Detection of Two Distinct Malignant B Cell Clones in a Single Patient Using Anti-Idiotype Monoclonal Antibodies and Immunoglobulin Gene Rearrangement

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Immunoglobulin gene rearrangement analysis and somatic cell hybridization techniques were used to examine the malignant cell population in an unusual patient with hairy cell leukemia and macroglobulinemia (N Engl J Med 296:92, 1977). Although previous investigations suggested that the IgM macroglobulin was secreted by the circulating leukemic cells, anti-idiotype monoclonal antibodies raised to the IgM macroglobulin failed to react with the malignant cells in the circulation and bone marrow. In contrast, approximately 50% of the mononuclear cells from an enlarged inguinal lymph node reacted strongly with the anti-idiotype antibodies. Subsequent reanalysis of all cell populations demonstrated that whereas the circulating and bone marrow cells were IgM-expressing, the macroglobulin was IgM-bearing and the lymph node cells were evenly divided among IgM-bearing and IgM-nonbearing. Immunofluorescence flow cytometry indicated that those lymph node cells that reacted strictly with the anti-idiotype antibody were IgM-bearing, demonstrating that they were the source of macroglobulin. An analysis of immunoglobulin gene DNA confirmed the coexistence of two distinct malignant B cell populations in the lymph node and indicated that the IgM-bearing lymph node cells were identical to the circulating and bone marrow leukemic cells.

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B CELL-DERIVED leukemias and lymphomas evolve from a single clone and therefore bear a unique immunoglobulin on their surface membrane.1-4 Because these cells are monoclonal, immunoglobulin that is secreted or membrane-bound is restricted to expressing a single κ or λ light chain and a single variable region on both the heavy and light chains. Because these determinants are unique to the malignant cells and are extremely rare among normal cells, they represent an ideal target for antibody therapy as well as a powerful tool for studying the clonal origin of human B cell malignancies.4

Anti-idiotype antibodies have been prepared against idiotypic determinants present on lymphocytes from individuals with lymphoid leukemia,4 macroglobulinemia,9 and multiple myeloma.6,10 The potential clinical utility of anti-idiotype antibodies has been demonstrated by Miller and co-workers using a murine monoclonal anti-idiotype antibody to treat a patient with diffuse poorly differentiated lymphocytic lymphoma.11

In the present study, we have used somatic cell hybridization techniques to prepare anti-idiotype monoclonal antibodies against the malignant cell population for a patient with concurrent macroglobulinemia and hairy-cell leukemia. This patient was reported as being the first case of hairy cell leukemia in whom the malignant cells appeared to secrete a paraprotein.12 Five anti-idiotype clones representing five murine immunoglobulin isotypes were produced, using the circulating paraprotein as the immunogen. These antibodies were then utilized to analyze this patient's leukemia cells.

MATERIALS AND METHODS

Case history. The patient is a 68-year-old man who is medically knowledgeable and who first noted an erythrocyte sedimentation rate of 31 mL/h from a previous mean of 11 mm/h in 1959.13 He also noted that the protein paper electrophoresis showed a small M component in the fast γ region. In 1965, splenomegaly and bone marrow lymphocytosis were documented. In January 1976, his hemoglobin was 7.5 g/dL, and he had a WBC count of 47,000/μL. He was referred to UCLA for additional studies. The liver was not enlarged, but the edge of the spleen was palpable 10 cm below the left costal margin. Approximately 20% of the peripheral blood lymphoid cells showed fine, hairlike projections. Platelet count was 104,000/μL and reticulocyte count was 6.8%. The bone marrow, although difficult to aspirate, showed almost complete replacement of the normal architecture by lymphoid cells. Protein electrophoresis disclosed an M component, immunoelectrophoresis showed an atypical IgM, and quantitative immunoglobulins demonstrated an IgM level of 1.025 mg/dL.

The malignant cells stained prominently for acid phosphatase which was tartrate resistant, and confirmation as isoenzyme 5 was confirmed by polyacrylamide gel electrophoresis (PAGE). Scanning electron microscopy demonstrated prominent and numerous cytoplasmic projections, and he was diagnosed as having hairy cell leukemia. Immunologic studies on the cells at that time showed that 90% of the patient's peripheral blood lymphocytes expressed surface IgM, and 81% contained cytoplasmic IgM. Tests for κ and λ light chains were not performed. It was also determined at that time that the hairy cell leukemia cells in vitro could secrete an IgM immunoglobulin.12

Due to continued fatigue and anemia, the patient underwent a splenectomy in 1980. Following the splenectomy, his hemoglobin increased and stabilized at 10 g/dL; WBC count was 10,000/μL with 60% lymphocytes and 40% neutrophils. His paraprotein was 1000 mg/dL by quantitative immunoglobulin, and electrophoresis continued to show an M component. The spleen weighted 860 g and microscopic examination of the spleen demonstrated an expanded red pulp with a diffuse infiltration of small round lymphoid cells.

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occupying sinusoids and cords of Bilroth. The regional lymph nodes demonstrated a similar infiltration, whereas a liver biopsy revealed no evidence of leukemic infiltrate. He has continued at full activity and has only been troubled by inguinal and femoral lymphadenopathy that causes some peripheral edema.

He was referred to the National Cancer Institute for the purpose of developing anti-idiotype monoclonal antibodies for therapy of his hairy cell leukemia. The bone marrow was almost entirely replaced by a monotonous population of cells which also represented >90% of the peripheral blood cells. Biopsy of an inguinal lymph node demonstrated effacement of the normal architecture with diffuse infiltration, with well-differentiated lymphocytes having plasmacytoid features without characteristic features of hairy cell leukemia cells. His WBC count was 9,600/μL with 63% lymphocytes, 34% neutrophils, and 3% monocytes; hemoglobin was 9.5 g/dL; platelets, 163,000/μL; and paraprotein was 3,700 mg/dL. Informed consent for all biopsy specimens was obtained.

Preparation of monoclonal anti-idiotype antibodies. Purified IgM from the patient’s serum was prepared by repeated euglobulin precipitation (dialysis against a 200-fold volume excess of distilled water), followed by passage of the IgM-enriched fraction over Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ). BALB/c mice were injected intraperitoneally with 100 μg of purified patient IgM emulsified in Freund’s complete adjuvant (Sigma Chemical Co, St Louis, Mo). A single booster injection of 100 μg was administered intravenously (IV) after seven days. Four days after the final immunization, the mice were killed, and the spleen cells were fused with the P3-X63-Ag8.653 (6.53) or NS-1/Ag4 (NS-1) mouse myeloma cell line as previously described.13

Ten to 14 days after fusion, culture supernatant fluids were screened for anti-idiotype activity using an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with purified patient or control immunoglobulin. Free sites were blocked with phosphate-buffered saline with 5% chicken serum, after which test supernatant fluids were added and incubated for one hour at 4°C. Appropriately diluted peroxidase-labeled goat anti-mouse IgG and IgM (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, Md) was then added and incubated for one hour at 4°C. The substrate, 2,2′ azino-di-(3-ethyl benzthiazoline sulfonic acid) (Sigma), was added at a concentration of 0.22 mg/mL in citrate-phosphate buffer, pH 4.0. Absorbance was read on a MicroELISA reader (Dynatech Laboratories, Inc, Alexandria, Va) 30 minutes later.

Hybridoma supernatant fluids that reacted with patient IgM and not the control IgM myeloma pool were further tested by ELISA against a panel of purified human myeloma proteins, a pool containing a minimum of 5 IgM myeloma proteins of both light chain types (Cappel Laboratories, Inc, Cochranville, Pa), and a pool of normal human serum (Flow Laboratories, Inc, McLean, Va, and Microbiological Associates Bioproducts, Inc, Walkersville, Md). Subcloning of idiotype-specific clones was performed by limiting dilution into wells containing normal mouse thymus cells as feeders. Isotyping of selected clone supernatant fluids was performed as described elsewhere.14 Five clones designated 17D443 (IgG2a); EC232 (IgG2a); 5F1-11 (IgG2a); 8A2-2 (IgG2a); and 5F8-1 (IgM) were selected for further evaluation on the basis of isotype and rate of immunoglobulin production.

Immunofluorescence and immunoperoxidase staining procedures. Mononuclear cells from peripheral blood, bone marrow, and lymph node from patient and/or control individuals were isolated by separation on a Ficoll-Hypaque gradient. Binding of the anti-idiotype and control antibodies was assessed by flow cytometry as previously described.14

Immunoperoxidase staining of frozen sections was performed using an indirect, two-stage procedure which has been described elsewhere,10 using peroxidase-conjugated rabbit anti-mouse heavy chain- and light chain-specific antisera (Dako, Accurate Chemicals, Westbury, NY).

Analysis of immunoglobulin gene rearrangements. DNA was purified from peripheral blood and lymph node mononuclear cells as described previously.13 All procedures for the Southern blot hybridization assay of immunoglobulin gene rearrangements, including a description of heavy chain- and light chain-specific DNA probes, have been presented in detail elsewhere.16,17

RESULTS

Production of monoclonal antibodies with specificity for the circulating paraprotein. Following fusion of the NS-1 or 8.653 myeloma cell lines with splenocytes from mice immunized with the patient’s paraprotein with NS-1 or 8.653, hybridoma growth was evident in >90% of the seeded wells by day 14 in culture. Thirty-nine percent of the NS-1 hybridomas and 35% of the 8.653 hybridomas exhibited murine immunoglobulin secretion. However, once testing was completed, only four NS-1 hybridomas (1.8%) and five 8.653 hybridomas (1.5%) were found to have anti-idiotype specificity (reacting exclusively with our patient’s serum IgM). Two clones (17D443 and EC232) originating from the NS-1 fusion and three clones (5F1-11, 8A2-2, and 5F8-1) derived from the 8.653 fusion were expanded and recloned. The antibodies from the resulting clones continued to react specifically with the patient’s IgM (Table 1).

Reactivity of anti-idiotype antibodies with patients tumor cells. By means of immunofluorescence flow cytometry, peripheral blood and bone marrow mononuclear cells were tested against the anti-idiotype antibodies. Although >90% of the peripheral blood and bone marrow mononuclear cells represented a morphologically uniform population of hairy cell leukemia cells, only 4% were weakly reactive with clone 17D443 (Fig 1). The circulating and bone marrow cells reacted with the anti-κ reagent 32% and 20%, respectively, while anti-λ reactivity was negligible. The macroglobulin was then tested by ELISA and by two-dimensional immuno-

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Table 1. Results of the ELISA to Determine Selectivity of Murine Monoclonal Anti-idiotype Antibodies

<table>
<thead>
<tr>
<th>Clone (Isotype of Antibody)</th>
<th>17D443</th>
<th>5F1-11</th>
<th>EC232</th>
<th>8A2-2</th>
<th>5F8-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal human serum</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>0.000</td>
<td>0.000</td>
<td>0.032</td>
<td>0.010</td>
<td>0.003</td>
</tr>
<tr>
<td>G. D. (IgM)</td>
<td>0.000</td>
<td>0.004</td>
<td>0.001</td>
<td>0.000</td>
<td>0.063</td>
</tr>
<tr>
<td>D. M. (IgM)</td>
<td>0.000</td>
<td>0.002</td>
<td>0.000</td>
<td>0.000</td>
<td>0.005</td>
</tr>
<tr>
<td>H. M. (IgM)</td>
<td>0.987</td>
<td>1.097</td>
<td>1.295</td>
<td>1.005</td>
<td>0.875</td>
</tr>
<tr>
<td>L. H. (IgM)</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.016</td>
<td>0.000</td>
</tr>
<tr>
<td>Meloy (IgM, α)</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.032</td>
<td>0.010</td>
</tr>
<tr>
<td>Cappel (IgM, λ)</td>
<td>0.010</td>
<td>0.059</td>
<td>0.001</td>
<td>0.003</td>
<td>0.005</td>
</tr>
</tbody>
</table>
| Polyvalin chloride microtitre plates were coated with purified human immunoglobulin or a 1:100 dilution of pooled normal human serum. Supernatant fluid from the listed hybridoma clones were added. Following extensive washing and blocking, peroxidase-conjugated goat anti-mouse IgG + IgM (H + L) antibodies were added and incubated for one hour at 4°C. Following washing, the chromogenic substrate 2,2′-azino-di-(3-ethyl benzthiazoline sulfonic acid) was added. Results are reported as the optical density at A 405 following a 30-minute incubation at room temperature.
Tumor cells from an inguinal lymph node were prepared as a single-cell suspension and tested by immunofluorescence flow cytometry, and 56% reacted strongly with the 17D443 anti-idiotype antibody (Fig 2A). Similar results were obtained with the other four anti-idiotype antibodies (data not shown). The 17D443 clone was also tested against a variety of malignant B cells from other patients and was unreactive (data not shown). The patient's lymph node cells were 51% \( \kappa \) positive, 40% \( \lambda \) positive, and 77% \( \mu \) positive. Mixture of the anti-\( \kappa \) and anti-\( \lambda \) reagents with the lymph node cells increased the percentage of staining to 90% (Fig 2B), demonstrating that these were indeed two separate populations of cells. Combining the anti-\( \kappa \) reagent with the 17D443 clone also increased the percentage of staining to 90%. However, the addition of the 17D443 antibody and the anti-\( \lambda \) reagent to the lymph node cells resulted in 55% staining with a pattern similar to that seen with the anti-idiotype reagent alone (Fig 2B). This suggested that the same population of cells reacted with the anti-\( \lambda \) reagent and the 17D443 anti-idiotype antibody.

**DISCUSSION**

The generation of anti-idiotype monoclonal antibodies was first reported by Hatsubai and co-workers. Immunoglobulin was "rescued" from the tumor cells by somatic cell hybridization techniques, and the human immunoglobulin generated was used as an immunogen. Murine anti-idiotype monoclonal antibodies specific for the lymphoma-associated immunoglobulin were generated and were later used to treat the patient. The patient entered a complete remission and has remained in remission for >3 years.

In this report, we have generated five anti-idiotype antibody clones that reacted quite specifically with IgM paraprotein of patient H. M., but which were unreactive against a large panel of IgM myeloma proteins and pooled human serum. Based on the original case report of this patient,
demonstrating 4% weak reactivity, which was only mini-

describing hairy cells producing an IgM paraprotein, we

It was therefore concluded that it was this second clone of IgMλ-bearing cells that produced the paraprotein.

One criticism of this study is that if light chain testing had been carried out initially, it would have been obvious that two separate clones were present. However, there exist data suggesting that two light chains may be generated from the same clone. In two separately reported cases of multiple myeloma and one case of B-cell leukemia, both κ and λ light chains were identified in the same cells by double immunofluorescence.22-24 Because a clone of cells may be defined by its idiotype, the use of monoclonal anti-idiotype antibodies should prove to be an important tool in the study of the clonal origins of B cell malignancies. Furthermore, an analysis of patterns of immunoglobulin gene rearrangements may be an even more powerful tool for the study of clonality.25 Two recent reports describing biclonal B cell lymphomas, using both of these techniques, suggest that biclonality may be more frequent than previously envisioned.26,27 In light of these findings, statements on the clonal origins of B-cell malignancies based exclusively on comparisons of paraprotein or surface membrane immunoglobulin light chains may be inappropriate.

We have also developed anti-idiotype antibodies to the IgMκ present on the surface of our patients circulating leukemia cells. Our plan was to use it for therapy in conjunction with the anti-idiotype antibody developed against the IgMλ paraprotein. We decided to begin therapy in our patient due to progressive inguinal adenopathy with swelling of his legs. Our antibodies were not yet ready, and the patient did not wish to receive more chemotherapy. Because of reported high response rates to interferon-α for patients with hairy cell leukemia28,29 and non-Hodgkin’s lymphoma,30 a decision was made to begin therapy with recombinant interferon-α. Shortly after starting interferon, the patient developed a severe hemolytic anemia that was refractory to steroids. Treatment with 2′-deoxycoformycin was also unsuccessful and the patient died.

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