

CONCISE REPORT

Monoclonal Immunoglobulin Rescue From a Patient With Chronic Lymphocytic Leukemia and Autoimmune Hemolytic Anemia

By Karol Sikora, John Krikorian, and Ronald Levy

In order to test whether autoimmune hemolytic anemia in a patient with chronic lymphocytic leukemia was due to autoantibody production by the neoplastic clone, somatic cell hybrids were constructed between the patient's leukemic cells and a mouse myeloma line. Light-chain-restricted human immunoglobulin was secreted by 4

of 11 of the established hybrid lines. No binding of this immunoglobulin to red cell surface antigens could be detected. Thus, we conclude that the autoantibody in this patient represented a concomitant reactive response and was not the product of the malignant clone of B cells.

MOST CASES of chronic lymphocytic leukemia (CLL) result from the neoplastic proliferation of B lymphocytes. These abnormal cells possess the ability to synthesize immunoglobulins that are inserted into the cell's surface membrane. Immunofluorescence with suitable antisera can demonstrate that both cytoplasmic and surface immunoglobulin is restricted in light chain type within all the neoplastic cells¹—a feature to be expected if CLL results from the clonal expansion of a single transformed cell. The failure to detect free monoclonal immunoglobulin in the serum in most cases implies that such immunoglobulin is either not secreted or is rapidly removed by absorption or some other mechanism. Clinically significant autoimmune hemolytic anemia (AIHA) occurs in about 5% of patients with CLL.² Red cells from a further 5% will give a positive direct Coomb's test in the absence of significant anemia. The source of the autoantibody and the mechanisms involved in its production are unknown. It is possible that this abnormal antibody could be the monoclonal product of the leukemic cells. Alternatively, the presence of the neoplasm could disrupt the intricate control mechanisms of the immune network with resultant polyclonal autoantibody production by normal cells. This question could be resolved if sufficient quantities of monoclonal immunoglobulin could be obtained from the leukemia cells. The recent advances in the techniques of somatic cell hybridization allow the rescue of large quantities of surface immunoglobulin from human neoplastic B lymphocytes.³ This involves the fusion of patient's neoplastic cells with a non-immunoglobulin secreting mouse myeloma line in the presence of polyethylene glycol (PEG). Fusion products are

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then selected by growth in appropriate tissue culture media and monoclonal human immunoglobulin-producing lines can be established. In this experiment, such hybrids were constructed using lymphocytes from a patient with CLL complicated by AIHA. The rescued immunoglobulin was then tested for its ability to bind to a panel of erythrocytes.

MATERIALS AND METHODS

Case Report

A 66-yr-old female presented with persistent chest infections, generalized lymphadenopathy, and a mildly elevated peripheral lymphocyte count. Bone marrow and lymph node biopsies were compatible with CLL. Serum immunoglobulins were normal with no evident monoclonal band detected by electrophoresis. A direct antiglobulin test on her red cells was initially negative. She was followed without treatment for 3 yr when a falling hematocrit and platelet count, together with a rising lymphocyte count, prompted therapy with chlorambucil and prednisone. At that time her direct antiglobulin test was positive. Following chemotherapy her lymphocyte count gradually fell, and the thrombocytopenia and anemia corrected. Six months following treatment, her Coomb's test became negative at a time when her lymphocyte count had fallen to its level at presentation (Fig. 1). Her autoimmune hemolytic anemia had therefore occurred at the time when the tumor load was greatest.

Red Cell Studies

Ten cubic centimeters of blood was drawn (with the patient's consent) and anticoagulated with EDTA. Red cells were washed and tested for agglutination using standard anti-human globulin antisera. Surface-bound immunoglobulin was released by acid elution following digitonin hemolysis using well established techniques.⁴ The eluted immunoglobulin was then tested for binding to a panel of red cells in low ionic strength solution.⁵

Somatic Cell Hybridization

Leukemic cells from 20 cc of freshly drawn blood were isolated by Ficoll-Hypaque sedimentation⁶ and used for somatic cell hybridization after storage in 10% dimethyl sulfoxide in a liquid nitrogen freezer. A non-immunoglobulin secreting mouse myeloma cell line, P3/NS1/1-Ag 4, an 8-azaguanine-resistant mutant of MOPC 21,⁷ was used for hybridization with the CLL cells. Polyethylene glycol (Baker, 1540) was used as a fusing agent. Hybridization was performed at room temperature. Human leukemic cells (5×10^7) and 2×10^7 mouse myeloma cells were centrifuged into a common pellet and washed twice in protein-free Dulbecco's Modified Eagle's Medium (DME). The pellet was gently resuspended in 2 ml of 40% PEG and centrifuged for a total of 6 min in the presence of the PEG, gradually bringing the speed

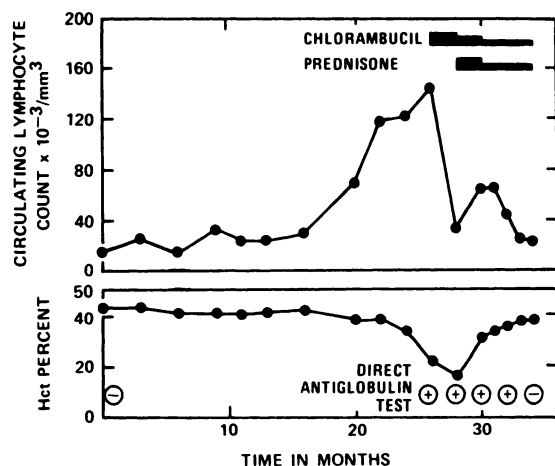


Fig. 1. Clinical course and therapy of a patient with CLL and AIHA.

of centrifugation up to 400 g. The PEG was removed, and the pellet was slowly resuspended in 12 ml protein-free DME. The cells were repelleted, suspended in 50 ml of DME containing 15% FCS and HAT,⁸ and dispensed into 96-well tissue culture plates (Linbro). After 2–4 wk, colonies of hybrid clones became evident.

Immunoglobulin Production Assay

A radioimmunoassay was used for the detection of immunoglobulin secretion by cells in culture.³ Fab₂ fragments of purified goat anti-human κ or λ antibodies, at a concentration of 2 $\mu\text{g}/\text{ml}$, in phosphate-buffered saline (PBS) were used to coat the surface of flexible vinyl microtiter plates (Cooke 1-220-24B) for 3 hr at 20°C. The plates were then washed with 2% bovine serum albumin (BSA) in PBS, and ¹²⁵I-labeled FAB₂ from normal human immunoglobulin was added to the wells in the presence of various concentrations of unlabeled standard competitors or culture supernatants. The plates were incubated a minimum of another 3 hr at 20°C, and then washed with 2% BSA in PBS, cut with a hot wire device, and the wells dropped into tubes for gamma counting.

Red Cell Binding Assay

The ability of secreted immunoglobulin to bind to red cells was determined by a double sandwich binding assay. Serial dilution tissue culture supernatants from immunoglobulin-secreting hybrids were incubated in vinyl microtiter plates with 10⁵ "panel-10" red cells (Biological Corporation of America, Port Reading, N.J.) per well. After 3 hr at room temperature the cells were washed 3 times in PBS with 2% BSA and incubated with goat anti-human κ or λ antisera (Tago Inc., Burlingame, Calif.) at a dilution of 1/400 for 3 hr. Following a second set of washes, the cells were incubated with affinity-purified ¹²⁵I-labeled rabbit anti-goat antibody. The plates were incubated for another 3 hr before being washed a third time, cut with a hot wire device, and the wells counted on a gamma counter. Typing sera currently in routine use in the blood transfusion laboratory at Stanford against anti-E and anti-e were used to check the specificity and sensitivity of the binding assay.

RESULTS

Red Cell Studies

The patient's red cells were strongly agglutinated by standard anti-human globulin antisera at the time of the lymphocyte hybridization. The agglutination was of the warm antibody type occurring with antiimmunoglobulin but not anticomplement antisera. Eluted immunoglobulin was found to agglutinate all members of a panel of red cells indicating nonspecificity. Further analysis showed the red cell binding immunoglobulin to be of the IgM type, similar to that on the leukemic cell surface.

Somatic Cell Hybridization

Within 4 wk of the hybridization, 11 hybrid lines were established that grew well in culture. After at least two changes in culture medium, the supernates were removed and assayed for immunoglobulin production. Four of the 11 supernates contained significant quantities of immunoglobulin, all bearing the light chain of the λ type (Fig. 2). (The patient's CLL cells all bore surface immunoglobulin of the λ type.) Supernates from the hybrids that produced the most immunoglobulin were removed at a time of high cell density and tested for binding to "panel 10" erythrocytes. That hybridization had occurred was evident from the morphology of the resultant cells and the subsequent demonstration by karyotypic analysis of both mouse and human chromosomes.

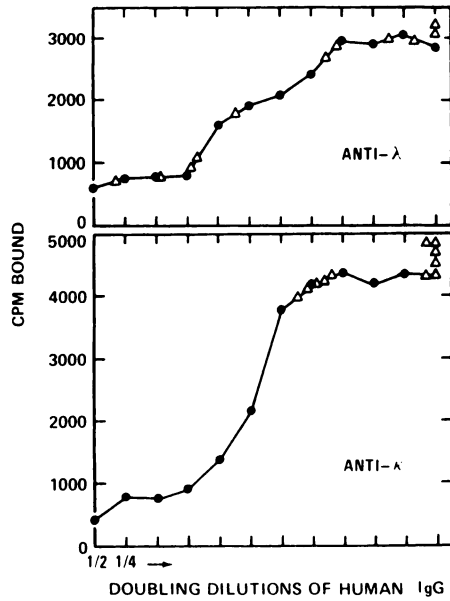


Fig. 2. Radioimmunoassay for human λ and κ chains by inhibition of radioligand binding. The wells of microtiter plates were coated with Fab'_2 of purified goat anti-human κ and λ . The binding of ^{125}I -human Fab'_2 was inhibited by increasing concentrations of unlabeled normal human immunoglobulin. Eleven different hybrid lines gave different degrees inhibition, some of them equivalent to $10\ \mu\text{g}/\text{ml}$ of human immunoglobulin. (●) Doubling dilutions of unlabeled human IgG. (△) Supernatants from human mouse hybrids added undiluted.

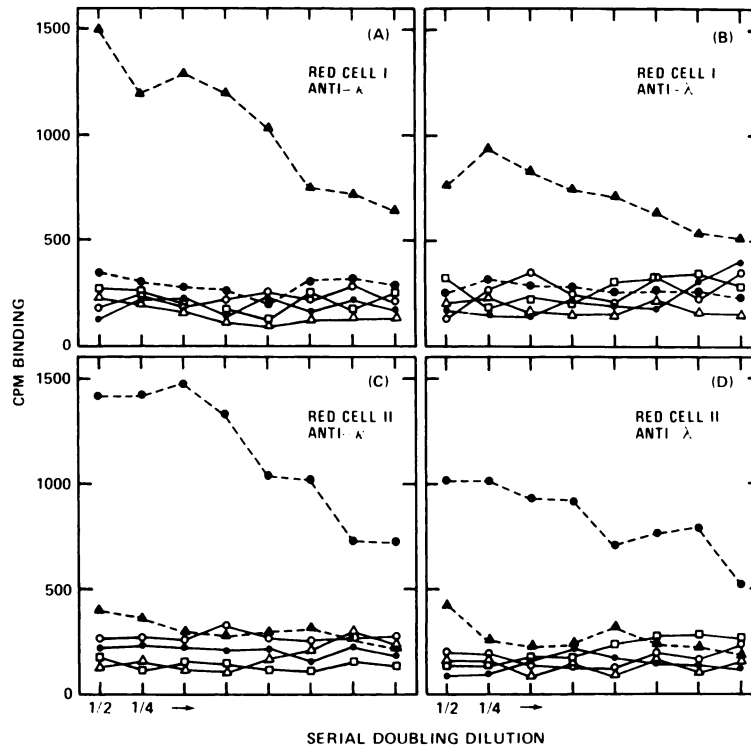


Fig. 3. Assay for binding of immunoglobulin to red cells. After incubating with serial dilutions of test immunoglobulin, the cells were washed and goat anti-human κ or λ antibody added. After a further wash, ^{125}I -rabbit anti-goat antibody was added. Binding of this reagent was determined by a gamma counter. (▲—▲) Anti- κ and (●—●) anti-E, starting at dilution of 1/20. All other symbols represent hybrid supernatants.

Red Cell Binding

Immunoglobulin eluted from the patient's red cells was shown by a standard antiglobulin agglutination test to bind to both R₁R₁ and R₂R₂ erythrocytes in the commercial panel 10. These two cells were used as antigens in the radiolabeled binding assay. The sensitivity and specificity of the assay was tested by the use of anti-e and anti-E antisera from patients who had received mismatched transfusions. As expected, anti-e bound only to the R₁R₁ cells and anti-E to R₂R₂ cells. Both κ and λ light chains were found to be binding, reflecting the polyclonal nature of the immune response to incompatible transfused cells. None of the immunoglobulin secreted by the hybrid cell lines showed any evidence of binding even at low dilutions (Fig. 3). The concentration of immunoglobulin present was of the order of 10 μ g/ml in the supernatant of the hybrid cell lines, a concentration at which red cell binding of antibody would have been easily detected.

DISCUSSION

B-cell neoplasms synthesize immunoglobulins. The quantities and fate of these monoclonal products depend on the lymphoma type. In lymphocytic and histiocytic lymphomas, immunoglobulin is found in the cytoplasm and in many cases on the cell surface. In multiple myeloma, sufficient quantities are secreted by the cell to be detected easily by electrophoresis in the serum of most patients. Some of these immunoglobulins have been found, by random screening, to possess antibody activity directed against blood group substances, bacterial polysaccharides, serum glycoproteins, and haptens.⁹ It is possible that chronic stimulation, either by foreign antigens or altered self-components, could play a part in triggering the process of neoplastic transformation of a B-cell clone, resulting in a lymphoma. The occurrence of AIHA in significant numbers of patients with CLL could thus result from production of anti-red cell antibody as a monoclonal product of the CLL cell. In a series of four such patients, Leddy and Bakemeier¹⁹ found light-chain restriction in the red-cell-bound immunoglobulin suggesting monoclonality. In our study, a direct relationship between tumor burden and Coomb positivity was noted, suggesting that the autoantibody might well be a tumor cell product. However, the rescued monoclonal immunoglobulin did not bind to a red cell panel, which did bind to antibody eluted from the patient's own erythrocytes. Thus, it would seem likely that the hemolytic anemia in this patient with CLL was due to a widespread disturbance in immune function and not due directly to monoclonal antibody secretion.

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