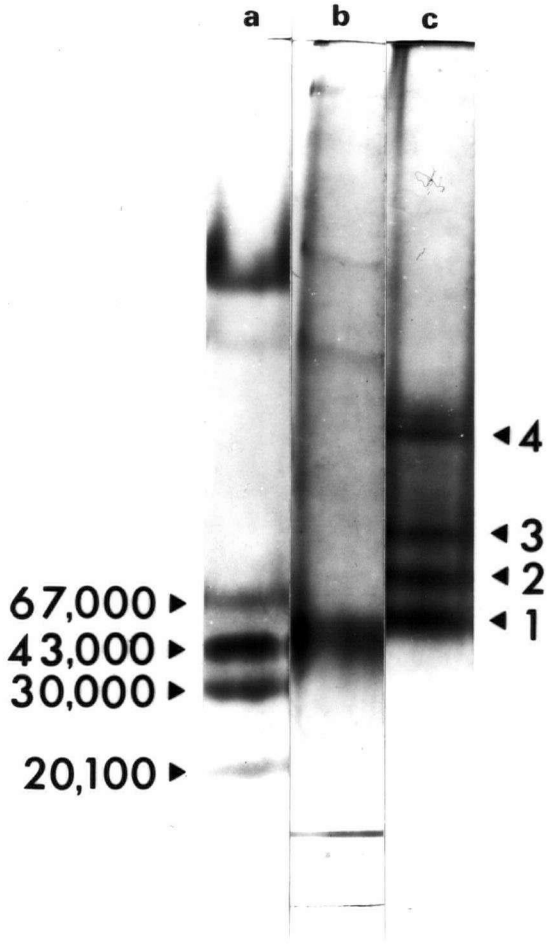
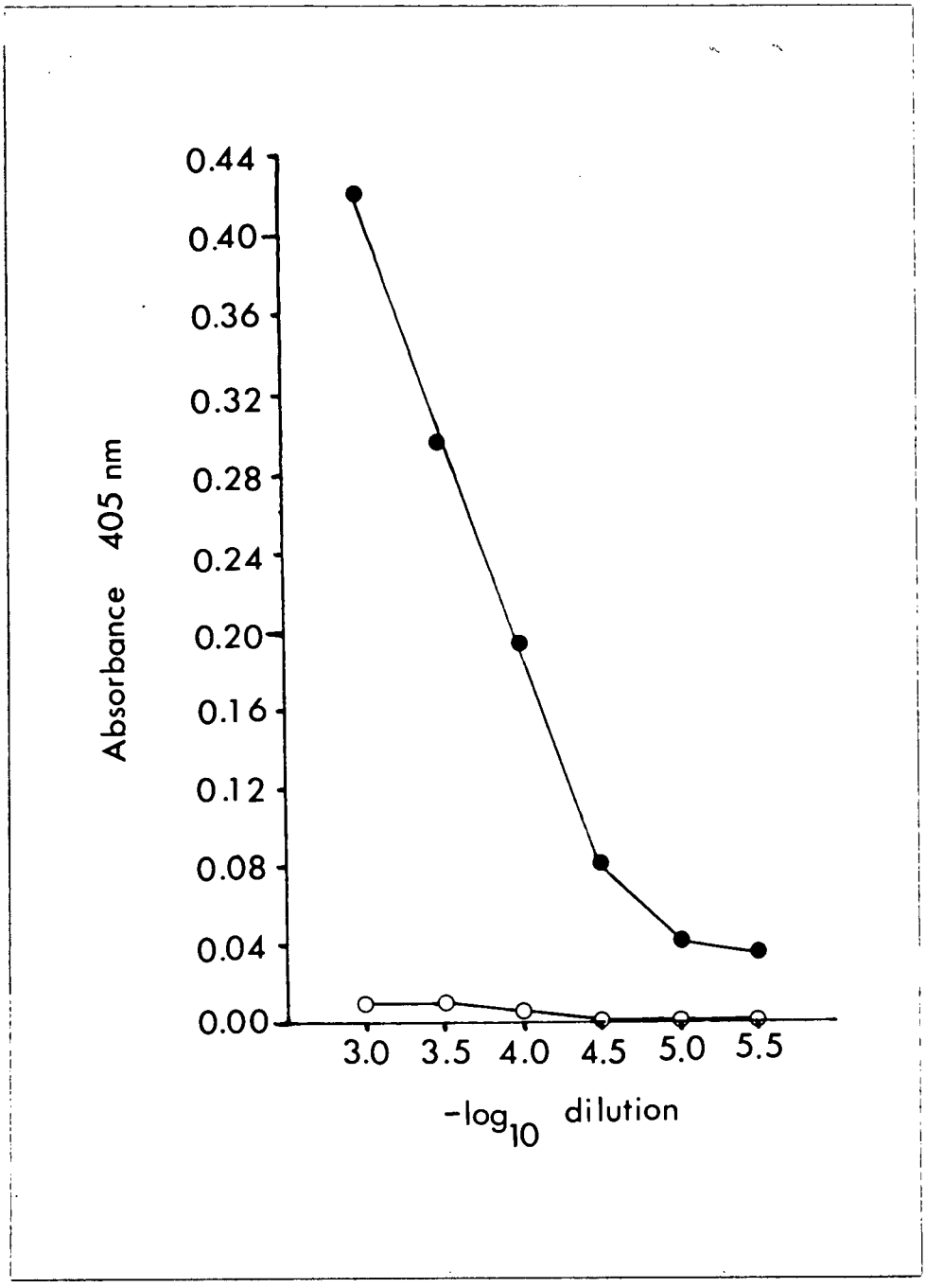

a
b
c
67,000
43,000
30,000
20,100
4
3
2
1

Figure 3. ELISA analysis of anti-AML antiserum to band 3. Antigen concentration was at 300 ng/ml and readings were made 45 min after substrate was added to the plates. ●———●, reaction of the antiserum with the AML extract; O———O, reaction of the antiserum with noraml PBL extract.


0.44
0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
Absorbance 405 nm
3.0
3.5
4.0
4.5
5.0
5.5
-log10 dilution

gels, it was possible that all the bands contained the same antigen. This was subsequently established by demonstrating that absorbed extracts of cells from a variety of AML patients, when run on reducing PAGE, all demonstrated the presence of band 1 (the unique component with the lowest molecular weight) but did not show the presence of either band 2 or 3 (Shipman, Malcolm and Levy, British J. Cancer, In press). Serologically, this observation was substantiated when we observed that antisera raised in rabbits to bands 1 and 2 each demonstrated complete cross reactivity in the ELISA with each other as well as band 3 (R. Shipman, M.Sc. Thesis, 1982). Similarly, complete identity between the three antisera was observed when they were compared for their ability to react with markers on the surface of cells from AML patients as demonstrated by FACS IV analysis.

Because band 1 (AML-1) antigen appeared to be the simplest common antigen, antisera was raised to it. This antiserum was used in the following study of marker detection on the surface of AML and CML patients' cells by FACS IV analysis.

The ability of anti-AML-1 to precipitate AML-1 antigen was tested by immunoprecipitation (Kurth et al., 1979). Iodinated AML-1 was precipitated with rabbit anti AML-1 and protein A and run on a polyacrylamide gel (Laemmli et al., 1970; Gold et al., 1976). The results (Figure 4) show that the antiserum precipitates predominantly AML-1 material and is active up to a dilution of 1:8000.

Figure 4. Immunoprecipitation of ^{125}I-labelled AML-1 with rabbit antiserum.

Lane 1 - normal rabbit serum + 50,000 cpm of antigen

2 - 1:2 dilution of anti-AML-1 + 50,000 cpm of antigen

3 - 1:8 dilution of anti-AML-1 + 50,000 cpm of antigen

4 - 1:32 dilution of anti-AML-1 + 50,000 cpm of antigen

5 - 1:128 dilution of anti-AML-1 + 50,000 cpm of antigen

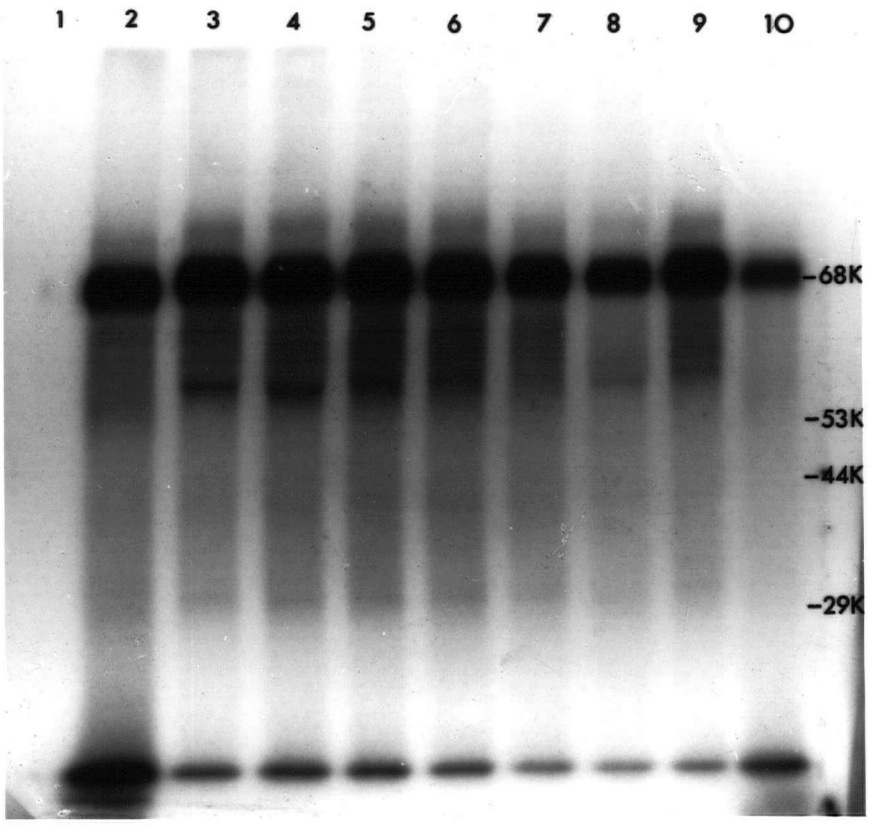
6 - 1:512 dilution of anti-AML-1 + 50,000 cpm of antigen

7 - 1:2048 dilution of anti-AML-1 + 50,000 cpm of antigen

8 - 1:8192 dilution of anti-AML-1 + 50,000 cpm of antigen

9 - AML-1 alone, 50,000 cpm.

10 - AML-1 alone, 25,000 cpm.


1
2
3
4
5
6
7
8
9
10
68K
53K
44K
29K

Reactivity of the Rabbit-anti-AML-Serum in FACS IV Analyses.

To test the specificity of this antiserum on clinical material, a series of experiments were carried out using bone marrow aspirates from patients with a variety of disorders. The data shown in Table 1 are the results of FACS IV analysis of bone marrow samples from patients shown to have AML. The results of representative individual tests as well as the averaged results of the total number of tests run are shown. Fluorescence profiles of these cells from two representative patients are shown in Figure 5, and the numbers corresponding to those profiles are shown in Table 1. These results were obtained by determining the number of cells fluorescing beyond the point at which the profile of cells constituting the negative control (NRS) intersected the profile of cells treated with the positive control serum. It can be seen that the number of cells fluorescing with the test (anti-AML) antiserum was frequently almost as high as the number fluorescing with the positive control (anti-normal) antiserum, and is always significantly higher than the negative control (NRS).

The results of PBL cells from a number of patients presenting with AML is shown in Table 2. It can be seen that in all cases, a high percentage of the cells fluoresced positively with the anti-AML-serum. The results obtained with the bone marrow aspirates and PBL were essentially the same (Tables 1 and 2). It is interesting to note that the number of cells fluorescing with anti-AML showed no correlation with the number of blast cells found in either bone marrow or PBL but is usually consistently higher. Furthermore, relative blast cell numbers did not correlate to relative fluorescence, sample 5 of Table 1 with an 8% blast

Table 1. FACS analyses of bone marrow cells from untreated patients with acute myelogenous leukemia

Diagnosis		% blasts	Number of cells fluorescing with various treatments*			% positive cells**
			Anti-normal human	NRS	Anti-AML	
1.	AML-M2****	43	23,920[1a]	1,553	22,299[1b]	92.7
2.	AML-M2	13	18,008	513	12,183	66.7
3.	AML-M2	15	24,446	623	10,366	40.9
4.	AML-M2	13	23,276	4,116	15,083	57.2
5.	AML-M2	8	15,096	983	15,457	>100
6.	AML-M2	74	17,266	2,395	16,191	92.9
7.	AML-M2	76	20,635	4,725	12,762	50.5
8.	AML-M2	11	19,670[2a]	2,332	21,628[2b]	>100
9.	APML-M3	8	16,789	3,444	15,096	87.3
10.	APML-M3	6	23,395	1,275	7,577	28.5
11.	AMML-M4	49	22,435	2,338	14,609	61.1
12.	AMM1-M4	10	23,413	2,492	19,672	91.7
	AML		-	-	-	72.46± 24.8***

* A total of 25,000 cells were analysed in each case.

** Percent positive = $\frac{\text{number of cells fluorescing with anti-AML - number fluorescing with NRS}}{\text{number of cells fluorescing with anti-normal - number fluorescing with NRS}} \times 100$

*** Averaged results ± standard deviation of 12 bone marrow samples.

**** FAB classification (see over).

1a,1b,2a,2b: See fluorescence intensity profiles in Fig. 5.

FAB Classification

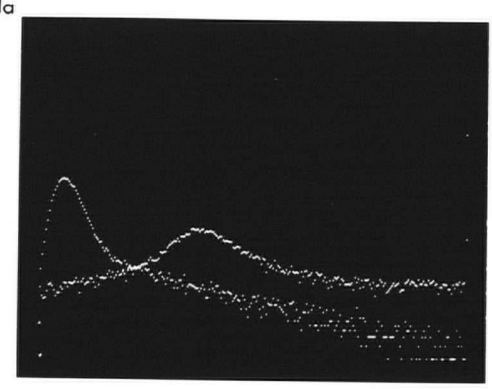
The FAB (Franco-American British Co-operative Group) classification is shown on all the tables.

Classification	Cell type
1. AML-M1	myeloblastic without maturation
2. AML-M2	myeloblastic with maturation
3. APML-M3	hypergranular promyelocytic
4. AMML-M4	myelomonocytic
5. AML-M5	monocytic
6. AML-M6	erythroleukemia
7. AML	in the cases when no FAB classification could be given by the pathologist

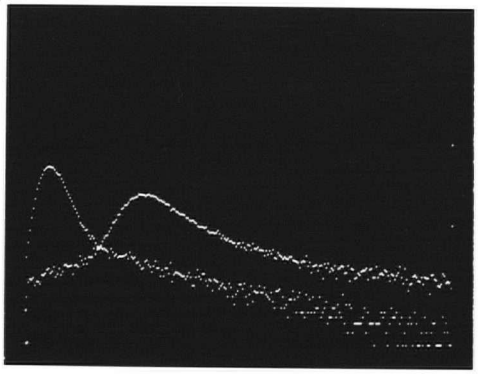
Figure 5. Fluorescence intensity profiles of bone marrow cells from two AML patients.

Vertical axis, relative number of cells in log scale. Horizontal axis, relative fluorescence intensity. Nonspecific fluorescence is demonstrated by the cells treated with NRS and these profiles are shown in a and b in all cases. Superimposed upon the NRS controls are the fluorescence intensity profiles of cells treated with anti-normal human antiserum (positive control) shown in a and the fluorescence intensity profiles of the same cells treated with anti-AML antiserum, shown in b.

1a



1b



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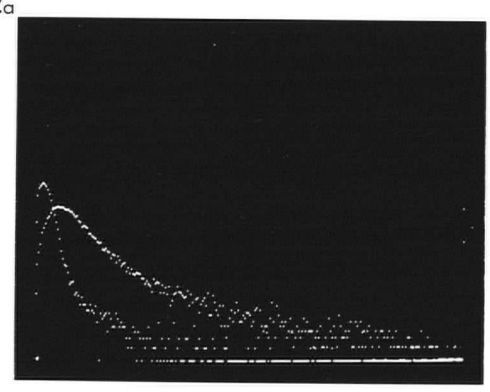

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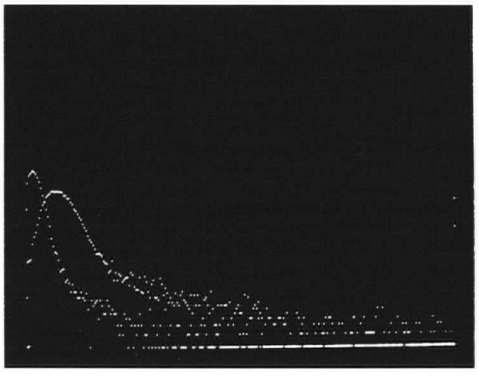

Table 2. FACS analyses of peripheral blood leucocyte cells from untreated patients with acute myelogenous leukemia

	Diagnosis	% blasts	Number of cells fluorescing with various treatments*			
			Anti-normal human	NRS	Anti-AML	% positive cells
1.	AML-M2	55	23,368	1,641	22,275	91.9
2.	AML-M2	40	24,895	38	22,685	91.1
3.	AML-M2	26	21,052	5,687	20,154	94.2
4.	AML-M2	77	20,900	4,840	8,252	21.2
5.	AML-M2	45	21,283	1,213	17,680	82.0
6.	APML-M3	93	23,120	280	21,021	90.8
7.	AMML-M4	60	20,705	2,790	20,480	98.7
8.	AMML-M4	23	18,796	4,306	10,476	42.6
9.	AMML-M4	50	24,541	937	23,803	96.8
10.	AML-M5	60	18,806	914	22,608	>100
	AML		-	-	-	80.93± 26.8**
11.	HL-60*** APML-M3 cell line		21,304	1,764	17,844	82.3

* A total of 25,000 cells were analysed in each case.

** Averaged results ± standard deviation of 10 PBL samples.

*** Acute promyelocytic leukemia cell line.

cell population had greater fluorescence intensity with the anti-AML-serum (>100) than the anti-normal human serum, however sample 7, with a blast cell population of 75% showed only 50% relative fluorescence. These results indicate that the antigen is not only on blast cells but is also expressed on other cell populations.

It is also interesting to note that the human cell line HL-60, described as promyelocytic leukemia line, also fluoresces with the anti-AML-serum (Table 2, sample 11).

A similar series of analyses were carried out on patients who were in clinical remission from acute myelogenous leukemia after chemotherapy. The results are shown in Tables 3 and 4. It can be seen that in both bone marrow and PBL, there are still a significant number of cells showing positive fluorescence with the anti-AML-serum, even though the clinical state in each case is clearly one of remission. The variation between cases is somewhat greater than that seen in clinical disease. Whether or not this is related to prognosis is uncertain at this time. This data indicates that, in remission patients, normally differentiating cells are expressing this cell surface antigen (malignancy marker).

One long-term remission patient, #4 in Table 3 with a remission period of 3-1/2 years at the time of testing, does show a decrease from the average % positive anti-AML cells (38.4% as opposed to 73.6% average). However, very few long-term remission patients have been available for testing to date. It is clear however, that the AML patient

Table 3. FACS analyses of bone marrow cells from patients in clinical remission from acute myelogenous leukemia after chemotherapy

Diagnosis	% blasts	Number of cells fluorescing with various treatments*			% positive cells
		Anti-normal human	NRS	Anti-AML	
1. AML-M1	3	16,176	1,573	7,325	39.4
2. AML-M2	2	17,852	480	13,362	72.2
3. AML-M2	1	24,106	1.197	6,067	21.3
4. AML-M2	0	17,940	1,338	7,716	38.4
5. AML-M2	1	14,405	935	15,254	>100
6. AML-M2	occasional	21,001	4,923	20,491	96.8
7. AML-M2	1	22,400	1,775	19,548	86.2
8. APML-M3	1	12,546	1,970	15,308	>100
9. APML-M3	3	11,845	2,160	12,141	>100
10. AMML-M4	1	16,934	2,087	10,426	56.0
11. AMML-M4	0	18,426	5,541	15,308	75.8
12. AMML-M4	3	23,832	1,547	13,335	49.5
13. AML-M5	4	24,469	2,041	23,401	95.2
14. AML-M5	2	14,268	290	17,975	>100
AML		-	-	-	73.6± 27.7**

* A total of 25,000 cells were analysed in each case.

** Averaged results ± standard deviation of 14 bone marrow samples from remission patients.

Table 4. FACS analyses of peripheral blood leucocyte cells from patients in clinical remission from acute myelogenous leukemia after chemotherapy.

	Diagnosis	% blasts	Number of cells fluorescing with various treatments*			
			Anti-normal human	NRS	Anti-AML	% positive cells
1.	AML-M2	0	19,980	2,437	12,060	54.8
2.	AML-M2	occasional	21,283	1,213	17,680	82.0
3.	AML-M2	0	14,967	1,080	5,192	29.6
4.	APML-M3	0	20,347	3,151	20,956	>100
5.	AMML-M4	occasional	23,827	2,277	11,775	44.1
6.	AML-not FAB classified	0	22,028	1,783	11,364	47.3
	AML		-	-	-	59.63± 26.2**

* A total of 25,000 cells were analysed in each case.

** Averaged results ± standard deviation of 6 PBL samples from remission patients.

presents the "malignant" antigen both during the acute phase and remission period. I have had the opportunity to test one AML patient over an extended period of time, results shown in Table 5. During the acute phase (10% blasts) he had 45.5% positive anti-AML cells, 4 months later now being in remission (occasional blasts in B.M.) he had 15.2% positive cells, 2 months following this, while still in remission (no blasts in B.M.) he presented 23.8% positive cells and 6 months later when in relapse (with 9% blasts in B.M.) a 66.2% positive cell population was noted. It is clear that many more patients (as they become available) should be tested through the phases of the disease; however this result seems promising in terms of its potential usefulness in predicting imminent relapse.

The observation that some PBL samples which are enriched for mononuclear cells, also showed a high number of fluorescing cells suggested the possibility that lymphoid, as well as myeloid cells, might be expressing this antigen. Since the involvement of lymphoid cells has never been implicated in human acute myelogenous leukemia, Dr. Patricia Logan and myself wished to establish the gross morphology of those cells showing the highest relative positive fluorescence with the anti-AML, and to determine whether this population did, in fact, include lymphocytes. The FACS IV was used to collect and sort PBL from a remission patient whose cells had been analysed as significantly positive, with the anti-AML-serum. Cytology was performed on that 25% of the cell sample showing the lowest positive relative fluorescence and on that 25% of the same sample showing the highest positive relative fluorescence.

Table 5. FACS analyses of bone marrow cells from an acute myelomonocytic leukemia patient with active disease, while in remission, and during relapse.

	Diagnosis	% blasts	Number of cells fluorescing with various treatments*			
			Anti-normal human	NRS	Anti-AML	% positive cells
1.	AMML-M4 (active disease)	10	24,862	3,112	13,011	45.5
2.	AMML-M4 (in remission)	occasional	19,055	1,371	4,056	15.2
3.	AMML-M4 (in remission)	0	22,734	3,255	7,884	23.8
4.	AMML-M4 (in relapse)	9	19,099	2,880	13,622	66.2

* A total of 25,000 cells were analysed in each case.

Similar sorts were carried out with the positive control antiserum (anti-normal-human). The results shown in Table 6, were obtained using PBL from the remission patient, which had been subjected to a one-step Ficoll-Hypaque separation (mononuclear enriched). As can be seen, a majority of the cells showing the highest level of fluorescence were lymphoid. These results indicate that in remission patients, cells of both lymphoid and myeloid lineage are expressing this antigen.

When bone marrow aspirates or PBL of patients in relapse with acute myelogenous leukemia were examined with the anti-AML-serum, it was again seen that a majority of the cells showed positive fluorescence, and again, the % positive cells did not correlate with the number of blasts present, indicating that this antigenic marker is present on cells other than blasts. The results are shown in Table 7.

A number of bone marrow and peripheral blood samples from patients with CML were also analysed. The results are shown in Tables 8 and 9. It can be seen that in this condition also, a high percentage of the cells analysed show positive fluorescence. These results were somewhat surprising because all these patients were being successfully treated at the time the bone marrow aspirates were taken. The possible implications of these data and the observation that the numbers of blast cells in the marrows of AML patients did not correlate with the numbers of cells fluorescing is included in the discussion.

Studies were carried out on bone marrow cells taken from patients with disorders other than AML (shown in Table 10). Representative fluorescence profiles of cells from patients with acute lymphocytic

Table 6. Microscopic analysis of cells from a remission patient with APML after labelling with either anti-normal human or anti-AML and after analysis and sorting on the FACS IV

Test performed	Test antiserum used: Anti-normal human		Anti-AML	
FACS IV analyses (number of cells fluorescing)	20,347		20,956 (100%)	
FACS IV sort	% cells in high relative* fluorescence population	% cells in low relative** fluorescence population	% cells in high relative* fluorescence population	% cells in low relative** fluorescence population
neutrophils	2	occ***	3	-
lymphocytes	95	98	92	97
monocytes	3	2	5	3
red blood cells	-	occ.	-	-
eosinophils	-	-	occ.	-

* Those cells sorted as the 25% of the population having the highest relative fluorescence.

** Those cells sorted as the 25% of the population having the lowest relative fluorescence.

*** Occasional.

Table 7. FACS analyses of cells from relapsed patients with acute myelogenous leukemia following chemotherapy.

Diagnosis	Cell population	% blasts	Number of cells fluorescing with various treatments*			% positive cell
			Anti-normal	NRS	Anti-AML	
AML-M2	bone marrow	13	19,167	5,903	15,753	74.3
	PBL	13	21,726	6,353	21,461	98.2
AML-M2	bone marrow	67	18,576	5,008	12,992	58.8
	PBL	34	23,276	775	17,218	73.0
AML-M2	bone marrow	40	24,826	2,834	22,298	88.5
AML-M2	bone marrow	91	24,469	2,041	23,401	95.2
AMML-M4	PBL	60	13,604	4,481	14,278	>100
AMML-M4	PBL	49	22,435	2,338	14,609	61.0
AMML-M4	PBL	9	18,988	1,753	20,710	>100

* A total of 25,000 cells were analysed in each case.

Table 8. FACS analyses of bone marrow cells from patients in the chronic phase of chronic myelogenous leukemia.

	Diagnosis	Number of cells fluorescing with various treatments*			
		Anti-normal human	NRS	Anti-AML	% positive cells
1.	CML	24,856	75	21,716	87.3
2.	CML	23,703	341	15,607	65.2
3.	CML	22,810	801	23,576	>100
4.	CML	22,128	358	17,861	79.6
5.	CML	23,411	900	24,601	>100
6.	CML	18,189	911	12,583	67.6
7.	CML	22,986	1,699	15,826	66.4
8.	CML	24,583	1,375	21,103	85.0
9.	CML	24,055	351	7,598	30.6
	CML	-	-	-	75.7± 21.6**

* A total of 25,000 cells were analysed in each case.

** Averaged results ± standard deviation of 9 bone marrow samples.

Table 9. FACS analyses of peripheral blood leucocyte cells from patients in the chronic phase of chronic myelogenous leukemia.

Diagnosis		Number of cells fluorescing with various treatments*			
		Anti-normal human	NRS	Anti-AML	% positive cells
1.	CML	22,418	2,005	13,607	57.0
2.	CML	22,986	1,699	15,826	66.3
3.	CML	14,421	196	7,156	41.9
4.	CML	22,586	1,375	21,103	85.0
5.	CML	20,610	575	3,693	17.9
6.	CML	21,020	1,480	17,361	81.3
7.	CML	24,493	330	16,851	70.1
8.	CML	22,904	923	10,432	43.3
9.	CML	21,797	671	6,743	28.7
	CML	-	-	-	54.6± 23.3**

* A total of 25,000 cells were analysed in each case.

** Averaged results ± standard deviation of 9 peripheral blood leucocyte samples.

Table 10. FACS analyses of bone marrow cells from patients with disorders other than AML

Diagnosis	Condition of Bone Marrow	Number of cells fluorescing with various antisera			
		Anti-normal human	NRS	Anti-AML	% positive cells
1. ALL	remission patient - normal marrow	23,657[1a]	348	456[1b]	0.3
2. ALL	acute disease - 82% blast cells	21,575	236	243	0.03
3. ALL	occasional blast cells	24,179	728	430	<0
4. ALL	acute disease - 92% blast cells	24,231	745	427	<0
5. ALL	congenital - 8% blast cells	24,882	2,118	3,535	6.2
6. CLL	lymphocytosis	23,516	687	753	0.32
7. CLL	lymphocytosis, pleomorphic population consistent with CLL	24,165	347	457	0.46
8. CLL	lymphocytosis, abnormal cells	23,472	3,242	3,112	<0
9. Lymphoma	6% blasts	23,519	5,168	4,070	<0
10. lymphoma	2% blast cells, no evidence of major infiltration	23,911[2a]	199	309[2b]	0.5
11. Lymphoma	no infiltration, normal marrow	21,088	1,453	773	<0
12. Hodgkins Lymphoma	no infiltration, normal marrow	21,897	2,431	2,377	<0
13. Hodgkins Lymphoma	no infiltration, normal marrow	24,572[3a]	3,463	4,257[3b]	3.8
14. Myeloma	no infiltration, normal marrow	20,071	2,331	2,173	<0
15. Idiopathic thrombocytopenic purpura	no blast	24,093	1,818	2,395	2.6

* A total of 25,000 cells were analysed in each test.

1a, 1b, 2a, 2b, 3a, 3b: See fluorescence intensity profiles in Fig. 6.

leukemia (ALL), lymphoma, and Hodgkins disease are shown in Figure 6. The representative samples shown in this figure are designated in Table 10. It is clear from these experiments that the anti-AML did not bind significantly to the marrow cells of any of these patients at measurable levels above background obtained with NRS. It appears that the anti-AML is recognizing a cell surface antigen common to AML and CML patients' cells, which is not present in detectable amounts on bone marrow cells of patients with other disorders.

Further studies with PBL from patients with leukemias of non-myelogenous origin are shown in Table 11. As seen with the bone marrow samples, the anti-AML-serum did not bind to these cells.

To determine whether the antiserum was detecting markers on mature cells, a series of normal individuals' PBL were analysed on the FACS IV with anti-AML-serum. None have shown any reactivity above background (Table 12). A number of normal bone marrow samples have also been tested. These were obtained from potential bone marrow donors or from patients with suspected P. vera who were subsequently shown to have normal marrows. These results are also presented in Table 12, in which it can be seen that these materials also showed no significant reactivity with the anti-AML. Also, Ficoll-Hypaque-enriched populations of either lymphocyte-monocyte cells or granulocytes from normal individuals were examined. A representative result is shown in Table 13 and confirms that the anti-AML-serum has no significant reactivity with these cell populations. The fluorescence intensity profiles of these samples are shown in Figure 7.

Figure 6. Fluorescence intensity profiles of bone marrow cells from patients with disorders other than AML.

Conditions are identical to those described for Figure 5. a. Profiles of cells treated with either NRS or anti-normal human serum; b. profiles of cells treated with either NRS or anti-AML serum. Sample 1 represents cells taken from a patient with ALL, Sample 2, cells from a lymphoma patient and Sample 3 from a patient with Hodgkins.

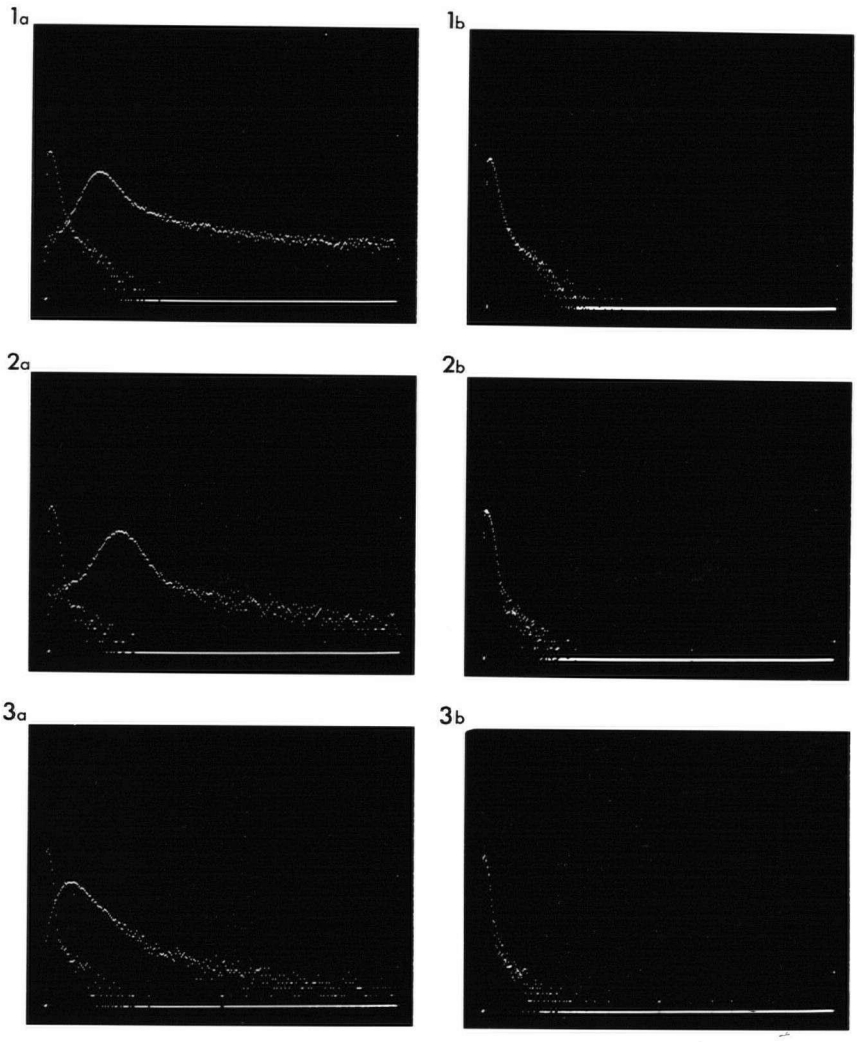
1a
1b
2a
2b
3a
3b

Table 11. FACS analyses of peripheral blood leucocyte cells from individuals with leukemias of non-myelogenous origin.

Diagnosis	Number of cells fluorescing with various treatments*			
	Anti-normal human	NRS	Anti-AML	% positive cells
1. ALL-remission	23,733	1,413	1,538	0.5
2. ALL-remission	21,197	958	1,227	1.3
3. ALL-remission	24,416	327	593	0.1
4. ALL 27% blasts	15,627	1,108	2,178	7.4
5. ALL-remission (same patient when in remission)	22,310	1,157	1,140	<0
6. Idiopathic thrombocytopenic purpura	19,788	597	370	<0
7. CLL	18,608	1,421	983	<0

* A total of 25,000 cells were analysed in each case.

Table 12. FACS analyses of cells from normal individuals

Cell population	Number of cells fluorescing with various treatments*			
	Anti-normal human	NRS	Anti-AML	% positive cells
1. bone marrow	24,650	277	415	0.5
2. bone marrow	16,105	91	101	0.06
3. bone marrow	22,898	143	420	1.2
4. bone marrow	14,082	2,463	2,023	<0
5. PBL**	24,604	263	712	1.8
6. PBL	24,783	519	272	<0
7. PBL	24,827	83	282	0.8
8. PBL	22,147	866	776	<0
9. PBL	19,870	354	196	<0
10. PBL	23,191	2,076	1,151	<0
11. PBL	22,541	1,768	1,675	<0
12. PBL	24,170	1,820	1,463	<0
13. PBL	24,397	2,930	1,153	<0

* A total of 25,000 cells were analysed in each case.

** PBL = peripheral blood leucocyte cells.

Table 13. FACS analyses of lymphocyte-monocyte and granulocyte-enriched population from a normal individual.

	Cell population	Number of cells fluorescing with various treatments*			% positive cells
		Anti-normal human	NRS	Anti-AML	
1.	Lymphocyte-monocyte enriched	24,455[1a]	852	1,191[1b]	1.4
2.	Granulocyte enriched	20,748[2a]	798	1,553[2b]	3.8

* A total of 25,000 cells were analysed in each case.

1a, 1b, 2a, 2b: See fluorescence intensity profiles in Fig. 7.

Figure 7. Fluorescence intensity profiles of Ficoll-Hypaque-enriched populations of either lymphocyte-monocyte cells or granulocytes from a normal individual.

Conditions are identical to those described for Figure 5. a. Profiles of cells treated with either NRS or anti-normal human serum; b, profiles of cells treated with either NRS or anti-AML-serum. Sample 1 represents the lymphocyte-monocyte enriched population, Sample 2 represents the granulocyte enriched population.

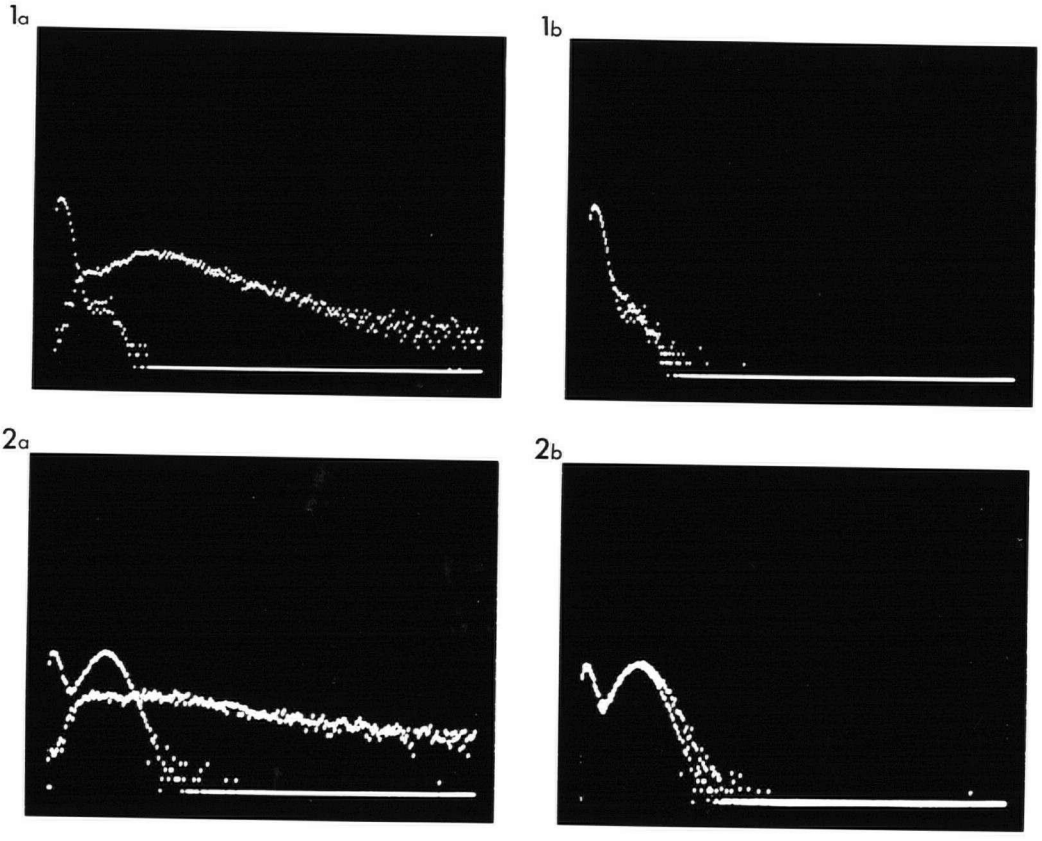
1a
1b
2a
2b

Table 14 shows the results of analyses of a bone marrow aspirate and peripheral blood leucocytes from an APML (acute promyelocytic leukemia) patient and the peripheral blood leucocytes from her normal identical twin. The APML bone marrow aspirate, peripheral blood leuocyte sample, and the Ficoll-Hypaque-enriched populations of either mononuclear of granulocytic cells (samples 1,2,3 and 4) all showed a high percentage of cells reacting positively with the anti-AML-serum. Hence, both bone marrow and the peripheral blood cells from this APML patient have the AML malignancy marker. It is clear, however, that the anti-AML did not bind to the peripheral blood leucocytes, nor to the enriched mononuclear or granulocyte populations (samples 5, 6 and 7 respectively) of the normal identical twin. Representative fluorescence profiles of peripheral blood leucocytes from the APML patient (sample #2) and from the normal identical twin (sample #5) are shown in Figure 8. This test demonstrates that the normal identical twin sister did not have the AML antigen and hence would have been an ideal bone marrow donor for the APML sibling (58 yr, ♀), age being the limiting factor. This normal individual also represented an ideal control.

The results of fluorescent testing of cells from two patients who received allogeneic bone marrow transplants from tissue matched sibling donors as a treatment for AML are shown in Table 15. Donor cells had not been treated in any way to reduce the mature T cell population. Cells from patient 1 were taken 20 months after transplantation and the patient was doing well with no disease recurrence. The cells from this patient did not show significant fluorescence with the test anti-AML-serum. The

Table 14. FACS analyses of cells from a patient with APML and her identical twin

Diagnosis		% blasts	Number of cells fluorescing with various treatments*			% positive cells
			Anti-normal human	NRC	Anti-AML	
APML-M3	1 bone marrow	7.0	23,610	3,136	22,052	92.3
	2 peripheral blood	7.0	21,807[1a]	2,842	14,796[1b]	63.0
	3 peripheral blood (mononuclear enriched-96% pure)	N.D.	23,578	5,260	11,415	33.6
	4 peripheral blood (granulocyte enriched-98% pure)	N.D.	23,385	1,030	16,573	69.5
Normal (Identical twin)						
	5 peripheral blood	0	20,774[2a]	667	524[2b]	<0
	6 peripheral bllod (mononuclear enriched-96% pure)	0	24,455	852	1,191	1.4
	7 peripheral blood (granulocyte-enriched-98% pure)	0	20,748	798	1,553	3.6

* A total of 25,000 cells were analysed in each case.

1a, 1b, 2a, 2b: See fluorescence intensity profiles in Fig. 8.

Figure 8. Fluorescence intensity profiles of peripheral blood leucocytes from a patient with APML (1) and her identical twin (2).
Vertical axis, relative number of cells in log scale. Horizontal axis, relative fluorescence intensity. Nonspecific fluorescence is demonstrated by the cells treated with NRS and these profiles are shown in a and b in all cases. Superimposed upon the NRS controls are the fluorescence intensity profiles of cells treated with anti-normal human serum (positive control) shown in a and the fluorescence intensity profiles of the cells treated with anti-AML-serum, shown in b..

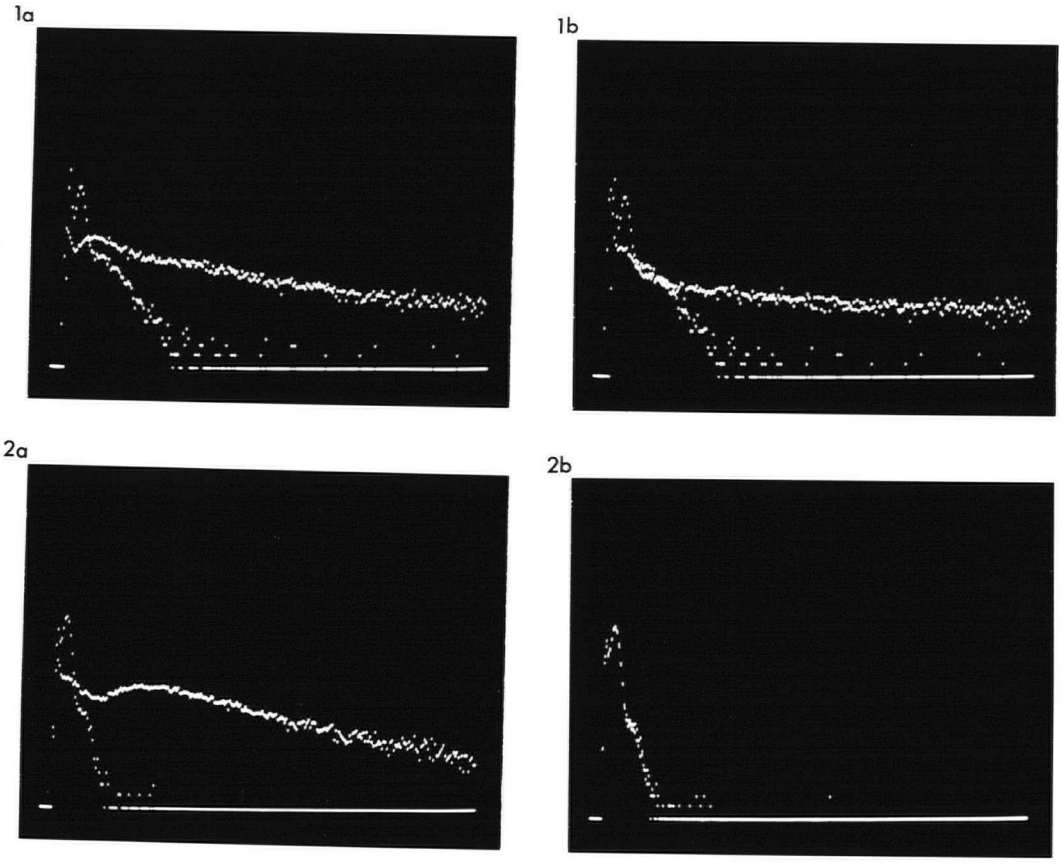
1a
1b
2a
2b

Table 15. FACS analyses of either bone marrow cells or PBL from patients with AML following bone marrow transplantation.

	Diagnosis	Time after transplant	Condition	Number of cells fluorescing with various treatments*			% positive cells
				Anti-normal	NRS	Anti-AML	
1.	AML bone marrow	20 months	remission	13,177	208	634	3.2
2.	AML-M5 PBL	2 weeks	remission (no blast cells)	23,761	2,128	23,759	99.9
	PBL	4 months	relapse 23% blasts	18,828	4,695	11,496	48.1
	bone marrow	4 months	relapse 23% blasts	20,736	2,439	14,618	66.5
3	Normal donor sibling of patient #2 (bone marrow)	-	normal	14,082	2,463	2,023	<0

* A total of 25,000 cells were analysed in each case.

cells from patient 2 were followed from shortly after transplantation. At no time (either 2 weeks after transplantation, when the patient was in clinical remission or 4 months later, when the patient was in relapse) did cells show negative reactivity with the anti-AML-serum. The donor (sample 3), on the other hand, had no measurable reactive cells in his bone marrow.

By and large, the anti-AML-serum under test here has shown absolute specificity with regard to reactivity with cells of patients with myelogenous leukemia. In all specimens tested, only 3 cases have not shown the expected reactions. These are shown in Table 16. Patient 1 was diagnosed as having ALL. At the time the bone marrow aspirate was tested, the patient was in clinical remission. However, a high percentage of the patients' cells fluoresced strongly with the anti-AML-serum. We have no explanation for this observation. Patient 2 was diagnosed as ALL. However, the clinical reports show that while bone marrow colony growth suggested ALL, peripheral blood growth was suggestive of some type of granulocytic disease. It would appear that this patient's diagnosis may be somewhat questionable. Patient 3 is a juvenile (10 year old) who had been diagnosed as having AML (see discussion).

Table 16. FACS analyses or cells from three patients whose cells yielded anomalous results with regard to their diagnosis.

Diagnosis	% blasts	Cell population	Number of cells fluorescing with various treatments*			% positive cells
			Anti-normal human	NRC	Anti-AML	
1. ALL**	remission (no blasts)	bone marrow	23,207	1,992	13,777	55.6
2. ALL***	92	bone marrow	20,505	1,683	17,142	82.2
	41	PBL	24,375	301	18,606	76.3
3. AML-M2****	42	bone marrow	18,244	4,495	4,718	1.6
	42	PBL	19,628	6,911	6,311	<0

* A total of 25,000 cells were analysed in each case.

** This patient has been diagnosed as a typical ALL in remission.

*** This patient record shows that although bone marrow growth suggests ALL, peripheral blood cell colony growth better fits some type of granulocytic disease.

**** This patient is a 10 year old juvenile AML case.

Discussion

There are a number of questions which are raised by the results reported here. It has been shown that an antiserum raised in rabbits to an antigen purified on preparative polyacrylamide gels from membrane extracts from cells of AML patients reacts strongly in FACS IV analysis with a cell surface antigen found on 39 of 40 samples taken from patients diagnosed as having acute myelogenous leukemias. Cells taken from individuals with no known disorders, did not react with this antiserum. It would thus appear that the isolated antigen represents what could be termed a tumor associated antigen (TAA) or malignancy marker in that it does not appear to be present, at detectable levels, on normal cells. This is in agreement with previous findings in this laboratory, and as reported in Chapter I, that the antigen could not be detected in extracts from normal cell membranes when tested with the antiserum in the ELISA (Al-Rammahy et al., 1980). Whether or not the antigen is present on a small population of normal bone marrow cells is the subject of ongoing studies in the laboratory.

It was also reported here that this AML-associated antigen is also detectable on the cells of patients with CML. Of 15 samples taken from patients with CML, all samples showed significant numbers of positively fluorescing cells (at least 20% positive) in both bone marrow and PBL materials. These results indicte that a common TAA is present on the cells of patients diagnosed as having either AML or CML. These results

may imply a common event in the onset of both of these conditions. This has not been suggested in previous studies, even though both conditions involve proliferative disorders of nyeloid cells.

It has been a consistent observation in these studies that the numbers of cells reacting with the anti-AML-serum in either bone marrow aspirates or PBL in AML patients do not correlate with the number of blast cells present in the sample. This would imply that the antigen is present on cells other than blasts. This is explicitly demonstrated by the observation that patients in clinical remission from AML (Tables 3 and 4) showed a high percentage of positively fluorescing cells. These findings are in agreement with those of Metzgar and Mohanakumar (1978) who found that simian anti-AML antisera reacted positively with 40% of cells from a patient in remission from AMML.

In patients with active disease, these findings indicate that this marker (antigen) may be present on a pluripotent stem cell. This finding also supports the possibility that there may be clonal dominance of a potentially malignant stem cell, probably pluripotential, in the bone marrow and peripheral blood of AML patients (Wiggans et al., 1978; Fialkow et al., 1979). The observation, in remission patients, that lymphoid as well as myeloid cells may be expressing this antigen, suggests that: a pluripotent stem cell is expressing the antigen, and cells expressing this antigen can differentiate, albeit transiently, in normal fashion. Indeed, Fialkow (1982), in his isoenzyme studies with glucose-6-phosphate dehydrogenase, demonstrated a single-enzyme G6PD phenotype in leukemic blast cells, suggestive of a clonal disorder. When studying remission

patients, however, there was a return of a normal double-enzyme phenotype in granulocytic cells. This observation suggests that normal stem cells were present, but not expressed during the acute phase of the leukemia. At first, one might conclude that Fialkow's findings contradict the AML remission date reported here. However, it may be that the malignancy marker in remission patients exists on potentially malignant and normal cells. At this time, however, it would be premature to conclude clonal dominance in AML. It is essential, initially, that a large number of remission patients be studied, to determine if predominance of this marker on normally differentiating cells is an indicator of the length of remission. At this stage, long term remission patients would be of great interest, but such individuals are rare, and it has been difficult to accumulate enough data to evaluate the prognostic value of this marker.

The question could be raised as to whether the antigen, detected on lymphocytes of remission patients, is actually synthesized by these cells or whether it is adsorbed from other sources. At this time, there is no definite proof as to the origin of the antigen on lymphocytes. Because the patients are in clinical remission it is unlikely that the amounts of antigen observed in these cells are derived from a few occult malignant cells. However, the possibility that the antigen is derived from a normally differentiating myelocytic population cannot be ruled out. The results of extensive FACS IV sorting studies on peripheral blood and bone marrow cells from patients with myeloproliferative disorders is one of the subjects of Dr. Logan's doctoral programme.

In 39 of 40 diagnosed cases of AML, the anti-AML used here detected antigen on the surface of a significant number of either bone marrow cells or PBL of these patients. The one case of diagnosed AML whose cells did not react with the antiserum, was a 10 year old juvenile patient. There is no explanation for this, however, it may be that a small number of AML patients do not express antigen on their bone marrow cells, or it may be that AML in children involves a series of cellular changes distinct from the adult disease. It is hoped that subsequent studies, as patient samples become available, will clarify these possibilities. Fialkow (1979, 1982) has shown differences in elderly and juvenile AML patients. Single-enzyme phenotypes were found in blast cells, erythrocytes and platelets of elderly patients, which is suggestive of clonal dominance. With the juvenile patients, the single-enzyme was found only in cells with restricted differentiative ability. These findings may reflect age-related variations in clinical features, or that different oncogenic events may be involved.

It was also found that cells from two patients diagnosed as ALL had cells (either bone marrow or PBL) which reacted strongly with the antiserum. One patient, who had a significant number of blast cells in both bone marrow and PBL had a disease picture which was somewhat ambiguous in that bone marrow cultures indicated ALL, whereas PBL cultures indicated a myeloproliferative disorder. The second patient was diagnosed as ALL but was in clinical remission at the time that cells were tested. At this time, it is only possible to state that the antigen is present on cells from a high percentage of patients diagnosed as AML (or other

myeloproliferative leukemias) and may be expressed on cells of a small number of patients with ALL. It has not been observed on cells of patients with other lymphoid disorders.

To date, cells from two patients who have received bone marrow transplants as treatment for AML have been examined. One patient who was doing well and was still in clinical remission after 20 months had essentially no cells in a bone marrow aspirate which reacted with the anti-AML-serum. The second patient, who was followed from 2 weeks after transplantation, at all times showed a significant number of cells reactive with the antiserum. This patient relapsed at 4 months post-transplant. It is possible that such monitoring may be of prognostic value in this form of treatment.

It has been shown that the antiserum described herein is detecting an antigen which is present on the surface of a high percentage of both bone marrow cells and PBL of most patients with AML (FAB M1 to M5) or CML, whether they are presenting with the disease, in remission or in relapse. It is not present, at detectable levels, on normal bone marrow cells or normal PBL, nor is it present, at detectable levels, on the cells of most patients with lymphoproliferative disorders. It is possible that this antigen may be expressed on a small population of normal cells of bone marrow origin. Because in FACS IV analysis, we observe variation of backgrounds ± 5% against NRS controls, we will not be able to determine if a small number of normal cells do have the antigen on their surfaces. Microscopic examination of individual cell populations using either fluorescent labelling or immunoperoxidase staining are the subject of

ongoing studies in the laboratory. Nonetheless, it may be concluded that this marker (antigen) may be of clinical significance in both prognosis and diagnosis of myelogenous leukemia.

CHAPTER III

Monoclonal Antibody (MAL-1) Specific for Myelogenous Leukemia

Introduction

Until recently, there have been relatively few reports on monoclonal antibodies reactive with membrane determinants of myeloid or myelogenous leukemia cells. In contrast, there are a large number of publications on monoclonal antibodies with reactivity for lymphocyte and lymphocytic leukemia surface antigens; i.e. common acute lymphocytic leukemia antigen (CALLA) (Greaves et al., 1980, 1981a, 1981b; Liszka et al., 1981; Navarrete et al., 1981; Newman et al., 1981; Mulder et al., 1981; Lebacq et al., 1982; Boucheix et al., 1982). Katz et al. (1981) have studied the chromosomal control of several monoclonal antibody-defined ALL cell surface antigens. Procedures have been developed for the sub-typing of ALL with monoclonals (null ALL, common ALL-cALL, pre-B-ALL, B-ALL and T-ALL) (Greaves et al., 1981; Poulik et al., 1981; Foon et al., 1982).

Non-lymphoid cells can also be characterized with monoclonal antibodies directed against cell surface differentiation antigens. Knapp et al. (1981) have a number of monoclonal antibodies directed against myeloid differetiation antigens which have varied specificities and may prove of use in the characterization and subdividing of myelocytic leukemias. Some investigators have used HL-60 cells as immunogens to produce myelocyte-specific monoclonals. One group (Peng et al., 1982) has produced a monoclonal which recognizes a differentiation antigen on early haemotopoietic cells of the myeloid and erythroid lineages (monoclonals Pro-Im 1, Pro Im 2). Another monoclonal (HL-C5) shows reactivity

restricted to the myeloid lineage (Girardet et al., 1982). This monoclonal may be of particular use for the diagnosis of AML in that it reacts strongly with M3 (hypergranular promyelocytic AML-FAB classification) less strongly with M2 (meyloblastic with maturation AML) and not at all with M1 (myeloblastic without maturation AML). Also a monoclonal which was produced by immunizing with AMML (M4) cells and demonstrates AMML specificity has been isolated by Uchánska-Ziegler et al. (1982). Reading et al. (1983) have produced human monoclonal antibodies reactive with human AML cells, but not with remission cells. They used an Epstein-Barr Virus (EBV) transformed B cell population from an AML patient in complete remission. This particular remission AML patient was previously found to have serum antibodies which were reactive with his own AML cells which had been stored at presentation, but unreactive with his own bone marrow cells stored after he reached remission. Seven cell lines were established from the EBV transformed cultures, and all continued to produce the antibodies. Only one of these antibodies reacted with the patient's remission bone marrow cells in the ELISA. Studies looking at the selective loss or diminished expression of antigens recognized by monoclonals on the surface of AML cells may also be of some diagnostic value (Navarrete et al., 1982; Rumpold et al., 1982).

It is clear that monoclonals may have significant applications in the diagnosis of AML. The difficulties in distinguishing the undifferentiated forms of AML from ALL may be eliminated. As has been stated earlier, with AML, the dominant cell type may reflect a level of maturational arrest, however this arrest is not necessarily at the level at which the malignant

transformation has occurred. Monoclonal Ab might clarify the scheme of normal myeloid and malignant differentiation. Already some interesting work has been done on the staging of myeloid differentiation using monoclonal identification markers (Andrews et al., 1982; Ball and Fanger, 1983). It may also be possible to increase the accuracy of measuring haematologic remission with monoclonals to specific markers. Indeed, the identification of leukemia cell types may lead to a new immunotherapy regime, in which the objective would be to encourage maturation of leukemia cells as has been done with the HL-60 cell line with DMSO and phorbol esters (Collins et al., 1978). Myelogenous leukemia specific monoclonals may in the future be used to treat remission bone marrow cells and hence have an even greater therapeutic value in autologous transplants (Fitchen et al., 1981). Indeed, J5 monoclonal Ab (anti-CALLA) has been used to successfully treat the bone marrow of ALL patients with no histocompatible donor. In one study, 7 of 13 patients treated this way have remained in a second remission for over 2 years (reported by Dr. J. Ritz, Sidney Farber Cancer Institute, at the UCLA Symposium, Recent Advances in Bone Marrow Transplantation, Feb., 1983). This method of purging the bone marrow has some very positive advantages over bone marrow transplantation in that patients without a histocompatible donor can be treated; it avoids clinical graft vs. host disease, and it allows for the end of chemotherapy, therefore eliminating the immunosuppression which occurs in post bone marrow transplantation.

This chapter reports on the production and characterization of a monoclonal antibody raised to band 1 (AML-Ag). Data is presented which shows the specificity of this monoclonal in an immunofluorescence test in the FACS IV, the ELISA and by polyacrylamide gel electrophoresis of the affinity purified AML antigen.

Materials and Methods

Immunization and Fusion Procedures

Balb/c mice were given an intraperitoneal (i.p.) primary immunization of 20 µg of PAGE purified (described in Chapter II) myelogenous leukemia specific Ag (AML-Ag, band 1) in 50% complete Freund's adjuvant (CFA) and then re-immunized in one month by three consecutive daily intravenous (i.v.) injections of the AML-Ag alone in PBS. The NS-1 myeloma cell line was cultured in DME medium supplemented with 0.163 M sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 µg/ml streptomycin, 5 mM hepes, and 20 % FCS in a humidified 10% CO_2 incubator at 37^{o}C. To ensure that the NS-1 cells were in log-phase growth at the time of the fusion, they were used at a density of about 10^5 cells/ml.

The spleens were removed from the primed mice the day following the last i.v. injection and fused to NS-I myeloma parental cells with a 50% polyethylene glycol solution (Serva Feinbiochemica, Heidelberg). The conditions for fusion were the same as those described by Oi and Herzenberg (1971). A cell ratio of approximately 10^8 immune spleen cells to 2 x 10^7 NS-I cells were used for the fusion. A 0.1 ml volume of the fusion products (2 x 10^5 total cells per well), plus newborn outbred mouse thymocytes (2 x 10^5 total cells per well) to act as a feeder layer, were plated in 96 - well tissue culture plates (Costar #3596). The selection procedure, to yield stable monoclonal antibody-producing hybrid cell lines was based on the well known

Littlefield's (1964) hypoxanthine-aminopterin-thymidine (HAT) selective medium. The day after the fusion 0.1 ml of 2X HAT selective medium was added to each well. Supernatants from these primary tissue cultures of fused spleen with myeloma cells were screened for antibody production using the ELISA (Kelly et al., 1979). Cells from wells in which supernatant material reacted positively with the AML-Ag (band 1) on the ELISA plate were cloned. Again outbred mouse thymocytes were used as a feeder layer. These cloned cells were again tested after 7 days and positive wells, when confirmed to be only one clone by microscopic examination, were grown up in culture in DME + 20% FCS. An aliquot (10^6 cells) of the antibody-producing culture cells were injected into pristane treated Balb/c mice. These mice produced ascites usually within 2 weeks which was subsequently collected from the peritoneal cavity of these mice. The ascites cells were either injected into pristane treated sublethally irradiated (600 rads) outbred mice (these mice routinely produced more ascites than Balb/c mice) or prepared for freezing at $-70^{o}C$ in DME containing 20% FCS and 10% DMSO. The ascites fluid collected from these mice was again screened for specificity with the ELISA and also selected for specificity in the FACS IV (methodology described in next section).

Cells and Cell Labelling

The HL-60 cell line was cultured as described in Chapter II. The acute lymphocytic leukemia cell line, CCRF-SB-ALL-B was maintained in DME plus 20% FCS, while the CCRF-SB-ALL-T cell line was grown in MEM plus 20%

FCS in a humidified 37°C incubator in 10% CO_2. Bone marrow and peripheral blood samples from patients with leukemia and other malignant diseases were purified as described in Chapter II.

For labelling, cell pellets (10^6 cells) from either bone marrow, peripheral blood, or cell line samples were incubated for 1-1/2 h on ice with 0.2 ml of ascites monoclonal antibody diluted to 1/100 in PBS. A monoclonal directed to an unrelated antigen (ferredoxin) used at the same concentration as the AML-monoclonal was employed as a negative control. The cells were subsequently washed three times in PBS and 0.2 ml of fluoresceinated goat-anti-mouse-IgG (Cappel) at a dilution of 1/20 with PBS (+ 2% FCS) was added to each cell pellet. Cells were incubated for another 1.5 h at 0°C, washed once in PBS, and then centrifuged through 100% FCS. They were finally suspended in 1.0 ml PBS and 5% FCS for FACS IV analysis (FACS IV Analysis was carried out as described in Chapter II).

Affinity Chromatography

The anti-AML monoclonal was first purified over DEAE-Sephacel and then coupled to Sepharose-4B using the methods described by Cuatrecasas (1970).

Isolation and Elution of Antigen by Affinity Chromatography

This Sepharose-4B-anti-AML monoclonal Ab column was used to isolate band 1 (AML-Ag) from high speed supernatants of AML membrane sonicate preparations. The column was first equilibrated with several washes of

borate-saline (pH 8.5). The sample (at 4 mg/ml) was loaded onto the column (7 ml column volume) and re-cycled over this column for 30 min. Borate saline was washed exhaustively over the column to remove unbound material and the bound material was then eluted with 0.1 N HCl. Eluted materials were immediately neutralized and each fraction was monitored for protein content by the Lowry technique (Lowry et al., 1951) or by measuring the absorbance at 280 nm of the 1.0 ml fractions collected. Equivalent preparations of membrane extracts from normal pooled PBL were run over the immunoadsorbent in the same manner. Individual samples from eluted fractions were tested at various concentrations in the ELISA for the detection of material reactive with the monoclonal antibody.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was performed as described in Chapter II except that SDS and 2-mercaptoethanol were used. The gel was silver stained according to the method of Wray et al. (1981).

Results

Reactivity of MAL-1 in the ELISA

Eight single-cell derived monoclonal Ab-producing clones which had reactivity with band 1 in the ELISA were isolated. Only one of these however, reacted consistently strongly and quickly (within 20 minutes) on ELISA plates coated with band 1 or on plates coated with whole membrane extracts of AML blasts. The reactivity of this monoclonal, on an AML extract and with extracts of the CCRF-SB-ALL-B or the CCRF-SB-ALL-T cell lines (the preparation procedures as described in Chapter II) is shown in Figure 9. This monoclonal (hereafter called MAL-1) showed binding to band 1 Ag and to an AML cell extract with readable titres (absorption at 405 nm) to a 10^{-6} dilution and it showed no reactivity to ALL-B or ALL-T Ag extracts even at high concentrations. It also showed no reactivity with ELISA plates coated with normal human PBL extracts and demonstrated reactivity to a HL-60 (promyelocytic) cell line extract.

Reactivity of MAL-1 in FACS IV Analyses

To test further the specificity of MAL-1 on these cell lines, FACS IV analysis experiments were carried out. The data in Table 17 shows these results, the number of cells fluorescing with the monoclonal on a HL-60 cell sample is ten fold greater than the negative control (the ferredoxin monoclonal, control MoAb). The heterologous rabbit antisera were run as controls for the experiment. The anti-AML-serum bound 43.3% of the HL-60

Figure 9. ELISA results with hybrid to myelogenous leukemia (MAL-1). Titration curves of MAL-1 monoclonal Ab to myelogenous leukemia in the ELISA. Antigen concentration was at 300 ng/ml and readings were made 45 minutes after substrate was added to the plates.

Δ——Δ reaction of MAL-1 monoclonal with an AML extract,

O——O reaction of MAL-1 to an ALL-T cell line extract,

▲——▲ reaction of MAL-1 to an ALL-B cell line extract.

The MAL-1 monoclonal also demonstrated reactivity to a HL-60 (promyelocytic) cell line extract, and no reactivity to normal human PBL extracts (results not shown).

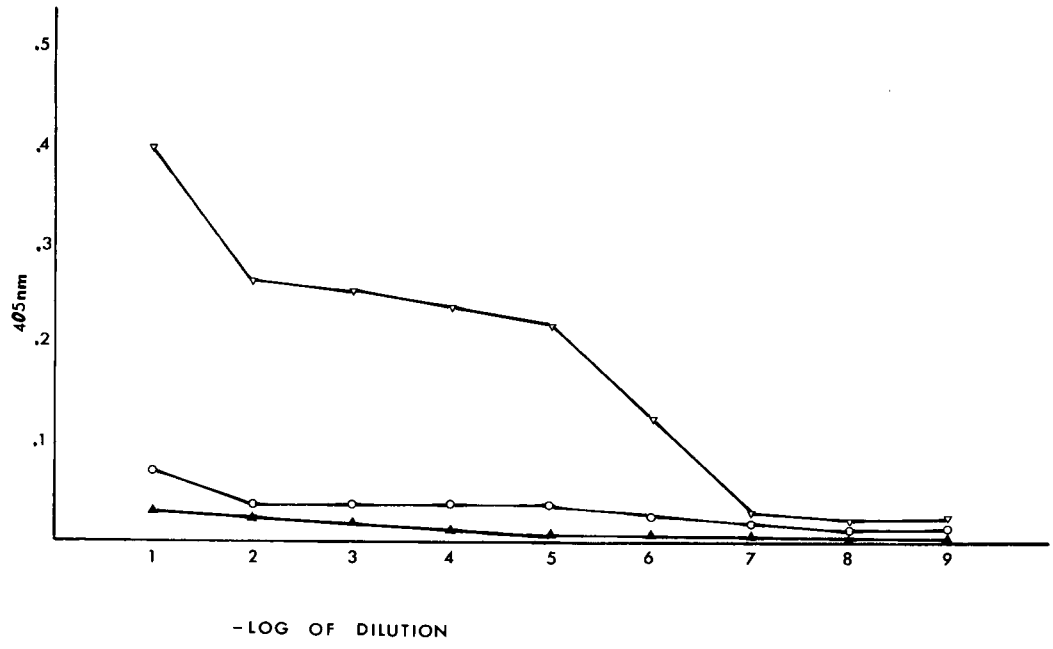
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Table 17. FACS analyses of human cell lines.

	Cell Population	Number of cells fluorescing with various treatments*				
		Anti-normal human	NRS	Anti-AML	Control MoAb**	MAL-1
1.	HL-60 (promyelocytic)	24,680	190	16,248	2,555	21,680
2.	ALL-B	24,233	561	524	3,797	3,381
3.	ALL-T	24,757	237	235	2,185	1,822

* A total of 25,000 cells were analysed in each case.

** Control irrelevant monoclonal antibody.

cells above background fluorescence. The monoclonal however, did not bind the ALL-B or ALL-T cell line. Similarly, the rabbit anti-AML-serum showed negative fluorescence with these two ALL cell lines. These FACS analysis are in agreement with the results found in the ELISA using MAL-1.

To test the specificity of MAL-1 on clinical material a series of experiments were carried out using bone marrow and peripheral blood samples. Table 18 shows the results of analysis of an AML (in active disease with 50% blasts) peripheral blood sample. With MAL-1 a considerable amount of binding above background was observed, approximately a 6 fold increase in relative fluorescence. As expected the anti-AML-serum bound these AML cells (87.4%). The bone marrow sample from this patient also exhibited high MAL-1 binding. A good negative control for the specificity of MAL-1 is the normal bone marrow from a bone marrow transplant donor (sample 3 in Table 18). A one year post-AML bone marrow transplant patient's peripheral blood sample (#4) also shows negative binding. In all cases the monoclonal showed a similar reactivity as the rabbit anti-AML-serum.

Table 19 shows the results obtained with MAL-1 on further testing of AML and some CML samples. Again, as with the rabbit anti-AML-serum, MAL-1 reacted substantially above background with these peripheral blood and bone marrow cells. It should be noted that MAL-1 reacted with both AML cells from patients in the active stage of the disease (patient samples 1 and 2) as well as with cells from AML patients in remission (samples 3 and 4). It also reacted with CML cells from chronic phase patients. The fact that MAL-1 reacted to remission cells shows that its activity does not

Table 18. FACS analyses of cells from acute myelogenous leukemia patients and from a normal individual.

Diagnosis	% blasts	Number of cells fluorescing with various treatments* Anti-normal human	NRS	Anti-AML	Control MoAb	MAL-1
1. AML-M2 (PBL sample)	50	21,183	2,122	18,791	2,684	16,860
2. AML-M2 (BM sample)	50	17,096	2,538	17,617	2,486	13,294
3. Normal BM (a BMT** donor)		23,559	1,629	1,859	1,575	1,540
4. AML - 1 year post-BMT recipient (PBL sample)	0	24,903	105	2,585	300	329

* A total of 25,000 cells were analysed in each case.

** Bone marrow transplant.

Table 19. FACS analyses of cells from acute and chronic myelogenous leukemia patients.

	Diagnosis	% blasts	Number of cells fluorescing with various treatments* Anti-normal human	NRS	Anti-AML	Control MoAb	MAL-1
1.	APML-M3-active disease (BM sample)	15	21,987	4,266	22,500	4,103	17,602
2.	APML-M3-active disease (PBL sample)	3	-	-	-	627	6,104
3.	AML-M2-in remission (BM sample)	0	-	-	-	722	5,938
4.	AML-M2- in remission (PBL sample)	0	20,124	1,174	10,567	2,006	3,611
5.	CML (BM sample)	chronic phase	-	-	-	1,788	5,875
6.	CML (PBL sample)- both granulocyte & lymphocyte populations	"	22,146	287	18,044	6,857	20,544
7.	CML (BM sample)	"	23,003	281	22,041	4,530	21,722
8.	CML (PBL sample)	"	24,703	48	18,765	534	6,934

* A total of 25,000 cells were analysed in each case.

correlate with the number of blast cells. Thus, MAL-1 shows similar binding properties to those exhibited by the heterologous anti-AML rabbit antiserum.

In Table 20 the mononuclear and granulocyte separated fractions of a CML patient (method as described in Chapter II) were labelled. MAL-1 bound both the granulocyte fraction and the mononuclear fraction. This result is also similar to that found with the rabbit anti-AML-serum. Furthermore, as previously demonstrated with the rabbit anti-AML-serum, MAL-1 also bound the granulocyte fractions more than it did the mononuclear sample. It is clear that MAL-1 reacts with other cell types (i.e. granulocytes, lymphocytes) and not only with the leukemic blast cell population. This phenomenon was also demonstrated with the rabbit anti-AML-serum, particularly with the FACS sorting study.

In Table 21, typical normal cell sample results are shown. MAL-1 exhibited no or very little binding to any of these samples. The low amount of fluorescence exhibited by some of these cells above background (i.e. sample 3) is probably due to non-specific sticking to the monocyte cell population. Also, as seen with the ALL cell lines, a bone marrow and a PBL sample from an ALL patient showed no fluorescence. The possibility still exists, however, that MAL-1 may recognize a differentiation antigen that may be expressed on only a small number of normal bone marrow cells, which the FACS IV analysis assay is not sensitive enough to recognize (see Discussion).

Table 20. FACS analyses of cells from a chronic myelogenous leukemia patient.

	Cell Population	Number of cells fluorescing with various treatments*				
		Anti-normal human	NRS	Anti-AML	Control MoAb	MAL-1
1.	Mononuclear enriched -96% pure	24,703	48	18,765	6,985	15,151
2.	Granulocyte enriched -98% pure	24,726	37	21,434	5,713	21,251

* A total of 25,000 cells were analysed in each case.

Table 21. FACS analyses from non-AML individuals.

Diagnosis	Number of cells fluorescing with various treatments*				
	Anti-normal human	NRC	Anti-AML	Control MoAb	MAL-1
1. ALL-BM** sample 99% blasts	18,775	3,017	2,210	1,670	1,543
2. ALL-PBL sample 90% blasts	18,460	2,898	2,610	3,480	3,642
3. Normal-PBL sample	22,121	629	302	2,776	993
4. Normal-PBL sample	23,820	414	235	5,833	6,225
5. Normal-PBL sample	23,442	1,615	1,166	909	1,334
6. Normal BM (for BMT)	18,847	1,303	1,487	1,260	1,232
7. Normal BM (for BMT)	23,840	276	330	586	427
8. Hodgkin's disease-BM sample	N.D.	N.D.	N.D.	1,478	6,227

* A total of 25,000 cells were analysed in each case.

** BM = bone marrow cells.

The monoclonal also showed binding to bone marrow cells of two Hodgkin's disease patients. In both cases the bone marrow reports were negative for Hodgkin's disease. The reports showed however, a marked hypercellular marrow with an increased myeloid to erythroid ratio, occasional promyeloblasts and blast cells. The fact that there were promyeloblasts and blasts present in these two cell populations may be of some significance. It is of course premature to speculate about this positive MAL-1 binding, which may indeed be a totally nonspecific phenomenon. It will be interesting to have follow-up bone marrow samples from these patients.

MAL-1 has shown less consistency in binding than the rabbit antiserum. MAL-1 showed erratic and on occasion no binding to AML cells which bound the rabbit anti-AML-serum (data not shown). The reason for this is unclear. It may be, however, that the epitope that MAL-1 is directed to, is internalized during a certain phase of the cell cycle, or it may be masked by other cell surface proteins or glycoproteins.

To investigate the possibility that cell density or growth might have some effect on the binding ability of MAL-1, HL-60 cells were labelled from cell cultures at different cell densities (i.e. actively dividing, log-phase growth, and at maximum density). Table 22 shows the results of these FACS IV analysis. It is clear from these results that the HL-60 cells expressed the epitope at all stages of cell growth.

A sandwich antibody was added to the indirect antibody technique to try to intensify and better clarify the binding of MAL-1. As a test for this technique, MAL-1 was added to HL-60 cells, then the cells were

Table 22. FACS analyses of HL-60 cells tested at different cell growth densities.

	Cell population	Number of cells fluorescing with various treatments* Anti-HLA** (tve control)	Control MoAb	MAL-1
1.	HL-60 5×10^4 cells/ml	24,332	736	15,608
2.	HL-60 5×10^5 cells/ml	22,414	1,423	14,932
3.	HL-60 1.5×10^6 cells/ml	23,009	2,001	16,101

* A total of 25,000 cells were analysed in each case.

** Anti-HLA = monoclonal antibody W6/32, against the 43,000 dalton molecular weight chains of HLA-A, B and C antigens. (Barstable, C.J., Bodmer, W.F., Brown, G., Galfre, G., Milstein, C., Williams, A.F. and Ziefler, A. (1978). Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens - New tools for genetic analysis. Cell 14:9.)

sandwiched with rabbit anti-mouse Ig (RaMIg) and finally with goat-anti-rabbit-FITC. This did successfully intensify MAL-1 binding as demonstrated in Figure 10 and Table 23. Although this particular series of experiments indicated that the cell line HL-60 expressed the antigen in a form recognized in cell suspension by MAL-1 at all stages of its growth; and that surface fluorescence could be intensified using a double sandwich, these results were not 100% reproducible. On occasion, MAL-1 would show no binding to HL-60 with or without the double sandwich technique. This inconsistency in cell surface binding by MAL-1 was also observed with some of the patient material with which it was tested. At this time, we have no definitive understanding of these observations but various possibilities will be discussed (see Discussion). It was felt that sufficient evidence was available to indicate that the epitope recognized by MAL-1 is exhibited on the surface of at least some myelogenous leukemia cells, and does not appear to be detectable on the surface of cells from normal individuals or patients with lymphoproliferative diseases.

Immunochemical Properties of MAL-1

MAL-1 was identified by immunodiffusion and a Protein A column as an IgG1. In order to investigate the nature of the binding properties of the monoclonal MAL-1, and the possibility that it might bind materials found in normal cells, an affinity column was prepared in which DEAE purified MAL-1 was conjugated to Sepharose-4B via cyanogen bromide. This immunoadsorbent column was used to absorb antigen from a membrane extract

Figure 10 Dot displays of the sandwich fluorescent technique on the HL-60 cell line.

1 (negative control) = HL-60 cells + control MoAb + rabbit anti-mouse + goat anti-rabbit - FITC.

2 (positive control) = HL-60 cells + anti-HLA + rabbit anti-mouse + goat anti-rabbit - FITC.

3 (MAL-1 indirect fluorescence) = HL-60 cells + MAL-1 + rabbit anti-mouse - FITC.

4 (MAL-1 sandwich fluorescence assay) = HL-60 cells + MAL-1 + rabbit anti-mouse + goat anti-rabbit - FITC.

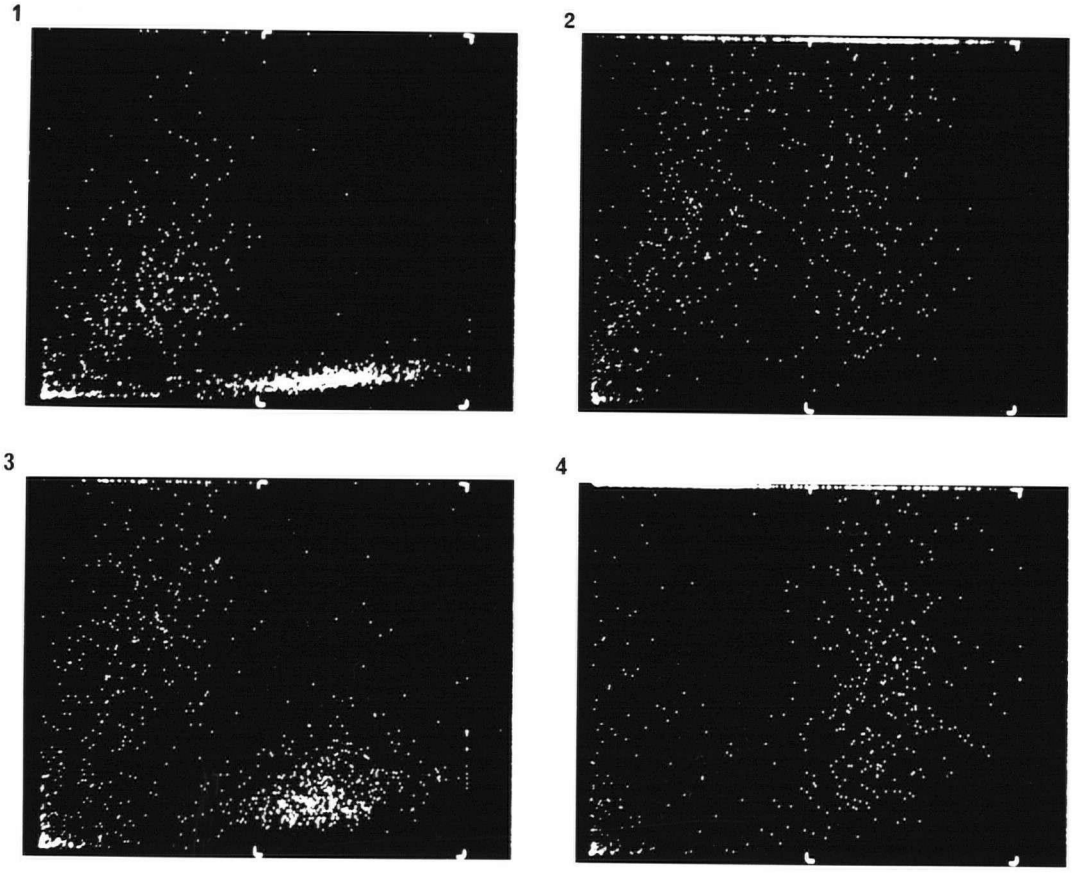

1
2
3
4

Table 23. FACS analyses of the sandwich fluorescent technique on the HL-60 cell line.

	Cell population	Number of cells fluorescing with various treatments* Anti-HLA (tve control)	Control MoAb	MAL-1
1.	HL-60 cells indirect assay	24,720	733	17,028
2.	HL-60 cells sandwich assay	23,078	2,188	24,893

* A total of 25,000 cells were analysed in each case.

prepared from blast cells of an AML patient. Absorbed material was eluted with 0.1 N HCl in 1.0 ml fractions. Each fraction was neutralized and an aliquot run on reducing PAGE. The results, shown in Figure 11, demonstrate that the major component eluting from the MAL-1 column present in the tubes is indistinguishable from the band 1 AML antigen to which the hybridoma was raised. No material was eluted or observed on the gel when an equivalent preparation of normal human PBL sonicate was run over the column.

In order to further determine whether the MAL-1 immunoadsorbent column would react specifically with material prepared from normal cell membranes, another experiment was run. In this case, an immunoadsorbent column, containing 7.0 ml of MAL-1-Sepharose was used to absorb 2.0 ml of AML-cell membrane extract at 7.5 mg per ml. Absorbed material was eluted in 0.1 N HCl in 1.0 ml aliquots and neutralized immediately. Over the same column, an equivalent preparation of normal cell membranes was absorbed. Eluted fractions were analysed for protein content and each fraction tested in the ELISA at 500 ng per ml with a standard dilution of 1:5000 of purified MAL-1. The results (Figure 12) show that the antigen with which MAL-1 reacts, while being present in the AML extracts is not detectable by this procedure in normal cell extracts.

Further absorptions were carried out using membrane extracts from cells of patients with CML, ALL or lymphnode cells from a patient with a lymphoma. Only material eluting from absorptions of CML membrane extracts gave positive reactive material either in the ELISA or on PAGE (Figure 13).

Figure 11. A sepharose-4B-MAL-1 column used to isolate band 1 (AML-Ag).

High speed centrifuged supernatants of AML and normal human PBL membrane sonicate preparations were run over the column. The bound material was eluted with 0.1 N HCl.

Electrophoresis was performed according to Laemmli et al., 1970.

Lane A : low molecular weight standards.

Lane B : purified AML antigen, band 1.

Lane C : fractions 2-5 of AML membrane extracts eluted from the MAL-1 column

Lane D : equivalent fractions eluted from the MAL-1 column after normal PBL extract had been passed over it.

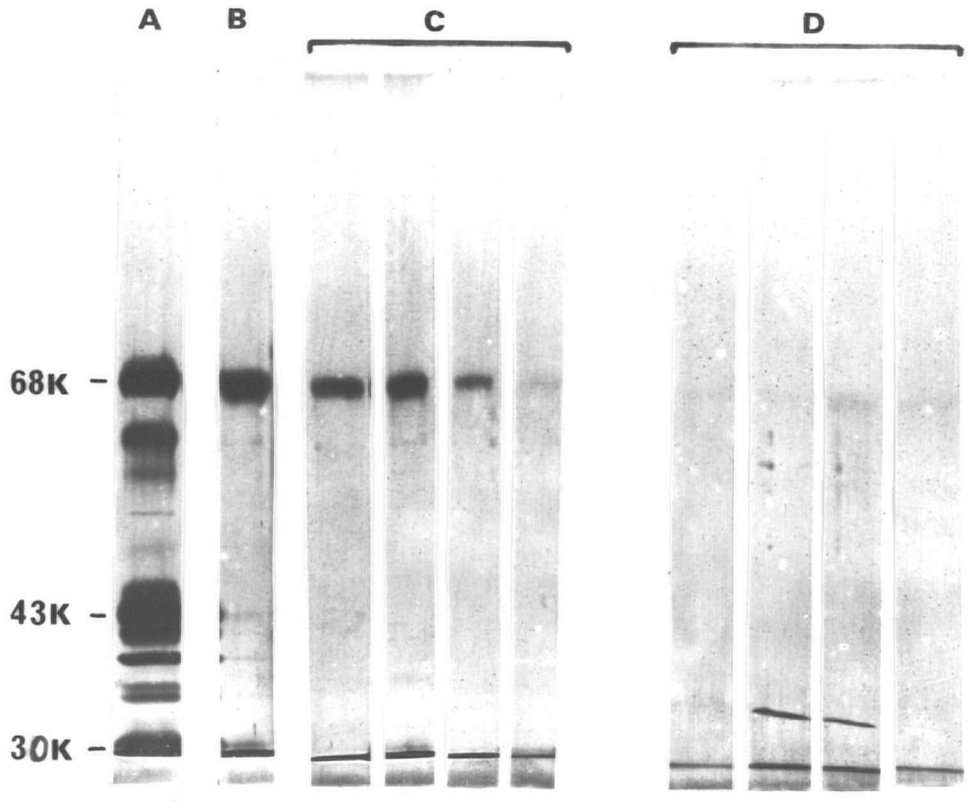

A
B
C
D
68K
43K
30K

Figure 12. ELISA results of an affinity purified AML antigen.

●——● reaction of MAL-1 monoclonal with antigen eluted from the Sepharose-4B column when an AML membrane sonicate was run over the column.

X——X reaction of MAL-1 monoclonal with material eluted from the Sepharose-4B column when a normal human extract was run over it.

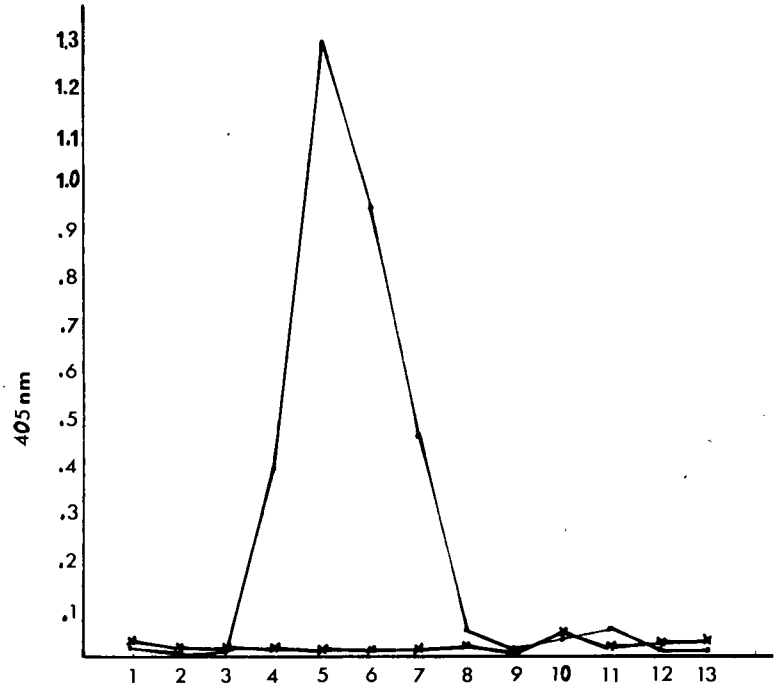
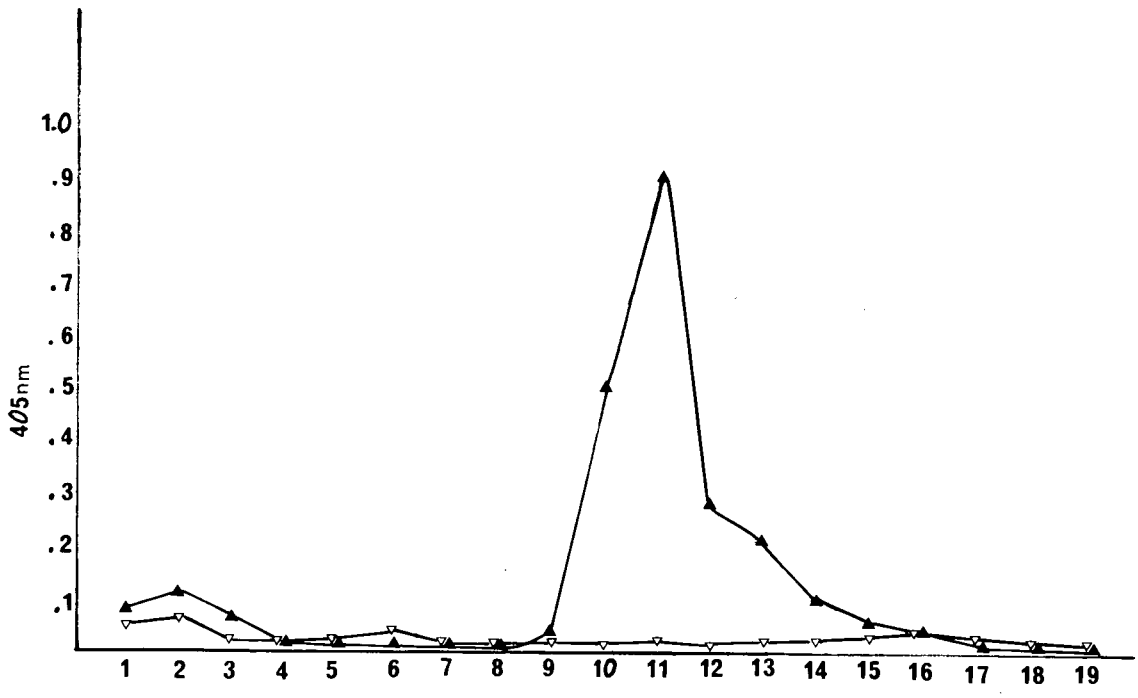

405 nm
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 13. ELISA results of an affinity purified CML antigen. ▲——▲ reaction of MAL-1 monoclonal with antigen eluted from the Sepharose-4B column when a CML extract was run over the column. Δ——Δ reaction of MAL-1 monoclonal with material eluted from the Sepharose-4B column when an ALL extract was run over it.

Negative ELISA results were also obtained when an extract of lymph node cells of a lymphoma patient was run over the column (not shown).


405nm
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

These results show clearly that MAL-1 recognizes and binds well to the major antigen detected by the heterologous rabbit antiserum described previously. The immunoadsorbent studies imply that this antigen is present at relatively high levels on and/or in cell membranes of patients with myelogenous leukemia but that it is not present at detectable levels in equivalent normal, ALL or lymphoma cell preparations. It should be emphasized that the tests reported here do not preclude the possibility that a small population of normal cells produces this antigen but that our methods may not be sufficiently sensitive to detect its presence in normal populations.

Discussion

It has been shown that MAL-1, raised to an antigen purified on preparative gels from membrane extracts of AML patients' cells, reacts in the FACS IV with a cell surface antigen found on cells of patients diagnosed as having AML. This AML-associated antigen was also detected by MAL-1 on the cells of patients with CML. It did not appreciably react in the FACS IV with cells from normal individuals. This finding is in agreement with that reported in Chapter II with the anti-AML-serum in which this antiserum reacted with most patient cells with AML or CML and not with cells from normal individuals or other patients. In addition, as seen with the rabbit anti-AML-serum, this monoclonal demonstrates positive fluorescence binding to the HL-60 cell line and no binding to the ALL-B and ALL-T cell lines. The MAL-1 monoclonal has also been shown to be specific in the ELISA and to be able to isolate the band 1 Ag when used on an immunoadsorbent column. The fact that MAL-1 maintains its band 1 binding capacity on an immunoadsorbent column is especially useful for isolating large amounts of band 1 Ag. Under equivalent conditions, no such material was demonstrated when extracts from normal cells were absorbed on the MAL-1 column. An ongoing study in this laboratory is to carry out sequencing studies on this antigen and to clone the putative malignancy marker gene.

The fact that MAL-1 bound to some Hodgkin's disease cells in the FACS IV study (Table 21, sample 7) is somewhat confusing. This was not seen with the rabbit anti-AML-serum. The fluorescence observed may be due to non-specific binding (i.e. sticky cells, Fc receptors). In the absence of more extensive data on either these particular patients or a larger number of Hodgkin's disease patients, it is impossible at this stage to interpret these results. As pointed out in the results, these patients had promyeloblasts present in the bone marrow aspirates, this may have some effect on the MAL-1 binding properties. As monoclonal technology is a relatively new science, there are many things that are not as yet understood about their reactivity. Many monoclonals which are commercially available bind a multitude of other cells and cell tissues as well as those against which they were initially stimulated.

One problem which exists with MAL-1 is that it does not show consistent binding properties in terms of its ability to bind to cells in suspension as determined by FACS IV analyses. The finding that MAL-1 acts as a suitable antibody for immunoadsorbent purification of the antigen indicates that MAL-1 itself has a relatively strong affinity for the antigen; therefore, it is unlikely that poor binding to cell surfaces is a consequence of low affinity. The determinants recognized by the rabbit and monoclonal reagents are probably not carbohydrate since treatment of the AML antigen with mixed glycosidases, or with periodic acid, or neuraminadase, or when reduced and alkylated, does not affect the binding ability of a MAL-1 column to the AML antigen (R. Shipman, personal communication).

The possibility that the nature of the AML antigen itself may constitute the problem must be considered. The AML antigen is a relatively hydrophilic molecule with a molecular weight of 68,000 daltons and a pI of 7.1 - 7.2 (Shipman et al., British J. Cancer, In press). This protein does not possess the expected characteristics of a membrane antigen in that it is readily soluble in aqueous solutions, contains a high percentage of aliphatic amino acids, and has been shown to be heavily glycosylated. It is possible, therefore, that the antigen itself, while being membrane associated, is not deeply embedded in the membrane. On this basis, it is possible to suggest that a heterologous antiserum directed to it (i.e., the rabbit antiserum), recognizing two or more epitopes, could effectively "freeze" or fix this antigen to the cell surface, thus making it easily detectable. Alternately, a monoclonal antibody, such as MAL-1, while recognizing the same antigen, can only bind a single epitope, and this in itself could serve to de-stabilize and release the antigen from the membrane. Indeed, Baker et al. (1982) have shown that AML cells shed compounds in vitro which can be precipitated with their anti-myeloblastic serum. Further studies, involving combinations of various monoclonal antibodies directed to the AML antigen, will address the possibility that this may be occurring under some circumstances.

Alternately, it is possible that the epitope recognized by MAL-1 is not readily accessible on the cell membrane, in that it may be masked by other membrane components. At this time, it is impossible to define more precisely the problems with obtaining reproducible membrane staining with

MAL-1. Studies in progress in this laboratory are to look at the distribution of this epitope by the immuno-gold staining method (DeWaele et al., 1981) and peroxidase direct slide staining (McMillan et al., 1981).

It is hoped that monoclonal antibodies such as MAL-1 can be used as "magic bullets" for cancer therapy. One monoclonal, anti-M-1 which is directed to a tumor specific antigen on the DBA/2J rhabdomyosarcoma M1, has been coupled with the photochemical haematoporphyrin and has been shown to exhibit significant in vivo anti-tumor activity without any visible side effects (Mew et al., 1983). MAL-1 when coupled with haematoporphyrin may show a similar specificity with myelogenous leukemia cells, or alternately such an assay may allow for a better understanding of the reactivity properties of MAL-1. Until the differentiation process is more precisely defined, it is very difficult to speculate on the nature, whether it be embryonic or differentiation or malignant, of the antigen which MAL-1 recognizes. Indeed, monoclonals such as MAL-1 may elucidate the differentiation process in haemopoietic cells. MAL-1 may be employed to detect a relapse in an AML remission patient or relapse in bone marrow transplant patients.

It is clear that MAL-1 shows specific reactivity with myelogenous leukemia cells. The specificity of MAL-1 on ELISA plates and its ability to isolate band 1 from AML blast cell and CML cell extracts and not from normal, ALL or lymphoma cell preparations on an immunoadsorbent column has been unequivocally shown.

CHAPTER IV

A Comparison of the Binding Ability to Myelogenous Leukemia Cells of the Rabbit Anti-AML-Serum with the Binding Ability of the MAL-1 Monoclonal

Summary Discussion

In this section, the binding ability of MAL-1 will be compared to the reactivity found with the rabbit anti-AML-serum. This comparison will be based on the percent positive cells reacting with MAL-1 or with the rabbit anti-AML-serum. For the purpose of this discussion, in the absence of a "positive control" for the monoclonal series (i.e., a monoclonal which would react with essentially all cells being tested) the percent of cells reacting with MAL-1 was estimated on the number of cells reacting with the rabbit anti-normal-serum. From Table 24 it can be seen that in most cases, both MAL-1 and the rabbit anti-AML-serum reacted better with bone marrow cells than they did with PBL. Both MAL-1 and the rabbit anti-AML did react with either AML or CML patients' cells regardless of status (acute, chronic or remission). The degree of reactivity did not correlate with the number of blast cells present; these antibodies are certainly not only reacting with the blast cell populations. In the FACS sort study, the fact that both a mononuclear enriched cell population as well as a granulocyte enriched population react equally well with these antibodies further support the theory of an "ubiquitous" nature for this malignancy marker. Furthermore, the findings of an ongoing FACS sorting study and immunoperoxidase cell staining study demonstrate the marker on virtually all cell types (Dr. Patricia Logan, personal communication). These studies may indeed give a better understanding as to the clonal nature of AML.

Table 24. Percent positive fluorescence of cell samples from patients with myelogenous leukemia when analysed using either the monoclonal MAL-1 or the conventional rabbit anti-AML serum.

	Sample	Diagnosis	% blasts	% positive with MAL-1*	% positive with Rb-anti-AML
1.	bone marrow	AML-M2	50	73.4	>100
2.	PBL	AML-M2	50	76.6	98.5
3.	bone marrow	APML-M3	15	75.5	>100
4.	PBL	AML-M2 remission	0	9.5	49.5
5.	bone marrow	CML	chronic phase	87.6	95.8
6.	PBL	CML	chronic phase	26.4	75.9
7.	PBL	CML	chronic phase	89.5	81.2
8.	PBL-mono-nuclear enriched	CML	chronic phase	76.0	75.9
9.	PBL-granulocyte enriched	CML	chronic phase	81.2	86.7

* Calculated upon the 100% level being the anti-normal human value (minus non-specific background).

With most cell samples the rabbit anti-AML reacted with a higher number of cells in the population than did MAL-1 (Table 24). This finding is not surprising, MAL-1 is directed to only one epitope, whereas the heterologous rabbit antiserum would recognize many epitopes.

As discussed in Chapter III, MAL-1 sometimes failed to react above background levels with cell populations of CML or AML patients with which the rabbit anti-AML reacted strongly (see Table 25). These findings are also not really surprising in that monoclonal antibodies are exquisitely specific regarding the epitope with which they react, and if that epitope is masked or unavailable on the cell surface, the monoclonal will fail to react. The rabbit anti-AML however, would contain antibodies of varied avidity and specificity, which may have a greater chance of binding to an antigen which may be oriented somewhat differently in some cells in comparison to others. Another possible explanation for the failure of MAL-1 to bind to cells with which the rabbit anti-AML bound may be related to the stability of the antigen-antibody complex. Because the monoclonal can bind only one epitope per molecule, this kind of one:one reaction may enhance sloughing off of antigens whereas multiple binding and possibly cross-linking by conventional antiserum may stabilize the complexes on cell membranes. These possibilities are currently being investigated by examining the ability of MAL-1 to bind cellular antigens in and/or on fixed cells, using the immunoperoxidase procedure. Of course, an ideal situation would be to have a "cocktail" of monoclonal antibodies, that is a mixture of monoclonals each directed to different epitopes of the AML malignancy marker. This would definitely yield more reproducible results

Table 25. Percent positive fluorescence of cell samples from patients with myelogenous leukemia which only bound the conventional rabbit anti-AML serum.

	Sample	Diagnosis	% blasts	% positive with MAL-1*	% positive with Rb-anti-AML
1.	bone marrow	AMML-M4	85	1.0	86.7
2.	PBL	AMML-M4		0	76.3
3.	PBL	AML-M2	35	0.06	67.8
4.	PBL	CML	chronic phase	0	65.2

* Calculated upon the 100% level being the anti-normal human value (minus non-specific background).

and more information as to the nature and orientation of this antigen.

At this time, the inability to demonstrate the presence of this antigen on and/or in cells other than those of patients with myelogenous leukemia does not establish the exclusive specificity of this antigen. It is indeed highly possible that when single cell populations are examined extensively, using more sensitive techniques than FACS analysis such as immunoperoxidase staining, we will be able to determine whether a small population of cells in normal individuals or patients with other conditions react with MAL-1 or the rabbit anti-AML.

It is clear that both the rabbit anti-AML and MAL-1 show specific reactivity with myelogenous leukemia cells. In particular, the usefulness of a monoclonal such as MAL-1 is indeed potentially great, both as a diagnostic and as a possible therapeutic tool. We are now studying the feasibility of using MAL-1 to purge bone marrow of AML candidates for transplantation with no histocompatible donors.

In summary, the data reported herein raise interesting questions regarding the events involved in leukemogenesis, and indicate some directions in which future research should be carried out. Our finding that cells of patients in remission from AML react (sometimes strongly) with both the rabbit antiserum and MAL-1, show that the "malignancy marker" continues to be expressed on normally differentiating cells. Preliminary experiments with cell sorting indicate that both lymphocytes and granulocytes are expressing the antigen. These observations at least suggest the possibility that the initial oncogenic event giving rise to

both AML and CML occurs in a pluripotent stem cell. The finding that the antigen continues to be expressed on normally differentiating remission cells has a number of implications. Are remission cells truly "normal" or do they constitute, in some patients, a dominant clone of malignant cells, which have been induced by chemotherapy to differentiate normally, at least for a time? Is the antigen expressed on the clonogenic cell in the leukemia patient, and if so, will it be possible to eliminate the cancer cells from bone marrow cells of patients who are subjects for autologous marrow transplantation? Do long term remission patients have a different distribution of the marker than do the ones studied so far (we have not had access to such patient material at this time)? Will it be possible to attribute a function to this antigen?

It is beyond the scope of this thesis to answer these important questions. However, the antisera and monoclonal antibody developed in the course of this research provide tools by which continuing investigation can address some of them.

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