Brief Definitive Report

p24: A HUMAN LEUKEMIA-ASSOCIATED AND LYMPHOHEMOPOIETIC PROGENITOR CELL SURFACE STRUCTURE IDENTIFIED WITH MONOCLONAL ANTIBODY*

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In mature mammals the bone marrow is the major site of development of lymphohemopoietic cells, including precursors of T and B lymphocytes. Acute lymphoblastic leukemia (ALL) in humans is generally considered to be a malignancy of lymphoid progenitor cells in bone marrow; a minority of patients have malignancies with a thymic phenotype and only rare patients have a mature T or B cell phenotype (1, 2). Cells from some ALL patients are known to have cytoplasmic immunoglobulin and to be of the pre-B phenotype (3, 4). Cell surface structures on ALL cells might provide markers for lymphoid progenitors. Two major cell surface glycoproteins associated with ALL have been characterized: the HLA-DR or Ia-like antigens (5, 6) and a 100,000-mol wt structure (gp100) (7, 8). A monoclonal antibody (9) against the gp100/common ALL antigen structure has been reported (10). Monoclonal antibodies are also available that identify monomorphic determinants of HLA-DR (11, 12). Both the HLA-DR and gp100 determinants can be demonstrated on terminal deoxynucleotidyl transferase (TdT)-positive lymphocytes in normal bone marrow, which are considered to be lymphocyte precursors (13).

In our study a monoclonal antibody raised against a pre-B form of ALL is described. This antibody precipitates an ~24,000-mol wt polypeptide (p24) on the surface of lymphoid leukemia cells; the polypeptide is detectable on the surface of bone marrow lymphohemopoietic precursors and in most cases of non-T, non-B ALL.

Materials and Methods

Cells for Immunization and Testing. Established human hemopoietic cell lines used in this study were obtained and grown as previously described (14). Fresh leukemic cells were obtained from samples submitted from multiple institutes for analysis at the University of Minnesota or the Imperial Cancer Research Fund (ICRF), London. Normal peripheral blood (PB) and bone marrow (BM) were obtained from volunteers. Heparinized PB and BM were generally separated using Ficoll-Hypaque (F-H) density sedimentation. Interface cells were washed and incubated at 37°C for at least 2 h to remove labile immunoglobulin before further study.

Hybridoma Production, Cloning, and Screening. NALM-6-M1 cells (15) were used for immunization of BALB/c mice as previously described (16). The BALB/c myeloma cell line P3-NSI-1-Ag4-1 (NS-1) was used for fusion. Hybridomas of interest were removed from the original fusion well and subcultured onto BALB/c nonadherent spleen cell feeders in round-bottomed micro-

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titer plates. Supernates were assayed for antibody reactivity against the immunizing cells; subcultures with the highest titers were cloned by limiting-dilution (0.5 cells/well) as previously described (14, 16). A one-step complement-dependent cytotoxicity assay (16) was used to screen culture supernates.

Immunofluorescence Assays. Indirect immunofluorescence was used to assess binding of monoclonal antibody to cell populations. Monoclonal antibody or control ascitic fluid was mixed with target cells and incubated for 30 min at 4°C. After washing, cells were stained with fluorescein isothiocyanate (FITC) -goat anti-mouse Ig (GAMG). FITC-GAMG was from the ICRF, or from Meloy Laboratories Inc., Springfield, Va. Antibodies were generally affinity purified $F(ab')_2$ fragments and were absorbed with insolubilized human Ig. The second incubation was continued for 30 min at 4°C, followed by washing, mounting, and examination with a Zeiss fluorescent microscope equipped with Ploem epi-illumination (Carl Zeiss, Inc., New York).

Double-Marker Analysis of Hemopoietic Cells. Double-marker analysis was performed in BM to determine the relationship between binding of the monoclonal antibody BA-2 and other markers. Cells were first stained with BA-2 and FITC-GAMG and then with tetramethylrhodamine isothiocyanate (TRITC) goat anti-human polyvalent Ig (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) for 30 min at 4°C or, incubated with sheep erythrocytes followed by centrifugation and incubation for 1 h at 4°C. In experiments to demonstrate phagocytosis, BM cells were mixed with latex particles and incubated at 37°C for 1 h and then stained with BA-2 and FITC-GAMG as described above. In some experiments cells were examined for the presence of nuclear TdT and the BA-2^+ cells. Cells were first stained in suspension with BA-2 antibody followed by FITC-GAMG, cytocentrifuged, fixed with cold methanol and incubated with rabbit anti-calf TdT, (kindly supplied by Dr. Fred Bollum, Uniformed University of the Health Sciences, Bethesda, Md.) for 30 min. Cells were then washed and incubated with TRITC goat anti-rabbit antibody for 30 min, washed, mounted, and examined using the fluorescent microscope.

Additional Antibodies Used in This Study. Monoclonal anti-HLA-DR monomorphic determinant antibodies were obtained from Dr. Walter Bodmer, London and Dr. John Hansen, University of Washington, Seattle and termed DA-2 (11) and 7.2 (12), respectively. A monoclonal antibody (J5) against gp100/cALLa was kindly provided by Dr. Jerome Ritz and Dr. Stuart Schlossman, Sidney Farber Cancer Institute, Boston, Mass. (10). Heterologous absorbed (rabbit) anti-gp100 and rabbit anti-HLA-DR were produced at the ICRF.

Radiolabeling, Immune Complex Formation, and Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE). Metabolic labeling of cells with [^{35}S]methionine as well as external labeling with ^{125}I using lactoperoxidase were conducted with standard methods as previously described (8). Immune complexes were precipitated using *Staphylococcus aureus* Protein A. Standard methods were utilized in SDS-PAGE which has been described previously in detail elsewhere (8).

Results and Discussion

Hybridoma Production. Cells from the pre-B ALL-derived cell line, NALM-6-M1, were used for mouse immunizations. NALM-6-M1 cells are CyIgM$^+$, SIg$^-$, HLA-DR$^+$, gp100/cALLa$^+$, TdT$^+$, T lymphocyte antigen$^-$, E rosette$^-$ (15). Mouse spleen cells were fused with NS-1 cells and colonies that appeared in culture wells were screened using NALM-6-M1 and the autologous Epstein-Barr virus-transformed B lymphoblastoid cell line, B85, allowing detection of antibodies recognizing leukemia-associated and/or progenitor antigens. Cells from a NALM-6$^+$ B85$^-$ fusion well were cloned at limiting-dilution and passaged in pristane-primed mice. The antibody product of one clone, BA-2, was shown to be a *Staphylococcus aureus* protein A-binding IgG antibody. The titer of BA-2 ascites fluid in indirect immunofluorescence was 1:1,000.

Immunochemical Characterization of the Structure Recognized by BA-2. NALM-1 cells (17)

were labeled with ^{125}I using the lactoperoxidase method. Cell extracts were incubated with BA-2, and immunoprecipitation and SDS-PAGE were carried out. Results of radioautographs using a 15% polyacrylamide gel are shown in Fig. 1. Lanes A–D are results obtained using increasing amounts of BA-2 antibody (2–20 μl of undiluted BA-2 ascitic fluid). A strong single band is demonstrated at ~24,000 mol wt, henceforth designated p24/BA-2. A similar band was observed when SIg^+ CLL cells were treated in a similar manner (data not shown). In contrast are shown results obtained with the rabbit heteroantiserum against gp100 (lane E) and the anti-gp100 monoclonal antibody J5 in lane F. Lanes E and F show the expected single band at ~100,000 mol wt; the absence of a band in lane F serves as a control indicating that BALB/c mouse ascitic fluid does not contain antibody that precipitates a structure of 24,000 mol wt. Rabbit HLA-DR antiserum was utilized in lane G and shows the expected bimolecular complex at ~34,000 and 29,000 mol wt. Experiments were performed using either reducing or nonreducing conditions; similar bands in both groups indicate that p24/BA-2 is a single chain polypeptide without internal disulfide bonds. The biosynthesis and shedding of p24/BA-2 by leukemia cells was also studied. NALM-1 cells were biosynthetically labeled with [^{35}S]methionine. Cell extracts and culture supernates were subjected to immunoprecipitation and SDS-PAGE. The p24 band was observed in both cell extracts and in cell supernates. These results provide direct evidence to indicate that p24/BA-2 is synthesized by the cells and not absorbed from the serum-containing tissue culture medium.

Distribution of p24/BA-2 on Normal Hemopoietic Cells. Normal hemopoietic cells were studied for the presence of p24/BA-2 positivity using indirect immunofluorescence. As shown in Table I, erythrocytes and granulocytes do not demonstrate p24/BA-2 in indirect immunofluorescence. Small numbers of $p24/BA\text{-}2^+$ cells were found in PB (mean of <1%) and larger numbers in the mononuclear populations in BM (mean of 3%). Analysis of $p24/BA\text{-}2^+$ cells in blood and BM using phase-microscopy indicated that these cells were small, round mononuclear cells that were brightly stained. Double-marker assays demonstrated that $BA\text{-}2^+$ PB and BM cells from normal adults were invariably E $rosette^-$ (E^-), surface Ig^- (SIg^-), and nonphagocytic. Double-marker assays in adult BM revealed that 40–60% (mean 50%) of the BM population positive for TdT was also $p24/BA\text{-}2^+$. These results are consistent with the hypothesis that the lymphohemopoietic progenitor population in human BM contains cells of the TdT^+ $p24/BA\text{-}2^+$ phenotype.

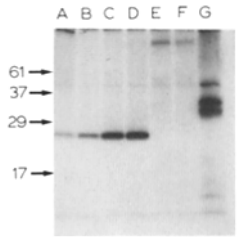

FIG. 1. Immune precipitation and SDS-PAGE with BA-2, anti-gp100 and anti-HLA-DR antibody. NALM-1 cells were externally labeled with ^{125}I using the lactoperoxidase method. Protein A-antibody-antigen complexes were denatured under reducing conditions and subjected to PAGE on a 15% polyacrylamide slab gel. In lanes A–D, BA-2 was used with increasing amounts of antibody, and in lanes E and F,(absorbed rabbit heteroantisera and mouse monoclonal antibody, respectively) anti-gp100 was used. Lane G shows results with rabbit anti-HLA-DR.

TABLE I

Distribution of p24 on Normal and Leukemic Lymphohemopoietic Cells

Cell type	Results using indirect immunofluorescent microscopy and flow cytofluorimetry
Normal lymphohemopoietic cells	
Erythrocytes	Negative
Granulocytes	Negative
PB Mononuclear cells (12 individuals tested)	Mean <1% (range 0–2%)
BM Mononuclear cells (11 individuals tested)	Mean 3% (range 2–5%)*
Leukemic cells	
Non-T, non-B ALL (including pre-B ALL) (SIg^- T antigen$^-$, E^-, $CyIgM^\pm$)	54 Positive samples‡ / 70 Total samples (77%)§
T ALL (E^+ and/or T antigen$^+$)	2 Positive samples / 11 Total samples (18%)‖
B CLL (SIg^+)	5 Positive samples / 10 Total samples (50%)‖

* Cells were nonphagocytic, SIg^-, E^-, small to intermediate size, frequently TdT^+, lymphoid cells.
‡ >10% positive cells.
§ Staining when present was bright to moderate.
‖ Staining when present was moderate to dim.

Distribution of p24/BA-2 on Leukemic Cells. We evaluated the expression of p24/BA-2 on cells from patients with ALL. Results were obtained using indirect immunofluorescence microscopy and in many instances confirmed using flow cytofluorimetry (FACS-I; BD FACS Systems, Mountain View, Calif.). Samples were reported as positive when >10% of cells bound BA-2. 70 cases of non-T, non-B, (including pre-B) common ALL were studied (Table I). 54 of 70 (77%) cases of non-T, non-B ALL bound BA-2 antibody in indirect immunofluorescence. Binding of BA-2 in non-T, non-B ALL as determined by intensity of fluorescence in the FACS and under the microscope, was moderate to bright. In contrast to results with non-T, non-B ALL were results from patients with ALL of the T phenotype (malignant cells were positive for E receptor or T antigen[s]) in which only 2 out of 11 (18%) were positive, and both showed moderate to dim intensity of fluorescence. Cells from 10 patients with SIg^+ chronic lymphocytic leukemia (CLL) were studied and in five cases (50%) binding was observed in 10–40% of cells; the binding was dim to moderate. No reactivity was observed in the other five cases of SIg^+ CLL.

Relationship between p24/BA-2 and Other Surface Markers. The relationship between p24/BA-2 and gp100/cALLa was evaluated further in 64 patients with ALL. 34 of 64 (53%) of cases of ALL had cells expressing both gp100 and p24. Conversely, cells from 15 of 64 (23%) had no detectable p24 or gp100. Discordance between the distribution of p24 and gp100 was evident in 15 cases, 9 of which were $p24^+$, $gp100^-$ and 6 of which were $p24^-$, $gp100^+$. HLA-DR and p24 were also compared; cells of 56 of 86 cases (65%) of ALL were positive for both p24 and HLA-DR. Conversely, 13 of 86 (15%) were negative for both and discordance between p24 and HLA-DR was observed in 17 cases (14 were $HLA\text{-}DR^+$, $p24^-$, and 3 cases were $p24^+$, $HLA\text{-}DR^-$). Although p24/BA-2 appears to be restricted primarily to progenitor cells and most non-T leukemias within the lymphohemopoietic system, prelimary evidence indicates that some epithelial malignancies (e.g., carcinomas) and neuroectodermal malignancies (e.g., neuroblastoma) bear p24/BA-2 (data not shown).

p24/BA-2 appears to differ from recently described antigens found primarily on human B lymphocytes. These determinants have mol wt of 54,000 (18), 65,000 (19)

and a dimer of 40,000 and 150,000 (20). We have produced a second monoclonal antibody after immunization with NALM-6-M1. This antibody (BA-1), brightly stains normal and malignant SIg^+ lymphocytes, including chronic lymphocytic leukemia (CLL); most cases of non-T, non-B ALL, and granulocytes but not normal or malignant T lymphocytes (16). Surface structures found on T lymphocytes were found on mature lymphocytes and have higher molecular weights than p24/BA-2 (21). A 25,000-mol wt determinant may be the human homologue of Thy-1 (22); this determinant is found on all human thymocytes and peripheral T cells. A number of lymphocyte membrane antigen groups including TL, Lyt, Lyb, and Qa have been described in the mouse. All have a different tissue distribution and a higher molecular weight (45,000–110,000) than p24/BA-2 (reviewed 23).

The function of p24/BA-2 on the surface of human lymphohemopoieitc cells is unknown. The demonstration of $p24^+$ on TdT^+ bone marrow cells suggests that p24 is a normal gene product of cells contained within the lymphohemopoietic progenitor populations. The stable expression of p24/BA-2 on most non-T, non-B ALL may be a reflection of the level of maturation arrest present in these leukemic cells.

Summary

This study was directed at surface structures that are found on human lymphohemopoietic progenitor cells in normal and leukemic bone marrow. A monoclonal antibody was produced against an acute lymphoblastic leukemia (ALL) cell line of the pre-B phenotype; this antibody (BA-2) was used to demonstrate a cell surface polypeptide of ~24,000 mol wt that migrates similarly in both reduced and nonreduced form. This polypeptide, p24/BA-2, was shown by immune precipitation and gel electrophoresis and cell distribution studies to be different from HLA-DR and gp100/cALLa. p24/BA-2 was present on the surface of 77% (54/70) of cases of non-T, non-B ALL; BA-2 staining was less bright or nondetectable in surface Ig^+ (SIg^+) chronic lymphocytic leukemia (CLL) and T ALL and nondetectable on peripheral T and B lymphocytes. Approximately 3% of bone marrow mononuclear cells were p24/BA-2^+, and these cells were E $rosette^-$, surface (SIg^-), and nonphagocytic. Marrow TdT^+ progenitor cells were frequently p24/BA-2^+. Results suggest that p24/BA-2 represents a surface structure present on lymphohemopoietic bone marrow progenitor cells and that most common types of ALL bear the p24/BA-2 structure.

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