Etiologic Aspects of Cold Agglutinin Disease: Evidence for Cyogenetically Defined Clones of Lymphoid Cells and the Demonstration That an Anti-Pr Cold Autoantibody is Derived From a Chromosomally Aberrant B Cell Clone

By L.E. Silberstein, G.A. Robertson, A.C. Hannam Harris, L. Moreau, E. Besa, and P.C. Nowell

This study investigated the clonal nature of cold agglutinin disease in a series of nine patients, which included the benign or idiopathic form as well as cases with an underlying lymphoma. Surface marker phenotyping and karyotypic analysis were performed on peripheral blood lymphocytes. An increased proportion of B cells was found in four cases and in three of these patients a monoclonal B cell population was identified with a mu, kappa phenotype. In the same three cases, as well as an additional patient, an aberrant karyotype was demonstrated. The cytogenetic abnormality present in all four cases included trisomy 3; two patients also had a trisomy 12. One of these four patients had a well-differentiated lymphoma and underwent a splenectomy. Splenic lymphocytes were transformed with Epstein-Barr virus and cultured en masse. Eight clones were established producing the same cold agglutinin with identical specificity as that present in the patient’s plasma. Five of these clones were studied cytogenetically, and all had the same abnormal karyotype (51,XX,+3,+9,+12,+13,+18) found in peripheral blood and splenic lymphocytes. Thus, in this case, the cold reactive autoantibody was produced by the chromosomally abnormal, neoplastic clone of lymphocytes. Our findings support the view that cold agglutinin disease represents a spectrum of clonal disorders.

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CHRONIC cold agglutinin disease may be idiopathic or associated with an underlying lymphoproliferative disorder. In both instances, hemolysis is usually caused by an IgM, kappa autoantibody that binds to red blood cells at low temperature. The cold agglutinin is considered to be a monoclonal antibody because it may form a homogeneous peak on the serum protein electrophoretic pattern and contains a single light chain. It has been generally assumed that the cold autoantibody is derived from a clone of B cells, which may or may not have expanded to the magnitude of clinical lymphoma, but there is little real evidence in support of this conclusion. In the idiopathic form of this disorder, a clone of lymphoid cells has not been specifically demonstrated, nor has the cold autoantibody been demonstrated to be derived from a defined clone of B cells in either form of cold agglutinin disease.

We studied a series of nine patients with cold agglutinin disease, which included cases of the idiopathic form as well as patients with an underlying lymphoma. Chromosomal data and monotypic cell surface immunoglobulin of lymphocytes in four of nine patients provided definitive evidence for the clonal nature of their disorder. In one of these patients we were also able to demonstrate the origin of the cold autoantibody from a chromosomally abnormal clone of B lymphocytes.

MATERIALS AND METHODS

The study included nine patients with cold agglutinin disease in the age group of 37 to 78 years. Clinical and laboratory data of these individuals were obtained at the time of entry into this study and are summarized in Table I. These patients had a prior history of cold agglutinin disease that ranged from 6 weeks to 10 years. All patients were considered to have idiopathic cold agglutinin disease except patients 1 and 3, in whom an underlying lymphoma was diagnosed. Three patients had evidence of organomegaly. The peripheral smear in all nine patients showed polychromasia of varying degree; spherocytosis was only present in patient 2. The hemoglobin ranged from 5.6 to 10 g/dL and was associated with an elevated reticulocyte count. The leucocyte count ranged from 5,400 to 18,600/mm³ and was not associated with an absolute lymphocytosis (>4,000/mm³) in any of the cases. In patients 1,2,3,4, and 8 an "M" component was noted on the serum protein electrophoretic pattern and identified as IgM, k by immunofixation.

Description of four patients with evidence for a circulating clone. Patient 1 is a 37-year-old woman with a 2-year history of cold agglutinin disease who was admitted to the hospital with a severe hemolytic episode. On physical exam, the spleen tip was palpable 3 finger breadths below the left costal margin. There was no peripheral lymphadenopathy. Splenomegaly was confirmed by ultrasound and computerized tomography scans that did not reveal abnormalities in the liver or lymphadenopathy. A bone marrow aspirate revealed erythroid hyperplasia but no increase in lymphocytes or plasma cells. Laboratory studies performed at that time are summarized in Table I. She was treated with red blood cell (RBC) transfusions, corticosteroids, and plasma exchange. Despite 8 weeks of therapy her hemoglobin level remained below 7 g/dL, and a splenectomy was performed. Light microscopic examination of splenic tissue and two splenic hilar lymph nodes was consistent with a malignant lymphoproliferative disorder. Frozen section analysis of the spleen and one node for cell surface antigens suggested a monoclonal population of B cells which stained positively with antibodies to B1, IgM, kappa, and HLA-Dr. The patient was diagnosed as having a well-differentiated lymphoma. Clinically she did not respond to splenectomy and is presently receiving combination chemotherapy consisting of cyclophosphamide, vincristine, and corticosteroids.

Patient 2 is a 62-year-old man who at the time of the present study, had a 2-year history of chronic cold agglutinin disease. Physical exam revealed hepatomegaly which was confirmed by liver spleen scan with a homogenous uptake of radionucleotide. A liver biopsy and bone marrow studies failed to show evidence of a lymphoproliferative disease process. Laboratory data obtained 2 years after initial diagnosis are illustrated in Table I. The patient...
disease. Physical exam revealed continued to sheets of small lymphocytes consistent evidence of acrocyanosis. Laboratory data are shown ties showed no underlying lymphoma. In an attempt to control the hemolytic process that is ascribed to an He is presently undergoing combination chemotherapy 6 weeks after diagnosis of cold agglutinin disease showed splenomegaly and blood transfusions. Physical exam, with lymphoma. The patient continued to have severe hemolysis and required frequent RBC transfusions. He is presently undergoing combination chemotherapy in an attempt to control the hemolytic process that is ascribed to an underlying lymphoma. Patient 4 is a 78-year-old woman with a 6-year history of idiopathic cold agglutinin disease. During the past 3 years she has required intermittent RBC transfusions for symptomatic anemia. By physical exam, chest x-ray, and bone marrow studies the patient has no evidence of an underlying lymphoma. She was admitted to our hospital for treatment of an exacerbation of her cold agglutinin disease. Physical exam revealed no organomegaly and the extremities showed no evidence of acrocyanosis. Laboratory data are shown in Table 1. She was transfused with 2 units of packed red blood cells and was discharged with a stable hemoglobin of 9.2 g/dL. During the next 4 months she did not require additional therapy and maintains a hemoglobin of 8 to 9 g/dL.

Serology. Cold agglutinin titers were performed at 4°C with measured quantities of group O normal adult red blood cells (RBC), L-antigen negative group O adult RBCs, group O cord RBCs, and autologous RBCs. Test erythrocytes were also treated with ficin and neuraminidase according to standard methods. The serum of all patients was also incubated with 0.01 mol/L of the IgM-reducing agent diethiothreitol (DTT) in phosphate-buffered saline and tested for residual cold autoantibody activity.

Isolation of cold autoantibodies. Equal volumes of saline-washed packed red blood cells and patient serum were incubated for two hours at 4°C with inversion of the tubes every ten minutes. The cells were centrifuged and the supernatants removed at 4°C. The cells were washed six times with normal saline (4°C) and a heat eluate prepared at 40°C. The cold antibody isotype was then evaluated by Ouchterlony analysis (Cooper Biomedical, Malvern, Pa).

Surface marker phenotyping of lymphocytes from peripheral blood and spleen. Lymphocytes, separated from peripheral blood and spleen in one case by density gradient centrifugation on Ficoll–Hypaque, were labeled by a double-antibody fluorescent staining technique. Cells were incubated for 30 minutes at 4°C with an appropriate dilution of mouse monoclonal antibodies directed against B- or T-cell surface antigens. Antibodies used were OKIa, OKT11, OKT3, OKT4, OKT8 (Orthomune, Ortho Diagnostic Systems Inc., Raritan, NJ); B1 (Coulter Immunology, Hialeah, Fla); and antihuman IgG, IgM, IgD, kappa and lambda light chains (Becton Dickinson Co, Sunnyvale, Calif). Labeled cells were washed three times in cold phosphate-buffered saline and stained with a second antibody, fluorescein-conjugated goat antimouse IgG (Tago, Burlingame, Calif). Cells were washed, fixed, and analyzed by cytofluorometry (Spectrum III, Ortho Diagnostics).

Karyotypic analysis. Mononuclear cells were cultured for 72 to 96 hours with a combination of a 1:1000 dilution of pokeweed mitogen (Gibco Laboratories, North Andover, Mass), and the phorbol ester TPA (Chemicals for Cancer Research, Eden Prairie, Minn, 0.01 to 0.05 mcg/mL) supplemented with crude preparations of interleukin-2 that contained B cell growth factor activity, as previously described.

Air dried slide preparations were trypsin-Giemsa banded by standard techniques. From 12 to 56 metaphases were counted, and at least three karyotype analyses were performed on each specimen. Additional karyotypic analyses were carried out when necessary to characterize chromosomally abnormal clones.

Establishment of Epstein-Barr Virus (EBV)–transformed cell lines. Splenic lymphocytes from patient 1 were isolated by Ficoll–Hypaque gradient centrifugation. Cells (10⁶) were suspended in 4 mL RPMI 1640 medium, supplemented with 10% fetal bovine serum and 10 μg/mL/gentamycin. The EBV-containing supernaant of the B95-8 Marmoset cell line was first freeze-thawed and subsequently filtered. One mL of this supernatant was added to the lymphocyte suspension. The lymphocytes were then cultured at 37°C in 5% CO₂ in 95% humidified air in 25 cm² tissue culture flasks for 6 weeks. Karyotypic analysis was performed on the cells, and the supernatant was tested for cold agglutinin activity against untreated and ficin-treated group O normal adult RBCs. The cells were subcloned by limiting dilution in 96 well plates. Statistical clones were then again analyzed by karyotypic analysis and their supernatant tested for cold agglutinin activity.

RESULTS

Serology of cold autoantibodies. The cold agglutinin of patient 1 was not reactive against enzyme-treated RBCs and was therefore classified as anti-Pr. The reactivity of the other eight patients' cold autoantibodies was enhanced with ficin-treated RBCs. The sera of patients 3, 4, 6, 7, 8, and 9 reacted stronger with adult RBCs than with cord RBC and thus demonstrated an anti-I specificity. The sera of patients 2 and 5 reacted equally strong with adult and cord RBCs.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Duration of Disease*</th>
<th>Organomegaly</th>
<th>Histologic Evidence for Lymphoma</th>
<th>Hgb (g/dL)</th>
<th>Ret. Ct. (%)</th>
<th>Titer</th>
<th>Paraprotein†</th>
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<td>16384</td>
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<td>—</td>
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<td>no</td>
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<td>9.5</td>
<td>1024</td>
<td>—</td>
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</table>

*At the time of entry into the study.
†The values are expressed as reciprocal of titer at 4 °C against adult red blood cells.
‡The nature of the paraprotein is determined in the serum by protein electrophoresis followed by immunofixation.

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Inactivation of cold agglutinin activity by the IgM-reducing agent dithiothreitol was observed in all patients' sera except patient 2. In the latter patient an RBC cold absorption of this patient's serum followed by heat elution identified both IgM and IgG in the eluate by Ouchterlony analysis. The presence of an IgG cold antibody or the possibility that the pathologic IgM cold antibody was, in part, monomeric could explain the resistance to dithiothreitol. The cold agglutinin titers in the patients' sera ranged from 1:512 to 1:16384 (Table 1) against normal group O adult RBCs at 4 °C. All the cold agglutinins were reactive at 25 °C although weaker than at 4 °C. No cold agglutinin activity was observed at 37 °C except in patient 8 whose serum had a titer of 1:4 at 37 °C.

Surface phenotype. Surface marker phenotyping was performed on seven patients (Table 2); in the other two patients, insufficient lymphocytes were isolated for complete phenotyping. All patients demonstrated circulating T cells with a normal ratio of OKT4:OKT8 positive cells (with the exception of patient 9 where the proportion of OKT8 cells was slightly elevated). Four of the seven patients (cases 1, 2, 3, 5) showed an increased proportion of B cells (determined by the sum of kappa and lambda light chains ≥ 30%), and in all of these there was a distortion of the normal 2:1 kappa-to-lambda ratio suggesting the presence of a monoclonal B cell population bearing kappa light chains. The heavy chain isotype was identified as mu in three cases (patients 1, 2, 3); adequate data were not obtained on case 5. Patient 3 also demonstrated staining for gamma heavy chains, but this was very weak and probably extrinsic. The other cases (patients 4, 7, and 9) of cold agglutinin disease showed no evidence of a circulating monotypic B cell population in the circulation.

Surface marker analysis of the splenic lymphocytes in patient 1 showed a predominance of B cells with the μ,κ phenotype, comprising 68% of all splenic lymphocytes.

Karyotypic analysis. In four cases (patients 1, 2, 3, 4), a clone with an aberrant karyotype was demonstrated (Table 3). In all four cases the cells were trisomic for chromosome 3, with this being the only abnormality in two instances (patients 2, 3); in patient 1 (Fig 1) and patient 4, abnormal clones also included trisomy 12 (Table 3). A cytogenetically aberrant clone was not detected in the other cases (patients 5 through 9) of cold agglutinin disease including patient 5 where a monoclonic B cell population had been identified in the circulation. Conversely, in patient 4 surface marker phenotyping of peripheral blood lymphocytes did not identify a monotypic B-cell population, while karyotypic analysis demonstrated the presence of a chromosomally abnormal clone.

Characterization of EBV-transformed splenic lymphocytes. After 6 weeks of culture, cytogenetic studies of the EBV-transformed splenic lymphocytes of patient 1 revealed the presence of both eukaryotic cells as well as the clone with the aberrant karyotype 51,XX,+3,+9,+12,+13,+18 (Table 3). Through subcloning by limiting dilution, eight clones were established that also produced the cold autoantibody with Pr specificity similar to the cold antibody present in plasma of the patient; the unconcentrated supernatants of the aberrant clones reacted with group O normal adult RBC (range of titer 1:2 to 1:8) and were unreactive with enzyme-treated RBC. The cold autoantibody produced by the clones was identified as IgM, kappa by immunoelectrophoresis and displayed similar electrophoretic mobility as the paraprotein present in the plasma of the patient. Five of these clones were studied cytogenetically and all had the same abnormal karyotype with 51 chromosomes. All transformed clones expressed EBV nuclear-associated antigen, which was demonstrated by the anticomplement immunofluorescence technique.

Table 3. Chromosomal Abnormalities in Four Patients With Cold Agglutinin Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tissue</th>
<th>Abnormal Cells</th>
<th>Karyotype of Abnormal Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blood</td>
<td>28/56</td>
<td>51,XX,+3,+9,+12,+13,+18</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>12/23</td>
<td>51,XX,+3,+9,+12,+13,+18</td>
</tr>
<tr>
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<td>Blood</td>
<td>6/16</td>
<td>47,XY,+3</td>
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<tr>
<td>4</td>
<td>Blood</td>
<td>13/84</td>
<td>48,XX,+3,+12</td>
</tr>
</tbody>
</table>

* Cytenetic studies on peripheral blood lymphocytes of patients 5 through 9 did not show evidence of a chromosomally abnormal clone.
† Abnormal cells = cells with karyotype of abnormal clone(s).
The majority of patients with chronic cold agglutinin disease have an associated monoclonal IgM antibody that is considered to be the pathologic cold agglutinin. The disorder is rare, having been reported primarily as isolated case reports or in small series of patients. Consequently little information is available regarding the natural history and prognosis. A recent series of a large group of patients illustrated the association of monoclonal cold autoantibodies with both the idiopathic form of cold agglutinin disease as well as a variety of lymphoid neoplasms. Based on this association it has been suggested that cold agglutinin disease represents a spectrum of disorders, ranging from a benign, autoimmune condition such as idiopathic cold agglutinin disease, to a more clearly malignant lymphoproliferative process. To date, a chromosomally aberrant clone of B cells has not been demonstrated in the idiopathic form nor in those cases of cold agglutinin disease with an underlying lymphoma. In the present study of nine patients we were able to identify a cytogenetically defined clone of B lymphocytes in 4 patients. Two of the four patients had an underlying lymphoma while the other two were considered to have the idiopathic disorder; the latter two patients had a long history of cold autoimmune hemolytic anemia and had no histologic evidence of lymphoma using conventional diagnostic methods including physical exam, computerized tomography, chest x-rays, and bone marrow studies.

It has been assumed that the cold autoantibody is produced by B lymphocytes representative of the underlying disorder, analogous to plasma cell disorders such as multiple myeloma, where substantial evidence exists that the monoclonal immunoglobulin is produced by the malignant plasma cells. Many other disease states, however, including nonlymphoid malignancies and several autoimmune diseases have also been described with associated M components. The specificity of the monoclonal antibodies associated with nonlymphoid neoplasms is unknown, but some authors have suggested that they may reflect a monoclonal or oligoclonal immune reponse to the underlying tumor.

Theoretically, a similar etiologic mechanism could be responsible for the M components in cold agglutinin disease, where, in some cases, the cold agglutinins have been shown to bind autologous and allogeneic lymphocytes, presumably because lymphocytes bear surface antigens of the I/i antigen system. Monoclonal cold agglutinins could therefore be produced by an autoreactive B cell clone in response to a lymphoid neoplasm expressing an epitope that is recognized by the cold antibody. Our data in patient 1, however, argue against this view. To address the question of cold autoantibody origin, the patient’s chromosomally abnormal clone of B cells was immortalized and the culture supernatants contained a cold agglutinin with the same IgM, kappa isotype, and Pr specificity as the antibody present in the plasma of this patient. Thus, this proves that the cold autoantibody was derived from a chromosomally aberrant clone of lymphocytes.

The present findings support the view that chronic cold agglutinin disease may include a range of clonal disorders of increasing severity. For instance, a clone of B cells is present, but not detectable by laboratory methods such as those used in this study, and patients may or may not have an associated M component (patients 6 through 9); or, a clone of B cells has expanded and undergone additional somatic genetic change that is now recognized cytogenetically but evidence for lymphoma is lacking with standard diagnostic measures (patients 2 and 4). Finally, a clone of B cells has expanded to the point that it can be diagnosed as a lymphoma (patients 1 and 3).

The cytogenetic abnormality present in all four patients with a karyotypically abnormal clone included trisomy 3. It was the only abnormality in cases 2 and 3. Trisomy 3 is uncommon in human neoplasia, but has been reported occasionally in various B cell and T cell disorders, including multiple myeloma, angioimmunoblastic lymphadenopathy, chronic lymphocytic leukemia (CLL), and adult T-cell leukemia. It almost always occurs in association with other cytogenetic alterations, suggesting that an extra copy of one or more genes on chromosome 3 confers only a slight additional growth advantage in a lymphoid clone. As with trisomy 12, which was observed in two of our cases and is common in B-CLL, the specific genes involved in the pathogenesis of these low-grade lymphoproliferative disorders remain to be identified.

The cytogenetic findings in this study do provide, however, the first indication of an unusual nonrandom chromosomal change, trisomy 3, associated with cold agglutinin disease, as well as additional data on the clonal nature of this disorder. The results of this study also demonstrate that, in one patient, the defined B cell clone was the source of the monoclonal cold autoantibody.

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