Spurious E Rosette Formation in B Cell Chronic Lymphocytic Leukemia Due to Monoclonal Anti-Sheep RBC Antibody

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The apparent simultaneous presence of surface markers characteristic of both B and T cells is a phenomenon being described with increasing frequency in patients with chronic lymphocytic leukemia (CLL). We describe a patient with CLL whose B lymphocytes possessed surface immunoglobulin reactive with neuraminidase-treated sheep erythrocytes (SRBCs) and produced E rosette formation. Cytofluorography using monoclonal antibodies demonstrated the B cell nature of these cells and the absence of the SRBC receptor. Further documentation that the binding of SRBCs was mediated through immunologic reaction included E rosette formation inhibition by monospecific antisera and hemagglutination of SRBCs by a paraprotein isolated from the patient’s serum. Fusion of the CLL cells with a human hypoxanthine-aminopterin-thymidine-sensitive plasma cell line resulted in the production of human hybridomas that secreted the SRBC-reactive IgM antibody. An analysis of clinical histories of CLL patients whose cells exhibited this phenomenon from both immunologic and clinical perspectives is presented.

LYMPHOPROLIFERATIVE disorders involve clonal proliferation of various lymphoid cell types that have undergone malignant transformation. Chronic lymphocytic leukemia (CLL) has been shown to be a disorder of B cells in the vast majority of cases. Surface immunoglobulin (Ig) has usually been of one or two heavy chain types (commonly IgM or IgD or both) with either κ or λ light chain present, indicating the clonal nature of the cells. The sera of 5% to 10% of patients contain monoclonal immunoglobulin, usually IgM, that is often idiotypically identical to the surface membrane-bound Ig of the malignant cells, or occasionally, the product of a second clone of Ig-secreting cells.

A small minority of cases of CLL have been documented to be T cell in origin. Classically, this has been demonstrated by the ability of T cells to spontaneously rosette with sheep erythrocytes (SRBCs). More rarely, but with an apparently increasing frequency, cases of CLL have been reported in which surface markers for T and B cells appear to be present simultaneously.

We have encountered such a CLL patient whose homogeneous population of lymphocytes displayed surface Ig and rosetted SRBCs spontaneously. Our analyses have indicated that these cells are of B cell origin, with a monoclonal Ig on the surface of the cells and in the patient's serum, which appears to be specifically directed against an antigen on the sheep erythrocyte, thus producing spurious rosette formation. Documentation of this phenomenon has included (1) inhibition of E rosette formation with monospecific antisera; (2) hemagglutination of neuraminidase-treated SRBCs by the patient's serum and isolated monoclonal IgMλ paraprotein; and (3) in vitro production of this hemagglutinating IgM through human hybridoma formation.

PATIENT HISTORY

The patient was a 57-year-old white male who presented in September 1974 with fatigue, marked splenomegaly, and an enlarged right axillary lymph node. His peripheral white count was 160,000/μL. Morphologically, 99% of the cells were mature lymphocytes. His hemoglobin was 11 g/dL and his platelet count was normal. A diagnosis of chronic lymphocytic leukemia was made.

He initially received intermittent prednisone and chlorambucil. In February 1975, he was given 1,740 rad in ten fractions to the thymus, with a dramatic decrease in splenic size, disappearance of the axillary adenopathy, and a fall of his WBC count from 185,000 to 4,300/μL (86% lymphs, 11% neutrophils). Maintenance chlorambucil was initiated two months later and was continued until October 1978, when he developed dyspnea and an open lung biopsy was suggestive of a drug-induced interstitial pneumonitis.

He received vincristine in early 1979, until a symptomatic neuropathy occurred. By August 1979, marked splenomegaly was again noted, with a recurrence of the right axillary lymphadenopathy, and monthly cycles of cyclophosphamide (300 mg/d for five days) and prednisone (20 mg/d for five days) were administered. His WBC rose and Adriamycin (30 mg) was added to his regimen each month.

He continued to show progressive disease that was intermittently responsive to splenic irradiation (three courses of 600 rad were given in December 1979 and in February and October 1981). However, in January 1982, shortly after completion of his third course, massive splenomegaly was noted and lab values were: WBC count, 442,000/μL (98% lymphs); hemoglobin, 9.2 g/dL; and platelet count, 347,000. The majority of these cells were small mature lymphocytes. However, at this time, 20% of the cells appeared to be prolymphocytes. These cells were larger, with a more blast-like nucleus possessing nucleioli. He was placed on a protocol to receive CP 46,665, a macrophage potentiator. It was at this time that surface...
marker analysis of his leukemic cells was first performed. Serum protein electrophoresis demonstrated diffuse hypogammaglobulinemia. A monoclonal IgM paraprotein present. Heterophil antibody was not detected.

In February 1982, he was admitted with acute abdominal pain, and splenectomy was performed for a suspected subcapsular hemorrhage.

He completed treatment with CP 46,665 in May 1982. Several more attempts were made to control his disease with experimental agents, but, despite this, he continued to have marked elevations in his WBC count and he died in May 1983.

**MATERIALS AND METHODS**

### E Rosette Formation

The mononuclear cell fraction of heparinized peripheral blood was isolated using a Ficoll-Hypaque gradient. SRBCs were tested with and without prior treatment at 37°C for one hour with 10 µg/10⁶ cells *Clostridium perfringens*-derived neuraminidase (Sigma Chemical Co, St Louis). Spontaneous rosette formation was tested using a modified version of the method of Jondal et al. Equal volumes of 2.5% SRBC suspension and a suspension of lymphocytes in Hank's balanced salt solution (HBSS)/5% fetal calf serum (FCS) at 2 x 10⁶ cells/mL in HBSS/5% FCS were mixed and centrifuged at 200 g for five minutes and incubated on ice for four hours. The pellet was resuspended in one half of the supernatant volume. Lymphocytes with three or more attached SRBCs were considered to form rosettes.

Immune inhibition of E rosette formation was tested by preincubating 2 x 10⁶ lymphocytes for one hour at 4°C with 200 µL of class- or type-specific rabbit antisera containing approximately 1.0 to 1.5 mg antibody/mL. The cells were washed free of antibody and tested as above.

### Membrane-Bound Surface Ig

The direct immunofluorescence method using fluorescein-conjugated antisera to human Ig, as well as to individual Ig chains (κ, λ, μ, γ, δ, ε) was employed for detection of surface Ig. Polyvalent Ig and heavy chain-specific antisera were obtained from Meloy Laboratories (Springfield, Va). The κ and λ antisera were F(ab')₂ fragments (Tiago, Burlingame, Calif). Positively staining cells were enumerated with immunofluorescent microscopy and by cytofluorography with an Ortho (Westwood, Mass) System 50 H cytofluorograph using a Spectra Physics argon-ion laser (488 nm excitation). Data were processed using an Ortho FC300 signal processor and 2150 computer system.

### Trypsinization to Remove Surface Ig

Lymphocytes were washed in HBSS (without Ca²⁺ and Mg²⁺), suspended at a concentration of 2 x 10⁶ cells/mL in HBSS containing trypsin (2.5 mg/mL), incubated at 37°C for 25 minutes, and washed free of trypsin with Dulbecco’s minimal essential medium (MEM)/20% FCS. Surface marker studies for Ig were done immediately after treatment and again following 24 hours of incubation at 37°C, 5% CO₂, to allow for de novo synthesis of surface Ig.

### Acetate Wash to Remove Cytophilic Protein

Lymphocytes at 2 x 10⁶ cells/mL were suspended in acetate buffer and incubated at 37°C for ten minutes. Cells were then washed in HBSS/5% FCS and incubated at 37°C for two hours and tested for surface Ig as above.

### Fc Receptors for IgG

Lymphocytes at 2 x 10⁶ cells/mL in HBSS/5% FCS were incubated with an equal volume of 2% EA γ-indicator cells (ox erythrocytes coated with rabbit anti-ox IgG) for 45 minutes on ice. Those lymphocytes that rosetted with three or more EA γ-indicator cells were considered to have Fc receptors.

### Complement Receptors (EAC)

Lymphocytes (2 x 10⁶ cells/mL) were mixed in HBSS/5% FCS with an equal volume of Zymosan (ICN Nutritional Biochemicals, Cleveland) that had been previously coated with human complement. After centrifugation at 200 g at 4°C for seven minutes, the pellet was resuspended in half the supernatant volume. Those cells rosetting with three or more particles were considered to possess complement receptors.

### Monoclonal Antibody Typing

Lymphocytes were analyzed by cytofluorography with indirect immunofluorescence using murine monoclonal antibodies and FITC-labeled anti-mouse Ig (Meloy). T cell antibodies tested were OKT3 (Ortho, Raritan, NJ), Leu-2a, Leu-3a (Becton Dickinson, Sunnyvale, Calif), and Lyt-1 and Lyt-2 (New England Nuclear, Boston). B cell antibodies included B1 (Coulterclone, Hialeah, Fla) and HLA-DR (Cappel, Downingtown, Pa). The J5 antibody (anti-common acute lymphocytic leukemia antigen [anti-cALLA]) (Coulterclone) was also tested.

### Isolation of Monoclonal Ig

The monoclonal IgM from the patient’s serum was isolated using ammonium sulfate precipitation followed by gel filtration with Sephadex G200 (Pharmacia, Uppsala, Sweden) in 0.1 mol/L Tris-HCl, pH 8.0.

### Hemagglutination

Hemagglutination of untreated and neuraminidase-treated sheep and ox erythrocytes was carried out in microtiter plates as described by Avrameas et al.

### Human Hybridoma Formation Fusion

A human hypoxanthine-aminopterin-thymidine (HAT) medium-sensitive plasmacytoid cell line (DHMC line) was used as the fusion partner. Peripheral blood lymphocytes from the patient and the DHMC cells were both washed in serum-free RPMI 1640 (GIBCO, Grand Island, NY) and the cells were combined at a ratio of 5:1 (five CLL cells per one plasma cell). One milliliter of 38% polyethylene glycol (PEG) was layered over the cells, which were incubated for one minute at 37°C and then washed free of PEG with RPMI 1640. Cells were suspended at 10⁶ total cells/mL in Dulbecco’s MEM/20% FCS and dispensed in 96-well round-bottomed trays (100 µL).

### Table 1: Percentage of Patient and Control Peripheral Blood Lymphocytes Expressing Various Surface Markers

<table>
<thead>
<tr>
<th></th>
<th>T Cell Markers</th>
<th>B Cell Markers†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient</td>
<td>Control</td>
</tr>
<tr>
<td>Eₐ rosettes</td>
<td>98%</td>
<td>76%</td>
</tr>
<tr>
<td>OKT3*</td>
<td>1%</td>
<td>74%</td>
</tr>
<tr>
<td>Leu-2a*</td>
<td>&lt;1%</td>
<td>20%</td>
</tr>
<tr>
<td>Leu-3a*</td>
<td>&lt;1%</td>
<td>52%</td>
</tr>
<tr>
<td>Lyt-3*</td>
<td>2%</td>
<td>82%</td>
</tr>
</tbody>
</table>

*Only determined by cytofluorography.
per well). After overnight incubation at 37 °C in humidified air with 5% CO₂, 100 µL of 2× strength HAT medium was added to each well. The trays were then returned to incubation.

Screening

Hybridoma clone supernatants were screened for the production of the patient’s monoclonal IgM using the hemagglutination assay as previously described.

RESULTS

Surface Marker Studies

The essential B cell nature of the patient’s leukemic lymphocytes was clearly demonstrated (Table 1). Surface membrane-bound Ig was detectable on 98% of the isolated peripheral blood lymphocytes by immunofluorescent microscopy or cytofluorography with FITC-labeled polyclonal anti-human Ig. Moreover, 91% of the cells were positive for the B1 antigen, and 94% possessed the HLA-DR antigen. The common ALL antigen (cALLA) was not present on the cells. The only detectable surface light chain was lambda. Greater than 90% of the cells possessed µ, δ, and γ heavy chains. This is a consistent finding, despite removal of cytophilic protein with an acetate wash. These immunoglobulins were found after overnight incubation in Dulbecco’s MEM with 20% FCS and after trypsinization followed by 24 hours of incubation.

The patient’s leukemic cells consistently were found to rosette sheep erythrocytes. In the standard assay, employing neuraminidase-treated SRBCs, 98% of the lymphocytes were positive. No rosette formation occurred with untreated SRBCs, however. Cytofluorography, after staining the cells with Lyt-3, a monoclonal antibody specifically directed against the SRBC receptor, demonstrated only 2% positivity, while 82% of normal peripheral blood lymphocytes reacted. Furthermore, only 1% of the cells were positive with OKT3, a pan-T cell marker (control peripheral blood lymphocytes, 74%). The suppressor T cell subset marker, Leu-2a, stained positively in < 1% compared to 20% of control cells, and Leu-3a, the helper T cell marker, was positive in < 1% compared to 52% in the control.

Inhibition of E Rosette Formation

Since the data indicated the E rosette formation to be a finding unassociated with a true SRBC receptor, we postulated that the patient’s clonally derived cells possessed surface antibody with a specificity for antigen (or antigens) on SRBCs. Lymphocytes were preincubated with a monospecific anti-Ig antisera prior to performing the E rosette assay with neuraminidase-treated SRBCs. Results in Table 2 show that there was nearly total inhibition of rosette formation only when anti-IgM and anti-λ were used. No inhibition of E rosetting was observed when control lymphocytes were so treated.

Hemagglutination

As noted, the patient’s serum contained a small amount of monoclonal IgM. It is likely that this was produced by his leukemic lymphocytes, as there was also surface IgM present on the cells.

Serial dilutions of the patient’s serum and a similar concentration of the isolated IgM paraprotein were tested for their ability to agglutinate RBCs in a hemagglutination assay. In each case, neuraminidase-treated SRBCs were agglutinated at a dilution of 1:1,000,000. In contrast, there was no agglutination observed of untreated SRBCs or of ox erythrocytes with or without neuraminidase pretreatment. Normal control sera and sera from three other CLL patients failed to show significant agglutination titers.

Hybridoma Formation

Further confirmation that the patient’s monoclonal IgM was indeed responsible for the spurious rosette formation and hemagglutination results was derived from the hybridoma studies. Peripheral blood lymphocytes fused with the human plasma cell HAT-sensitive line (DHMC line) produced clones of cells beginning four weeks after fusion. Clonal supernatants from twelve 96-well trays were screened for their ability to cause hemagglutination of SRBCs. Supernatants from

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**Table 2. Inhibition of E, Rosette Formation Following Incubation With Monospecific Antisera Prior to E, Rosette Assay**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>96%</td>
<td>63%</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>2%</td>
<td>53%</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>67%</td>
<td>57%</td>
</tr>
<tr>
<td>Anti-IgD</td>
<td>95%</td>
<td>54%</td>
</tr>
<tr>
<td>Anti-κ</td>
<td>93%</td>
<td>64%</td>
</tr>
<tr>
<td>Anti-λ</td>
<td>2%</td>
<td>59%</td>
</tr>
</tbody>
</table>

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**Fig 1. Immunoelectrophoresis of hybridoma supernatants.** The anode is on the right, anti-IgM antibody is in the center trough. The bottom well contains concentrated supernatant from the DHMC line alone. The top well contains concentrated hybridoma supernatant fluid. The arc with the slower cathodal migration has the same electrophoretic mobility as the patient’s IgM.
745 of these wells induced agglutination in titers ranging from 1:16 to 1:512. Concentrated supernatants from a number of these hybridomas, when tested by Ouchterlony double diffusion, contained only IgMλ. While the parent DHMC line secretes 10 to 20 μg/mL of IgM that does not agglutinate SRBCs, the hybridoma clone supernatants clearly contained two IgMλ components, demonstrable by an immunoelectrophoresis. The dominant component (40 to 60 μg/mL) of those tested to date have had the electrophoretic mobility identical to the patient's isolated IgM paraprotein (Fig 1). Similar to the patient's paraprotein, the hybridoma-produced IgM causes hemagglutination of neuraminidase-treated SRBCs, but not untreated SRBCs or ox erythrocytes.

DISCUSSION

Our patient's cells clearly possessed B cell surface characteristics. Surface membrane-bound immunoglobulin was present, and the majority of cells had receptors for Fcγ and complement. The use of murine monoclonal antibodies lent further proof by demonstrating the lack of true sheep RBC receptor and the presence of B cell antigens (B1, HLA-DR).

Our postulate that the patient's surface immunoglobulin (slg) and serum monoclonal IgM specifically bound SRBCs appears to have been demonstrated conclusively. Complete inhibition of E rosette formation occurred after preincubation of the CLL cells with anti-IgM or anti-l. The partial inhibition by anti-IgG is difficult to explain. There may have been a small amount of IgG with anti-SRBC specificity produced by the CLL clone, as IgG remained detectable on the cell surface following extensive acetate washing, an unusual finding for CLL cells in our experience.

The hemagglutination studies using the patient's serum and neuraminidase-treated SRBCs revealed a remarkably high titer of hemagglutinating ability that was attributable to the monoclonal IgMλ. A unique opportunity to substantiate our findings further was afforded by the hybridoma technique that led to clones of cells producing hemagglutinating antibodies.

Other cases of malignant lymphocytes possessing slg capable of inducing SRBC rosettes have been described. In one series of various malignant lymphomas, three cases were noted to be positive for lα, slg, and E rosette, and all appeared to bind SRBCs through the slg.23 Similarly, a B cell lymphoma line has been demonstrated to possess this activity.26 Interestingly, there has been an increasing number of cases of CLL reported with slg-positive, E rosette-positive phenotypes in recent years, indicating that this phenomenon may not be as rare as we originally suspected.

Review of the case histories of six of these patients is interesting. Four of the six patients, including our own, have in common prolymphocytic-type morphology, mixed with more mature-appearing lymphs. In one case with a markedly elevated WBC count of 800,000, the blast-like cells are reported as the predominant cell.17,19 Also, clinically, these four patients were similar in that all had hepatosplenomegaly without significant peripheral lymphadenopathy. Though this is not inconsistent with CLL per se, this appears to be an atypical picture compared to the majority of cases of CLL.

It should be noted that several groups have reported that 1% to 6% of normal peripheral blood lymphocytes possess slg, Fcγ receptors, and the ability to rosette SRBCs.27,28 Lymphocytes were stained with anti-Ig (to detect slg) or aggregated Ig (to detect Fcγ receptors), and then the standard E rosette assay was carried out. Fluorescent microscopy was used to evaluate rosetting cells for the presence of fluorescent antibody. The basis of this phenomenon is not clear, but it is possible that these are T cells with adherent cytophilic antibody, that they are antigen-activated T cells that have bound immune complexes,27 or that they represent a normal counterpart of the abnormal CLL cells found in our patient.

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