Etiologic Aspects of Cold Agglutinin Disease: Evidence for Cytogenetically 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 specific activity 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.

Chronically 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 the idiopathic form of this disorder, a clone of lymphoid cells has not been specifically demonstrated, nor does 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 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 1. 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; spherocytes 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 leukocyte 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, and 4, an "M" component was noted on the serum protein electrophoretic pattern and identified as IgM, k by immunofixation.
continues to have chronic hemolytic anemia, which is treated intermittently with corticosteroids.

Patient 3 was admitted to the hospital with the diagnosis of hemolytic anemia and was treated for 2 weeks with corticosteroids and blood transfusions. Physical exam showed moderate hepatosplenomegaly but no peripheral lymphadenopathy. Pertinent laboratory data are shown in Table 1. A bone marrow aspirate and biopsy performed 6 weeks after diagnosis of cold agglutinin disease showed sheets of small lymphocytes consistent with lymphoma. The patient continued to have severe hemolysis and required frequent RBC transfusions. He is presently undergoing combination chemotherapy for treatment of an exacerbation of her cold agglutinin disease.

Table 1. Clinical and Laboratory Data of Nine Patients with Cold Agglutinin Disease

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Duration of Disease* (yr)</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>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>splenomegaly</td>
<td>yes</td>
<td>7.4</td>
<td>16</td>
<td>512</td>
<td>IgM, k</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>hepatomegaly</td>
<td>no</td>
<td>7.0</td>
<td>12</td>
<td>16384</td>
<td>IgM, k</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>hepatosplenomegaly</td>
<td>yes</td>
<td>8.5</td>
<td>14</td>
<td>2048</td>
<td>IgM, K</td>
</tr>
<tr>
<td>4</td>
<td>78</td>
<td>6</td>
<td>no</td>
<td>7.0</td>
<td>18.2</td>
<td>8192</td>
<td>IgM, k</td>
</tr>
<tr>
<td>5</td>
<td>74</td>
<td>8</td>
<td>no</td>
<td>11</td>
<td>1.9</td>
<td>4096</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>10</td>
<td>no</td>
<td>5.6</td>
<td>10.6</td>
<td>4096</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>5</td>
<td>no</td>
<td>12</td>
<td>5</td>
<td>2048</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>70</td>
<td>5</td>
<td>no</td>
<td>8.4</td>
<td>17.6</td>
<td>16384</td>
<td>IgM, k</td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>½</td>
<td>no</td>
<td>10.1</td>
<td>9.5</td>
<td>1024</td>
<td>—</td>
</tr>
</tbody>
</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 was determined in the serum by protein electrophoresis followed by immunofixation.

<table>
<thead>
<tr>
<th>No.</th>
<th>g/dL</th>
<th>%</th>
<th>mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.2</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>2</td>
<td>9.0</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>8.9</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>4</td>
<td>8.8</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>5</td>
<td>8.7</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>6</td>
<td>8.6</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>7</td>
<td>8.5</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>8</td>
<td>8.4</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>9</td>
<td>8.3</td>
<td>80</td>
<td>300</td>
</tr>
</tbody>
</table>

Cold agglutinin titers were performed at 4°C with measured quantities of group O normal adult red blood cells (RBC), I-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 dithiothreitol (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 2 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, OKT1, 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-staining buffer and stained with a second antibody, fluorescein-conjugated goat antimouse IgG (Tago, Burlingame, Calif). Cells were washed, fixed, and analyzed by cytofluorimetry (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 supernatant 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.
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 (as 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, k 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 monotypic 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 RBCs (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>
<thead>
<tr>
<th>Patient</th>
<th>Tissue</th>
<th>Abnormal Cells/Total 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>Spleen (bulk EBV culture)</td>
<td>12/23</td>
<td>51,XX, +3, +9, +12, +13, +18</td>
</tr>
<tr>
<td>2</td>
<td>Blood</td>
<td>6/16</td>
<td>47,XY, +3</td>
</tr>
<tr>
<td>3</td>
<td>Blood</td>
<td>8/23</td>
<td>47,XX, +3</td>
</tr>
<tr>
<td>4</td>
<td>Blood</td>
<td>13/84</td>
<td>48,XX, +3, +12</td>
</tr>
</tbody>
</table>

*Results expressed as percentage of total lymphocytes.
†Very weak staining.
‡Not determined.

**Table 2.** Surface Phenotype of Lymphocytes in Seven Patients With Cold Agglutinin Disease

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

*Cytogetic 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).
oligoclonal immune response
authors have suggested that they may reflect a monoclonal or
associated with nonlymphoid neoplasms is unknown,

cold agglutinin disease with an
association it
clonal immunoglobulin is produced
disorder, analogous to plasmacytic disorders such as multiple
produced by B lymphocytes representative of the underlying
tumor.

discussion
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 patients31
illustrated the association of monoclonal cold autoantibodies
with both the idiopathic form of cold agglutinin disease as
well as with 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 lympho-
ma. 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 lympho-
ma 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 meth-
ods including physical exam, computerized tomography,
chest x-rays, and bone marrow studies.

It has been assumed that the cold autoantibody is pro-
duced by B lymphocytes representative of the underlying
disorder, analogous to plasmacytic disorders such as multiple
myeloma, where substantial evidence exists that the mono-
clonal immunoglobulin is produced by the malignant
plasma cells.12 Many other disease states, however, including
nonlymphoid malignancies13,14 and several autoimmune dis-
edes15,17 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,13 presumably
because lymphocytes bear surface antigens of the I/i antigen
system.14 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 autoanti-
body 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
clon 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 aberrant 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 occa-
sionally in various B cell and T cell disorders, including
multiple myeloma, angioimmunoblastic lymphadenopathy,
chronic lymphocytic leukemia (CLL), and adult T-cell leu-
kemia.19,20 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,22 the specific genes involved in the
pathogenesis of these low-grade lymphoproliferative disor-
der 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.

acknowledgement
The authors are grateful to the following individuals for their
referral of patients to this study: Drs A.J. Solan, K. Sethi, R.B.
Sklaroff, W. Kirkley, R. Steingart, R.M. Brantz, C.N. Liedman,
and L. Carty MT (ASCP), SBB.
REFERENCES


8. Tsai C-M, Zopf DA, Yu RK, Wistar R, Jr, Ginsburg V: A Waldenstrom macroglobulin that is both a cold agglutinating and a cryoglobulin because of a N-acetyneuraminyl residues. Proc Nat Acad Sci USA 74:4591, 1977


Etiologic aspects of cold agglutinin disease: evidence for cytogenetically defined clones of lymphoid cells and the demonstration that an anti-Pr cold autoantibody is derived from a chromosomally aberrant B cell clone

LE Silberstein, GA Robertson, AC Harris, L Moreau, E Besa and PC Nowell