Differential Expression of CD24-Related Epitopes in Mycosis Fungoides/Sézary Syndrome: A Potential Marker for Circulating Sézary Cells

By Samuel J. Pirruccello and Molly S. Lang

In the hematopoietic system, the B-cell associated antigen CD24 is expressed at high density on B cells. B-cell precursors, and B-cell malignancies as well as at low density on peripheral blood polymorphonuclear leukocytes. The 42-Kd sialoglycoprotein has not been previously demonstrated to be expressed on T cells, thymocytes, or T-cell malignancies. We identified three patients with mycosis fungoides/Sézary syndrome that showed low density expression of the CD24-related epitope recognized by antibody BA-1 on circulating T cells. All three patients had Sézary cells by morphologic assessment and clonal T-cell populations in the peripheral blood by gene rearrangement studies. In two of these patients, indirect immunofluorescence with a panel of six anti-CD24 monoclonal antibodies demonstrated reactivity for two of six antibodies in one case and only one of six antibodies in the other. The biologic significance of CD24-related epitope expression on circulating T cells in mycosis fungoides/Sézary syndrome is unclear. However, these findings suggest that differential, low density expression of CD24-related epitopes (BA-1’, OKB2’) may be a useful phenotypic marker for identifying circulating Sézary cells.

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T he B-CELL associated antigen CD24 is a 42-Kd sialoglycoprotein recognized by the prototypic antibody BA-1.1,2 The molecule has been successfully radiolaabeled only on carbohydrate residues either by surface reduction with trinitiated sodium borohydride or by metabolic incorporation of tritium-labeled glucosamine.2,3 CD24 has a broad banding profile on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), suggesting heavy glycosylation. The molecule also shows partial comigration with immunoglobulin G (IgG) heavy chains on SDS-PAGE, giving the impression of a multi-chain protein complex.2,3

The function of CD24 is currently unknown. Previous studies reported an inhibition of Ig synthesis by pokeweed mitogen-stimulated B cells and by the CESS cell line after cross-linking CD24 with antibody.4,5 However, Rabinovitch et al could show no change in B-cell free Ca2+ by cross-linking of CD24 alone or with costimulation of surface Ig. We found that CD24 antigen density increases as pre-B acute lymphoblastic leukemia (ALL) cells enter the cell cycle, but the molecule does not appear to deliver a proliferation-related signal when cross-linked by antibody.6

In the hematopoietic system, the CD24 antigen is expressed at high density on B cells and B-cell precursors as well as at low density on polymorphonuclear leukocytes.7 The antigen has proven to be a useful B-lineage marker in ALL and is a favorable prognostic indicator in pre-B ALL in children.8 The CD24 epitope recognized by monoclonal antibody (MoAb) BA-1 has also been demonstrated on renal tubular epithelium,9 on some nonhematopoietic tumors,10 and on some normal epithelium.11 In these tissues outside the hematopoietic system, it is currently unclear whether BA-1 positivity represents epitope mimicry or true CD24 antigen expression. We report here the finding of limited CD24-related epitope expression on peripheral blood T lymphocytes from three patients with mycosis fungoides/Sézary syndrome.

Materials and Methods

Antibodies. Antibodies used for these studies included T6 (anti-CDS), T11 (anti-CD2), T3 (anti-CD3), T4 (anti-CD4), T1 (anti-CD5), T8 and T8-phycocerythrin (PE) (anti-CD8), I2 (anti-HLADR), J5 (anti-CD10), B4 (anti-CD19), and B1 (anti-CD20) (Coulter Immunology, Hialeah, FL). Fluorescein isothiocyanate (FITC)-conjugated goat antimouse, anti-κ, and anti-λ were obtained from Tago (Burlingame, CA). The hybridoma secreting MoAb BA-1 was the kind gift of Dr Tucker LeBien (University of Minnesota, Minneapolis). The anti-CD24 MoAbs LC66, ML5, and VIB-E3 were obtained from the Fourth International Workshop on Leukocyte Differentiation Antigens. MoAb OKB2 was obtained from Ortho Diagnostics (Raritan, NJ). Antibody 32D12 was the kind gift of Dr Steiner Funderud (Norwegian Radium Hospital, Oslo). Negative controls included a polyclonal mouse IgG, and IgG1-PE (Coulter). The IgM myeloma MOPC 104E was obtained from the American Type Culture Collection (ATCC; Rockville, MD) tissue bank.

Immunofluorescence. Immunofluorescence assays were performed as described previously.2 Briefly, peripheral blood mononuclear cells were isolated from heparinized peripheral blood by Ficoll-Hypaque gradient centrifugation and washed twice with phosphate-buffered saline (PBS). The cells were washed with cold PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN3, and aliquoted at 2 × 106 cells per tube. The cells were incubated with primary antibody for one-half hour at 4°C in 100 μL PBS/BSA/NaN3. Cells were washed twice and incubated with FITC-goat antimouse (Fab) for one-half hour at 4°C. For two-color immunofluorescence, cells were first incubated with IgM or BA-1 followed by goat antimouse FITC and then IgG1-PE or CD8-PE, respectively. After washing, the cells were analyzed on an EPICS C Flow Cytometer at 488 nm with a 5-W argon ion laser. Fluorescence was measured either on linear or log scale and percent positive cells were calculated after background subtraction using Epics software.

Southern blotting. T-cell receptor gene rearrangement by Southern blotting2 was performed on Ficoll-Hypaque isolated mononuclear cells or buffy coat preparations by standard techniques. Briefly, genomic DNA was isolated from the white blood cell preparations by genomic DNA isolation using an automated DNA isolation system (20 g/L proteinase and 100 μL phenol/chloroform Isotex; Fungy, Inc., Rochester, NY).

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the method of Bakhshi et al. Aliquots of DNA were cut separately with EcoRI, BamHI, and HindIII and electrophoresed on 0.8% agarose gels at 40 V overnight. After denaturation in 0.2 N NaOH, 0.6 mol/L NaCl, and neutralization in 0.5 mol/L Tris, pH 7.5, 1.5 mol/L NaCl, the DNA was transferred to nylon membranes (Schleicher & Schuell, Keene, NH) by blotting in 20X SSC (3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7). The membranes were washed, baked, and then prehybridized with 5X SSC, 1X Denhardt solution, 0.02 mol/L sodium sulfate, and 50% formamide overnight at 42°C. The filters were then hybridized with a 770-bp Tβ constant region probe (Dr. Aneel Masih, University of Nebraska Medical Center, Omaha) labeled by random primer extension (Dupont/NEN, Boston, MA). After washing, the filters were sealed wet into plastic bags and allowed to expose x-ray film from 4 to 40 hours at −70°C. For delineation of germline patterns, placental DNA was used as negative control.

Patients. Patient 1 was a 56-year-old woman who presented with several nonhealing skin lesions in August 1986. Immunohistology of skin biopsy material yielded a working classification of peripheral immunoblastic lymphoma of T-cell type. The infiltrating lymphocytes had a cytotoxic suppressor T-cell phenotype being positive for CD2 (T11), CD8 (T8, Leu-2) CD3 (Leu-4), CD7 (Leu-9), HLA-DR, and CD71 (OKT9). The tumor cells were negative for CD4 (Leu-3, OKT4), CD1 (Leu-6), CD19 (Leu-12), and CD22 (Leu-14). The patient responded poorly to therapy and was later reclassified as mycosis fungoides on October 16, 1987.

Patient 2 was a 58-year-old man who presented in July 1988 with multiple skin lesions. Clinical workup yielded a working diagnosis of mycosis fungoides. Immunohistology of skin biopsy demonstrated a cytotoxic suppressor T-cell phenotype with expression of CD2 (Leu-5), CD5 (Leu-1), CD7 (Leu-9), CD8 (Leu-2), HLA-DR, and CD45 (UCHL-1). The infiltrating tumor cells were negative for CD4 (Leu-1), CD1 (Leu-6), CD8, CD19 (Leu-12), CD22 (Leu-14), and CD15 (Leu-MI).

Patient 3 was a 51-year-old woman who presented with multiple skin lesions in January 1989. Clinical workup yielded a working diagnosis of mycosis fungoides. Skin biopsy demonstrated infiltrating T cells with a helper inducer T-cell phenotype showing positivity for CD5 (Leu-1), CD3 (Leu-4), CD4 (Leu-3), CD8, HLA-DR, and CD71 (OKT9). The tumor cells were negative for CD8 (Leu-2), CD1 (Leu-6), CD7 (Leu-9), CD19 (Leu-12), CD22 (Leu-14), and CD15 (Leu-MI).

RESULTS

At the time of phenotypic analysis, all three patients had a high percentage of circulating lymphocytes with morphology consistent with Sézary cells on peripheral blood smear (not shown). All three patients had monoclonal rearrangements of the β gene of the T-cell receptor in DNA isolated from peripheral blood white cells isolated by Ficoll gradients or from buffy coat preparations. Representative blots are shown for patients 2 and 3 in Fig 1. These gene rearrangement studies confirmed the morphologic impression of a circulating population of malignant T cells.

The original phenotyping data for the patients are presented in Table 1. Patients 1 and 2 showed a predominance of cytotoxic suppressor T cells, and patient 3 showed a predominance of helper inducer T cells consistent with the T-cell tumor phenotypes demonstrated by immunohistology on skin biopsy. However, only patient 2 showed an increase in peripheral blood lymphocytes expressing HLA-DR, which was present on all three patients tumor cell populations present in the skin lesions. A clear discordance was evident in all three cases for the percent BA-1 (CD24) positive cells in comparison with the other B-cell markers CD19 and CD20, as well as the percentage of T cells present. On a linear fluorescence scale, the mean channel fluorescence for BA-1 was noted to be considerably lower than that of the other T- and B-cell markers (not shown). Log scale fluorescence

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<th>Table 1. Cell Surface Markers of Peripheral Blood Lymphocytes</th>
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<td>Ag (Ab)</td>
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<td>CD1 (T6)</td>
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<td>CD24 (BA-1)</td>
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*Percent positive cells measured on linear fluorescence scale.
CD24 EXPRESSION ON SÉZARY CELLS

histograms are shown for patients 2 and 3 in Fig 2 and illustrate the pattern of BA-1 immunofluorescence. The mean fluorescence intensities for CD2 (not shown), CD4, and CD8 were 1.5 to 2 logs brighter than that obtained with BA-1. For comparison with the low density expression of the BA-1 epitope, the lymphocytes from patients 2 and 3 were also examined for reactivity with the anti-CD24 MoAb OKB2. In contrast to the positive reactivity noted with BA-1, neither patient's lymphocytes showed reactivity with antibody OKB2 (Fig 2).

After demonstration of reactivity of BA-1 with lymphocytes isolated from patients 2 and 3, we performed a number of control experiments to rule out the possibility that agglutinated antibody was causing a false positive immunofluorescence signal. We performed indirect immunofluorescence of patients' lymphocytes after centrifugation of BA-1 ascites to remove potential aggregates, as well as assaying with separate aliquots of BA-1 ascites prepared both at different times and in different laboratories. All BA-1 antibody preparations showed similar fluorescence profiles on these two patients' lymphocytes. We also performed two-color immunofluorescence with BA-1-FITC and CD8-PE on the lymphocytes from patient 2 and could demonstrate co-expression of these two antigens (see Fig 3).

To further assess the selective CD24-related epitope expression on these malignant T cells, we performed immunofluorescence studies with six of the seven antibodies comprising the anti-CD24 panel of the Fourth International Workshop on Leukocyte Differentiation Antigens. As seen in Table 2, of the six anti-CD24 monoclonals, lymphocytes from patient 2 reacted only with antibody BA-1. In contrast, the lymphocytes from patient 3 showed reactivity with MoAb BA-1 as well as with antibody VIB-E3.

**DISCUSSION**

The findings reported here demonstrate an unusual selective expression of CD24-related epitopes on the surface of circulating T cells in mycosis fungoides/Sézary syndrome. Stockinger et al. reported expression of CD24 or the CD24-related epitopes recognized by VIB-E3 and HB8 on the surface of Sézary cells. In that study, the anti-CD24 monoclonals ALB9, LC66, CLB gran Blyl, VTB-CS, and ALla were all negative on the Sézary cells used for analysis. As with this previous report, it is unclear whether reactivity with antibody BA-1 represents expression of an altered CD24 molecule on these T cells or expression of CD24.
epitopes on other cell surface glycoproteins or glycolipids. The fact that VIB-E3 reacted with one of these patients' lymphocytes but not the other demonstrates heterogeneity of CD24 epitope expression even among this small sample of cases. We attempted radioimmunoprecipitation of the antigen recognized by BA-1 from tritiated sodium borohydride-labeled cell lysates from patient 3 but were unable to demonstrate a protein or glycoprotein antigen. We are not sure whether this was due to the very low density expression of the BA-1 epitope or whether the epitope was dependent on tertiary or quaternary structure at the membrane surface and was destroyed on cell lysis. These possibilities seem most likely since very few counts were precipitated with BA-1 in comparison with a radiolabeled pre-B ALL cell line lysate used as a positive control.

We have previously shown that all six antibodies used for these fluorescence studies radioimmunoprecipitate a glycoprotein consistent with CD24. The positive immunofluorescence with VIB-E3 on lymphocytes from patient 3 is consistent with the earlier report by Stockinger et al 8 and the CD24 epitope expression detected with antibody BA-1. We have assayed peripheral blood lymphocytes obtained from patient 3 on four separate occasions. The patient has maintained 75% or more CD4+ T cells, a consistent pattern of low density expression of the BA-1 epitope, and absence of the epitope recognized by antibody OKB2.

These findings provide some insight into the epitope specificity of recognized anti-CD24 MoAbs. Stockinger et al 15 organized a subclustering scheme of anti-CD24 antibodies based on differences in epitope expression on various cell lines and by cross-blocking studies. Engel et al 16 reported a clustering scheme of CD24 epitopes by measuring time-dependent epitope loss from B cells following activation. We previously reported that anti-CD24 MoAb 32D12 recognizes a distinct epitope from the other anti-CD24 MoAbs VIB-C5, VIB-E3, LC66, BA-1, ML5, and OKB2 based on immunofluorescence studies with the Nalm 6 cell line. We demonstrate here that antibodies BA-1 and VIB-E3 also recognize distinct epitopes based on the BA-1+, VIB-E3- and BA-1+, VIB-E3+ phenotypes in patients 2 and 3, respectively. Radioimmunoprecipitation studies suggest that CD24 represents a heavily glycosylated protein 12 and raises the possibility that differences in epitope expression may be the result of selective expression of specific glycosyl transferases. More rigorous studies of CD24 protein and carbohydrate structure will be required to address this issue.

These findings raise a more clinically relevant possibility related to immunophenotyping and monitoring of peripheral blood in mycosis fungoides. All three patients reported here demonstrated low density expression of the anti-CD24 epitope recognized by antibody BA-1. More detailed immunofluorescence studies on the lymphocytes isolated from patient 2 as well as repetitive studies on patient 3 showed a consistent BA-1 positive OKB2 negative phenotype. The malignant T cells demonstrated on skin biopsy in patients 1 and 2 were derived from a cytotoxic suppressor T-cell lineage, while those of patient 3 represented a helper inducer T-cell lineage. The malignant T-cell phenotypes were, except for HLA-DR expression in patients 1 and 3, concordant with the predominant T-cell subset present in the circulation. Further, we demonstrated coexpression of BA-1 on the CD8 positive peripheral blood T cells from patients 2. We have subsequently seen a fourth patient with a working diagnosis of mycosis fungoides/Sézary syndrome. In this patient we could also demonstrate a circulating helper inducer T-cell population that was BA-1+ and OKB2- (not shown). However, gene rearrangement studies have not been performed to confirm the presence of a circulating clonal T-cell population in this fourth patient. These findings suggest that regardless of the functional T-cell phenotype, differential expression of the CD24 epitopes recognized by antibody BA-1 and OKB2 may be a useful marker for circulating Sézary cells.

These chronic T-cell malignancies frequently express normal mature T-cell phenotypes. Confirmation of the presence of circulating malignant Sézary cells therefore requires the demonstration of a monoclonal population by T-cell receptor gene rearrangement. Involvement of the peripheral blood in mycosis fungoides has been shown to predict a poorer survival and a higher frequency of lymphomatous and visceral tumor involvement. A phenotypic marker for circulating Sézary cells would, therefore, be a useful diagnostic adjunct in the management of these patients.

We attempted to demonstrate the clonal nature of the CD24 positive T cells using cells stored on patient 3 by BA-1-immunomagnetic bead selection and Southern blotting. Unfortunately, the few cells surviving the freeze thaw in 10% dimethyl sulfoxide (DMSO) were greater than 80% CD24 positive and we could not obtain enough CD24 negative cells after separation for DNA extraction. The only other DNA source available on this patient for comparison was from a buffy coat preparation. Due to neutrophil contamination this DNA preparation was not adequate for demonstrating clonal enrichment after BA-1 selection. Therefore, the ultimate usefulness of this phenotypic trait will require confirmation of these findings in a larger series of mycosis fungoides patients by correlation with gene rearrangement studies and demonstration of true monoclonality in the CD24+ T-cell population.

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