

POLYMORPHONUCLEAR NEUTROPHILS EXPRESS THE
COMMON ACUTE LYMPHOBLASTIC LEUKEMIA ANTIGEN

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The antigen associated with most cases of non-T, non-B acute lymphoblastic leukemia, as first described by Greaves and referred to as the common acute lymphoblastic leukemia antigen (CALLA), is a 95,000–100,000-mol wt surface membrane glycoprotein identified by both heteroantisera and monoclonal antibodies, J5, VIL-A1, and BA-3 (1–5). Because of the occurrence of CALLA on the cells from most cases of non-T, non-B acute lymphoblastic leukemia (common ALL), it is a useful marker for the diagnosis of this disorder. In addition, J5 antibody has been administered as passive serotherapy in common ALL (6). Recently, this antigen has been detected on a variety of other cell types including some normal bone marrow cells, as many as 25% of cases of T cell acute lymphoblastic leukemia (T-ALL), Burkitt's lymphoma, follicular lymphoma, normal renal tubular and glomerular epithelium, fetal small intestine, and breast myoepithelium (7, 8). During the course of investigations on the expression of CALLA in a variety of human hematopoietic malignancies, we observed reactivity of monoclonal anti-CALLA antibodies with normal polymorphonuclear neutrophils (PMN). This reactivity appears to be specific and not mediated by Fc receptors. Since the presence of CALLA positive cells can be considered indicative of involvement by lymphoma or leukemia, the expression of CALLA by PMN could produce falsely positive results when CALLA is examined for in bone marrow aspirates, peripheral blood or other body fluids.

Materials and Methods

Cells. PMN were isolated from the peripheral blood of 13 healthy normal adult volunteers. The granulocyte- and erythrocyte-rich fraction was separated by gradient centrifugation with Ficoll-Hypaque (Accurate Chemical & Scientific Corp., Westbury, NY) (600 g, 30 min) then mixed 1:1 with Plasma-Gel (HTI Corporation, Buffalo, NY) for 30 min at 37°C to allow the erythrocytes to settle out. The granulocyte-rich supernatant was washed three times in medium RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and resuspended at 3×10^6 /ml. Purity of the preparations was documented morphologically on cytocentrifuge preparations. Cell lines studied included Raji (Burkitt's lymphoma), NALM-1 (lymphoblast crisis of chronic myelogenous leukemia), MOLT-4 (T ALL), and HL60 (promyelocytic leukemia). The induction of HL60 cells to more mature myeloid cells was performed with dimethyl sulfoxide (DMSO) (1.25%; Microbiological Associates, Walkersville, MD) and 12-tetradecanoyl-phorbol-13-acetate (TPA) (10^{-8} M; Sigma Chemical Co., St. Louis, MO) as previously described (9, 10). Cell suspensions were prepared from lymph nodes involved by follicular lymphoma (12 cases) by pressing teased-out cells through a stainless steel mesh and washing in RPMI 1640.

Immunostaining. Indirect immunofluorescence was performed by incubating 2×10^5 cells

with 100 μ l of primary antibody or control immunoglobulin in phosphate-buffered saline containing bovine serum albumin (2%) and sodium azide (0.2%) for 30 min on ice. After three washes, 100 μ l of the fluorescein-conjugated, affinity-purified IgG fraction of goat anti-mouse IgG (1:40, Kirkegaard and Perry Co., Kensington, MD) was added for another 30 min on ice. Murine monoclonal anti-CALLA antibodies included (a) J5 in three forms (1 μ g/ml): whole ascites and IgG-F(ab')₂ (kindly provided by Dr. Jerome Ritz, Harvard University) and IgG fraction (Coulter Corp., Hialeah, FL); (b) BA-3 antibody (ascites, 1:100) (4, 5) (courtesy of Dr. Tucker Le Bien, University of Minnesota); and (c) VIL-A1 antibody (3) (courtesy of Dr. Walter Knapp, University of Vienna). Other monoclonal antibodies included OKM1, OKT6 (Ortho Pharmaceuticals, Raritan, NJ), and Leu-1 (Becton, Dickinson & Co., Sunnyvale, CA); all were used at 1 μ g/ml. Additional controls included a series of nonreactive, purified murine monoclonal (hybridoma) IgG proteins that were substituted for primary antibody and included one each of the IgG classes IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ (all at 30 μ g/ml) (provided by Dr. Kenneth Schroer, National Institutes of Health). Nonreactive ascites from the IgG₁-producing murine myeloma P3 \times 63 (Bethesda Research Laboratories, Rockville, MD), was also used as a negative control. In some instances heat-inactivated (56°C, 30 minutes) normal human serum (1:4) or a human IgG₁ myeloma protein (30 μ g/ml, provided by Dr. Gerald Crabtree, National Institutes of Health) were added to PMN (37°C, 30 min) before immunostaining. Cells were subsequently washed in ice-cold buffer and stained as described above.

Fluorescence Detection. Fluorescence was assayed on a fluorescence-activated cell sorter (FACS-II, B-D FACS Systems, Becton, Dickinson & Co), at 500 mW of laser power (argon laser), 488 nm wavelength, 630 V photomultiplier tube sensitivity, and a fluorescence gain setting of 4 on a scale of 16. The data was recorded on a PDP-11/34 computer (Digital Electronic Corp., Marlboro, MA), 1023-channel histograms were generated and the percentage of fluorescent cells as well as the mean fluorescence intensity (mean channel number) were calculated. Fluorescence microscopy was performed on a Leitz Orthoplan equipped with epifluorescence at 450–490 nm excitation and 515 nm suppression filters.

Immunoprecipitation and Gel Electrophoresis. Cell surface membrane proteins or intact cells were labeled with ¹²⁵I using lactoperoxidase and hydrogen peroxide (11). The cells were then solubilized in 10 mM TRIS buffer (pH 7.4) containing 0.14 M NaCl, 1% Triton-x-100, and 100 μ g/ml phenylmethylsulfonyl fluoride. After centrifugation, these cellular extracts were then immunoprecipitated with J5 (2 μ g/5–10 \times 10⁶ cells) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (11). Parallel control immunoprecipitations were also performed with UPC10, a nonreactive murine IgG2a (Litton Bionetics, Bethesda, MD).

Results

The granulocyte fraction from all normal individuals tested stained with J5, VIL-A1, and BA-3 (mean 89%, range 55–99%). All three preparations of J5, namely, ascites, intact IgG, and F(ab')₂, reacted with granulocytes (Fig. 1 A, B). Greater than 90% of cells stained with OKM1, however, no fluorescence was observed with the nonreactive mouse ascites, purified murine IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, antibodies OKT6 and Leu-1, or after omission of primary antibody. Further, preincubation of cells with normal human serum or human IgG₁ did not affect either the percentage of cells stained by J5 or the intensity of fluorescence (Fig. 1 C). To determine whether anti-CALLA antibody could distinguish between neutrophils and eosinophils, the leukocytes from normal individuals and from a patient with hypereosinophilia were stained with the F(ab')₂ fraction of J5 and sorted into fluorescent-positive and -negative fractions on the FACS. The cells were collected, cytocentrifuged, stained with Wright's stain, and differential counts were performed. The positive fractions were greatly enriched for PMN (95 and 100% for normals and 86% for the hypereosinophilia patient), whereas the negative fractions were predominantly eosinophils, lymphocytes, and monocytes. The fluorescence intensity of neutrophils stained with

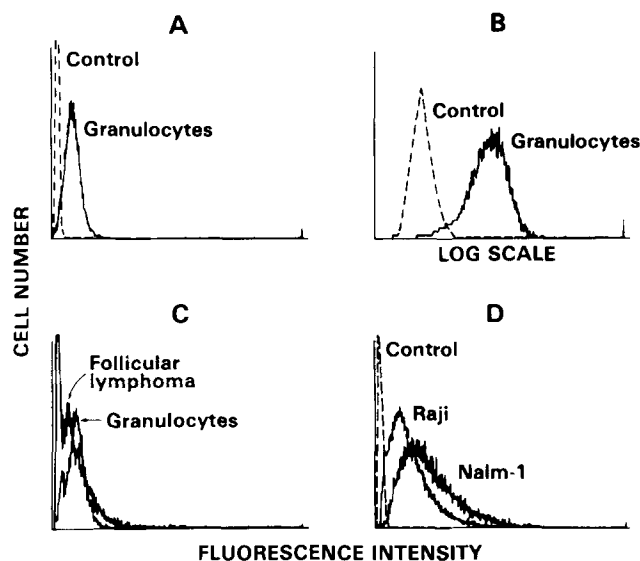


FIG. 1. (A) J5 staining of granulocytes as compared with control (murine IgG_{2a}) shows 98% of cells stained. Linear scale. (B) Log transformation of histogram from A amplifies the positive staining reaction of granulocytes with J5. Log scale (two decades). (C) Granulocytes and follicular lymphoma had comparable fluorescence intensity when stained with J5. These granulocytes were pretreated with normal human serum and showed no diminution of fluorescence intensity when stained with J5. Preincubation with human IgG1 myeloma protein or staining with the F(ab')₂ fraction of J5 showed similar fluorescence (not shown). The small negative peak in the granulocyte fraction was sorted and proved to be largely eosinophils. Linear scale. (D) The Burkitt's lymphoma cell line, Raji, and a common ALL cell line, NALM-1, reacted with J5 and the relative intensity of the fluorescence was greater than that of granulocytes. Linear scale. The control in all cases was a murine IgG_{2a} monoclonal protein. All other negative controls produced similar results (see text). Each curve includes 10,000 cells counted.

J5 was comparable to that observed with follicular lymphoma cells but less than that found with Raji cells (mean channel 160) and NALM-1 cells (mean channel 180) (Fig. 1 D). The average mean fluorescence channel was 83 for PMN (10 samples) and 102 for follicular lymphomas (12 samples). IgG-J5 also reacted with HL60; however, this interaction appeared to be mediated through Fc receptors since (a) staining was inhibited by preincubation with human serum or human IgG1 myeloma protein, and (b) F(ab')₂ J5 did not react with HL60. Addition of either TPA or DMSO to HL60 cells did not result in induction of specific J5 reactivity during a 5-d course.

The membrane determinant detected on PMN by anti-CALLA antibodies was biochemically characterized by cell surface labeling, immunoprecipitation, and SDS-PAGE gel electrophoresis. Autoradiographs of these gels demonstrated that J5 antibody identified a protein on the cell surface of PMN of ~95,000–110,000 mol wt, and was similar in size to that found on Raji cells (95,000–100,000 mol wt) (Fig. 2). These proteins recognized by J5 were not present in immunoprecipitates performed with control UPC10 IgG_{2a} monoclonal antibodies or on the T cell leukemia cell line, MOLT-4.

Discussion

These studies demonstrate that the anti-CALLA monoclonal antibodies, J5, VIL-A1, and BA-3 consistently react with human polymorphonuclear neutrophils but not

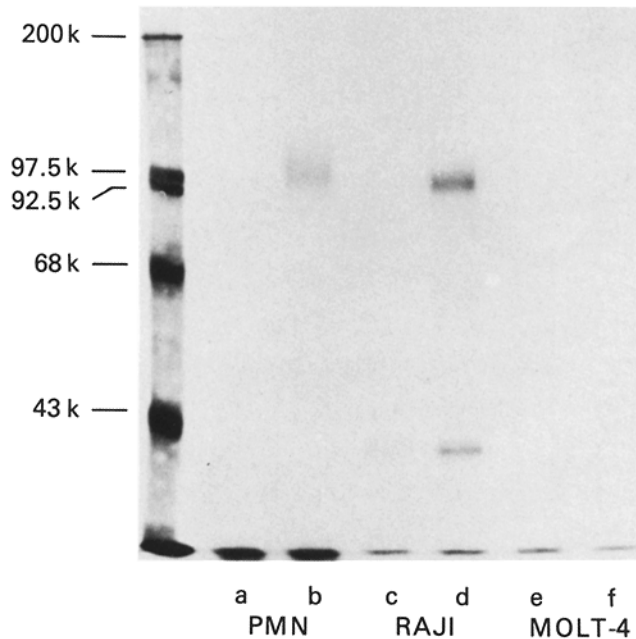


FIG. 2. SDS-PAGE analysis of ^{125}I -surface-labeled cells after immunoprecipitation. Molecular weight standards are shown in the left lane. PMN (a, b), Raji (c, d), and Molt-4 (e, f) were immunoprecipitated with a nonreactive murine IgG2a (UPC10) (a, c, e) or J5 (b, d, f). From PMN, J5 immunoprecipitated a single band that has a molecular weight similar to that of the band obtained with Raji cells. These proteins were not immunoprecipitated with Molt-4 cells or with UPC10 monoclonal antibodies.

with other leukocytes. This reactivity does not appear to be mediated by cell surface Fc receptors since staining persisted after saturation of these receptors with either human IgG1 myeloma protein or normal human serum. Furthermore, the $\text{F}(\text{ab}')_2$ fraction and the intact IgG form of J5 showed equivalent reactivity. Other murine IgG2a antibodies, including Leu-1, did not react with granulocytes. Preparative cell sorting of J5-positive and -negative fractions indicated that the J5-positive cells observed among granulocytes and unseparated peripheral blood leukocytes were, indeed, PMN.

The antigen detected on the cell membrane of PMN was similar in size to CALLA present on Raji cells (95,000–110,000 mol wt for PMN and 95,000–100,000 mol wt for Raji) and was immunostained by three different anti-CALLA antibodies. Thus, the antigen detected on PMN not only shares an epitope with CALLA, but these antigens may be identical. Although blasts from the lymphoblast crisis of chronic myelogenous leukemia (CML) are frequently CALLA-positive (2, 4, 7), CALLA may not be expressed on myeloid precursor cells since; (a) the cells of the stable phase of CML are reported to lack CALLA (7); (b) anti-CALLA (J5) treatment does not inhibit proliferation in myeloid clonal assays (12); and (c) as observed in the present study, the promyelocytic leukemia cell line, HL60, did not specifically stain with J5. Thus, CALLA appears to be acquired at a late stage of PMN differentiation. Although anti-CALLA antibodies have a role in the diagnosis of lymphoma and leukemia, admixed PMN in patient samples could masquerade as CALLA-positive malignant cells. As an example, the pleural effusion from a patient with follicular lymphoma

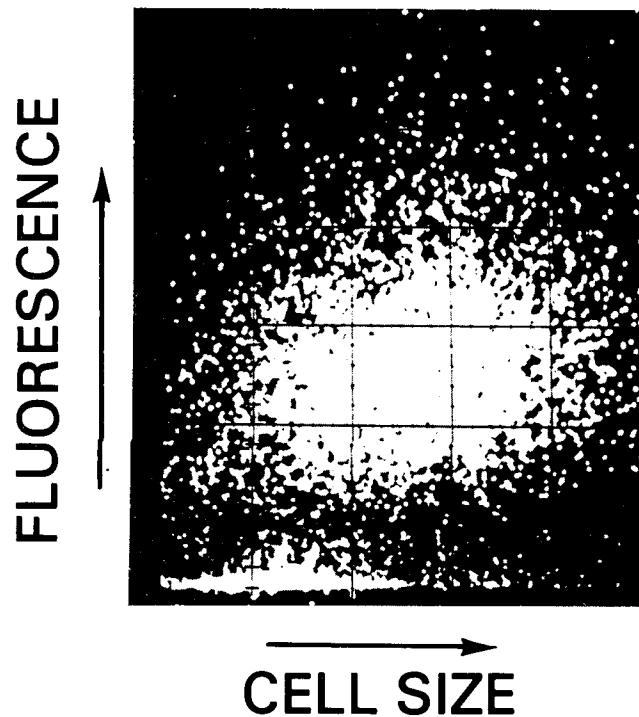


FIG. 3. Dot plot display of FACS-II analysis of a pleural fluid from a patient with follicular lymphoma stained with J5. A cluster of large, stained cells is seen in the upper right. This stained cell population was sorted and was found to consist of polymorphonuclear neutrophils.

contained 6% J5-stained cells (Fig. 3). This result was initially interpreted as indicating a low level of involvement by the neoplastic process. However, when these stained cells were sorted on the FACS, they proved to be entirely composed of PMN. Therefore, caution should be exercised when studying effusions, bone marrow aspirates, and especially unseparated leukocyte buffy coats as has been suggested for immunofluorescence analysis by flow cytometry (13).

Summary

Monoclonal antibodies J5, VIL-A1, and BA-3, known to react with the common acute lymphoblastic leukemia antigen (CALLA) were found to specifically stain normal human polymorphonuclear neutrophils (PMN). The antigen detected on PMN had a molecular weight (95,000–110,000 mol wt) close to that of CALLA (95,000–100,000 mol wt) and thus these surface membrane antigens are likely related, if not identical. The fluorescent staining intensity of PMN is comparable to that of CALLA-positive leukemic cells and the presence of PMN in patient samples could potentially produce false-positive results in diagnosis.

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