Lymphocytes Forming Red Cell Rosettes in the Cold in Patients With Chronic Cold Agglutinin Disease

By Ten Feizi, Peter Wernet, Henry G. Kunkel, and Steven D. Douglas

Lymphocytes that formed rosettes with human red cells in the cold were observed in the peripheral blood of four patients with chronic cold agglutinin disease. Similar rosettes were not observed in normals or other controls studied. Specificity for I and i red cells was demonstrated which paralleled that of the serum macroglobulins. The rosettes were inhibited by soluble I and i antigens in accordance with the specificity of the serum antibodies. Lymphocytotoxicity assays with idiotypic antisera made to the serum macroglobulins detected the same proteins on the lymphocyte surface. Evidence was obtained that the rosette test underestimated the number of cells bearing the specific cold agglutinin protein. Antisera against cold agglutinins as well as anti-IgM antibodies blocked the rosette formation. By all of these criteria, the surface lymphocyte receptors were indistinguishable from the serum antibodies of these patients.

It is now well established that lymphocytes with readily demonstrable surface immunoglobulin (Ig) are bone-marrow-derived (B) cells capable of differentiating into antibody-secreting cells. In chronic lymphocytic leukemia the leukemic lymphocytes usually carry a single immunoglobulin, usually IgM, and in Waldenström's macroglobulinemia it has been shown that a proportion of peripheral blood lymphocytes carry one type of surface IgM. By complement mediated lymphocytotoxicity assays we have recently demonstrated idiotypic determinants of IgM on the lymphocytes of two patients with monoclonal serum IgM components. A proportion of Waldenström's macroglobulins have readily demonstrable antibody activity. It was anticipated that examination of the lymphocytes from such patients would enable further comparison of surface antigen receptors with serum antibodies. In chronic cold agglutinin disease the monoclonal IgM proteins usually have specificities for the I and i antigens which are present on human erythrocytes and on certain glycoproteins related to blood-group substances.

This report describes the use of the immunocytoadherence (rosette) test to demonstrate the occurrence of specific I or i binding receptors on peripheral blood lymphocytes of four patients with chronic cold agglutinin disease. The...
specific inhibition of rosette formation with antimacroglobulin and anti-idiotypic antisera and with water soluble I-i antigens confirmed the close similarity of the surface receptors to the secreted proteins.

MATERIALS AND METHODS

Lymphocytes were isolated from heparinized venous blood of four patients with chronic cold agglutinin disease, of 12 healthy persons, and of four patients with Waldenström’s macroglobulinemia without cold agglutinin activity. All four cold agglutinins were IgM kappa proteins; in three of the patients, Phi,15,17 Low,20 and Matos, they had anti-I13 specificity and in the fourth, Nic,1,20 the specificity was anti-i.14 The 12 healthy persons and the four patients with Waldenström’s macroglobulinemia without cold agglutinin activity were tested as controls. The lymphocyte isolation procedure was carried out at 37°C as previously described9 by incubation in nylon columns and Ficoll gradient centrifugation. In some instances the nylon-column stage was omitted.

Red blood cells from an adult of blood group OI and an adult of Oi were used. The Oi cells (donor Osc) were supplied by Mr. Laurence Marsh, New York Blood Center.

Rosette Tests

The isolated lymphocytes were suspended in Hanks balanced salt solution at a concentration of 1.4 × 10⁶/cc. Erythrocytes of group OI or Oi were washed and suspended in Hanks solution at a concentration of 0.5%. Twenty microliters of lymphocyte suspension were mixed in Falcon plastic tubes with an equal volume of red cell suspension and with 10 μl of normal rabbit serum (NRS). The mixture was kept for 5 min at 37°C with occasional agitation and incubated at 4°C for 1 hr. In early experiments the cell mixture was centrifuged at 400 rpm for 5 min at 4°C prior to incubation for 1 hr. Rosette formation (greater than four erythrocytes adhering to a lymphocyte) was assessed in a hemacytometer at 4°C or at room temperature after fixation for 20 min with 4% of a 5%, glutaraldehyde solution in 0.1 M phosphate buffer pH 7.2 containing 6%, sucrose. A minimum of 2000 lymphocytes were counted. In tests of inhibition of rosette formation the NRS was replaced by anti-immunoglobulin sera or by water-soluble I and i antigens. These latter were the ovarian cyst glycoprotein OG 20%, 2 X., which has been shown to contain the several I and i determinants, and a milk glycoprotein which reacts well with only a minority of anti-I antibodies.15 17

For light microscopy, smears of unfixed rosette preparations were made in the cold and stained with Wright’s Stain.

For electron microscopy, rosette preparations from case Nic (studied on two occasions) were enriched by resuspension and gravity sedimentation at 4°C for 2 hr. The relative frequency of various cell types was assessed in rosette preparations without enrichment. The cells were fixed in cold 1.5%, redistilled glutaraldehyde buffered with 0.1 M sodium cacodylate containing 6%, sucrose, postfixed in cold Millonig’s osmium tetroxide, dehydrated. and embedded in Epon. Sections were prepared on an LKB ultrotome III, stained with uranyl acetate lead citrate and examined in a Siemens 101 electron microscope.

Antisera

Antisera were prepared in rabbits as previously described.21

1. Anti-IgM was against an IgM lambda Waldenström macroglobulin, Gray: after absorption with human Fraction II and a Bence Jones lambda protein, it showed IgM specificity by double diffusion.

2. Idiotypic anti-IgM Sto was an antiserum prepared against a Waldenström macroglobulin Sto7 without known antibody activity. After absorption with two other macroglobulins (IgM kappa and IgM lambda), with Fraction II and with normal human serum, this antiserum precipitated only with IgM Sto and not with other macroglobulins.

3. Anti-cold agglutinin sera (anti-CA) were raised against the isolated cold agglutinins of cases Nic and Phi and an additional anti-I cold agglutinin Sch.15,17 After absorption as in (b), these antisera showed individual specificity as well as reactivity with other IgM cold agglutinins.
All three antisera precipitated with the serum cold agglutinins of cases Nic, Phi, and Low but not with macroglobulins without cold agglutinin activity. These antisera will be described in greater detail separately. The antisera and the NRS were inactivated at 56°C and absorbed at 4°C with washed packed ABO cells to remove antihuman erythrocyte agglutinins.

Complement mediated lymphocytotoxicity tests with anti-IgM and antidiotypic antisera were performed by a modification of the microdroplet method as described previously.9

RESULTS

Rosette-forming cells (RFC) were observed only with the lymphocytes of the four patients with cold agglutinin disease and not with those of the four patients with macroglobulinemia without cold agglutinin activity, nor with those of the 12 healthy persons. With cases Phi, Low, and Matos, RFC were observed with Group OI erythrocytes but not with O1 erythrocytes; with case Nic, RFC occurred only when O1 erythrocytes were used. Thus the RFC reflected the specificities of the serum antibodies of these patients. In the unfixed state the rosettes were stable for 24 hr or longer at 4°C, but they dissociated immediately at 20°C. After glutaraldehyde fixation in the presence of NRS, the rosettes were stable at 20°C. In the absence of NRS the glutaraldehyde fixed rosettes were easily disrupted. The numbers of RFC were not affected by glutaraldehyde fixation or by centrifugation of the cell mixtures prior to incubation for 1 hr; after centrifugation the majority of RFC became surrounded by clusters of erythrocytes which concealed the central cell, and this step was therefore omitted after initial exploratory studies.

By light microscopy rosette-forming cells appeared to be small or medium-sized lymphocytes (Fig. 1). Electron-microscopic examination of enriched rosette preparations revealed that the vast majority of RFC had typical features of lymphocytes, namely, dense heterochromatic nuclei with small nucleoli and cytoplasm containing many single ribosomes, rare strands of rough-surfaced endoplasmic reticulum (RER), occasional mitochondria, and a small Golgi zone (Fig. 2). Although frequent plasmacytoid cells (approximately 10%), were observed in the non-enriched preparations, only rarely were such cells observed to form rosettes (Fig. 3). The zones of association between erythrocytes and rosette-forming lymphocytes usually involved numerous projections of the lymphocyte which were in close proximity to the erythrocytes. Frequently inter-
digitations between erythrocyte and lymphocyte projections occurred (Fig. 2). In some planes of section small erythrocyte fragments were found in proximity to lymphocyte plasma membranes; invaginations were also observed in cross section (Fig. 2). Rarely, broader zones of intimate association between erythrocyte and lymphocyte occurred. This latter type of association was more often observed with rosette-forming plasmacytoid cells (Fig. 3).

Table 1 shows the RFC counts observed with the four cold agglutinin patients. Serial observations with case Nic showed considerable fluctuations during an 8-mo period. The RFC counts tended to be higher when the nylon-column procedure was omitted. The counts ranged between 3 and 130 per 10^3 cells when nylon and Ficoll steps were used, and between 11 and 180 per 10^3 when the nylon stage was omitted. On the day when the peripheral blood RFC count was 79 per 10^3, a heparinized bone marrow sample from this patient was tested. Among the mononuclear cells isolated by Ficoll gradient, 13 rosettes per 10^3 were observed. The fluctuations in the numbers of peripheral blood RFC in this patient could not be correlated with changes in blood lymphocyte counts which ranged between 340 and 1300 per cu mm.

With cases Phi and Low more limited data are available. Major fluctuations were not observed and the RFC counts were of the order of 4-11 per 10^3. Case Matos was tested once only and had 18 RFC per 10^3 cells.
To investigate the possibility that the RFC were the result of adsorbed cold agglutinins on the surface of lymphocytes, heparinized blood of a healthy group O person was mixed with an equal volume of whole serum from patients Nic or Phi and incubated at 37°C for 6 hr. and the lymphocytes were isolated, washed, and tested with Oi or O1 erythrocytes as described above. No rosette formation was observed.

Inhibition of Rosette Formation

The results of rosette inhibition tests with cases Nic, Phi, and Low are shown in Table 2. The ovarian cyst glycoprotein OG 20°, 2X which contains I and i determinants was a potent inhibitor of rosette formation in cases Nic and Phi at the concentration used, 1.17 mg/ml. (Case Low was not tested.) The milk glycoprotein which reacts very weakly with the serum antibodies of these two patients had little or no inhibitory effect at a concentration of 1.2 mg/ml. Also shown on Table 2 are the results of inhibition tests with the anti-IgM antiserum and with the antisera against cold agglutinins Nic, Phi, and Sch and against macroglobulin Sto. Complete or almost complete inhibition was observed in all three cases with the anti-IgM serum. The idiotypic antiserum against IgM Sto, as expected, had no inhibitory effect, for this antiserum precipitated only with IgM Sto and not with other IgM proteins. The antiserum
Table 1. RFC at 4°C with Group Oi or O1 Erythrocytes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>Method</th>
<th>RFC per 10^3 Mononuclears</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nic (Oi)*</td>
<td>18 April 1972</td>
<td>F</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NF†</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>15 May</td>
<td>NF</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>8 Aug.</td>
<td>F</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>25 Aug.</td>
<td>F</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>5 Sept.</td>
<td>F</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NF</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>14 Sept.</td>
<td>F</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NF</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>29 Sept.</td>
<td>F</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>16 Nov.</td>
<td>F</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>21 Nov.</td>
<td>F</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>30 Nov.</td>
<td>F</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NF</td>
<td>12</td>
</tr>
<tr>
<td>Phi (O1)*</td>
<td>18 April 1972</td>
<td>F</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>14 Sept.</td>
<td>F</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>29 Sept.</td>
<td>F</td>
<td>4.5</td>
</tr>
<tr>
<td>Low (Oi)</td>
<td>21 July 1972</td>
<td>NF</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>3 Aug.</td>
<td>NF</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>8 Aug.</td>
<td>F</td>
<td>5.7</td>
</tr>
<tr>
<td>Matos (Oi)</td>
<td></td>
<td>F</td>
<td>18.0</td>
</tr>
</tbody>
</table>

*Type of erythrocyte used in rosette test.
†Method of leukocyte separation; F. Ficoll gradient; NF, nylon filtration followed by Ficoll gradient.

against cold agglutinins Nic, Phi, and Sch, in addition to showing individual specificity, showed cross specificity for other IgM cold agglutinins: Each of these three antisera precipitated with cold agglutinins Nic, Phi, Low, and certain other IgM cold agglutinins but not with IgM proteins without cold agglutinin activity. The high degree of rosette inhibition observed with the three anticolcold agglutinin antisera indicated that the lymphocyte surface cold agglutinin receptors carry the same cross-specificity determinants as the serum cold agglutinins.

Table 2. Inhibition of RFC With I-i Antigens, With an Anti-Human IgM and With Idiotypic Anseria

<table>
<thead>
<tr>
<th>Patient</th>
<th>RFC per 10^3 Mononuclear Cells</th>
<th>Anti-IgM</th>
<th>Anti-Ca*</th>
<th>% Inhibition of RFC</th>
<th>Anti-Ca*</th>
<th>Anti-Ca*</th>
<th>Anti-Ca*</th>
<th>Anti-idio Stof</th>
<th>Glycoprotein OG (l + i)</th>
<th>Milk Glycoprotein</th>
</tr>
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<tbody>
<tr>
<td>Nic</td>
<td>10.0</td>
<td>92</td>
<td>84</td>
<td>84</td>
<td>82</td>
<td>0</td>
<td>nt†</td>
<td>nt†</td>
<td>nt</td>
<td>nt‡</td>
</tr>
<tr>
<td></td>
<td>80.0</td>
<td>100</td>
<td>98</td>
<td>100</td>
<td>93</td>
<td>nt†</td>
<td>nt†</td>
<td>nt†</td>
<td>nt†</td>
<td>nt‡</td>
</tr>
<tr>
<td>Phi</td>
<td>4.5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>0</td>
<td>94</td>
<td>0</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>100</td>
<td>nt</td>
<td>nt</td>
<td>88</td>
<td>0</td>
<td>nt</td>
<td>nt‡</td>
<td>nt</td>
<td></td>
</tr>
</tbody>
</table>

*The absorbed anticold agglutinin sera, anti-CA Nic, anti-CA Phi, and anti-CA Sch, which in precipitation tests showed cross specificity for other IgM cold agglutinins, were potent inhibitors of all of the cold agglutinin rosettes.
†The idiotypic antiserum against Sto which precipitates only with IgM Sto and not with other IgM proteins caused no inhibition of rosette formation.
‡nt—not tested.
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Table 3. RFC at 4°C With Group Oi Erythrocytes, and Mean Cytotoxicity Values With Anti-IgM and Anti-cold Agglutinin Antisera. Lymphocytes Were From Patient Nic.

<table>
<thead>
<tr>
<th>Cells Killed* per 10⁵</th>
<th>RFC per 10⁶ Cells</th>
<th>Anti-IgM</th>
<th>Anti-CA Nic</th>
<th>Anti-CA Phi</th>
<th>Anti-CA Sch</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1401</td>
<td>80</td>
<td>50</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>164</td>
<td>109</td>
<td>44</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

* Mean values after subtraction of background killing with normal rabbit serum.
† Mean cytotoxicity values with cells from a healthy person ranged from 0 to 7 per 10⁵ with the absorbed anti-cold agglutinin sera.

The cytotoxicity values with anti-IgM represent the total number of IgM-bearing cells. The values with anti-CA Nic represent the number of cells carrying surface antibodies similar to or identical with cold agglutinin Nic. The cytotoxicity obtained with the other two anti-CA sera indicate that the membrane-bound cold agglutinins, like the serum antibodies, contain cross-specificity determinants.

Comparison of RFC Numbers and Lymphocytotoxicity With Idiotypic Antisera

In cytotoxicity tests the numbers of lymphocytes killed with idiotypic antisera were usually higher than the numbers of RFC. The results of two sets of experiments with Nic lymphocytes are shown in Table 3. The RFC counts were 10 and 39 per 10⁶ lymphocytes when the respective cytotoxicity values with anti-CA Nic were 80 and 109. With anti-CA Phi and Sch cytotoxicity values of 30–57 per 10⁵ were observed; these were interpreted as being due to cross specificity since the corresponding values with cells from a healthy person ranged from 0 to 7 per 10⁵. Further cytotoxicity experiments with the cold agglutinin antisera have been published previously.⁹

DISCUSSION

Although 64% of healthy persons have low titers of “naturally occurring” anti-I cold agglutinins,²³ no RFC were seen in tests with OI cells in 12 healthy donors; thus their incidence in peripheral blood must be extremely low. In patients with chronic cold agglutinin disease, however, peripheral blood lymphocytes have readily demonstrable surface receptors that react with erythrocyte bound and water-soluble I and i antigens in a manner similar to or identical with their serum antibodies. As with the serum antibodies, the reaction is stable only in the cold, but glutaraldehyde fixation renders the rosettes stable at 20°C and enables readings to be carried out conveniently at room temperature. The marked inhibition of rosette formation with anti-IgM and anti-cold agglutinin antisera and, in particular, the demonstration of cross-idiotypic specificity gave evidence that these receptors were structurally closely similar to the serum antibodies.

The light- and electron-microscopic studies indicate that the vast majority of RFC have the morphologic characteristics of lymphocytes. Very rare plasmacytoid²⁴ cells formed rosettes. These observations indicate that the majority of the RFC are not actively secreting Ig but are cells bearing membrane-bound IgM receptors. Furthermore, these findings are consistent with the view that cells, as they mature, tend to lose their surface Ig receptors.²⁵

The zones of contact between rosette-forming lymphocytes and erythrocytes
usually involved cytoplasmic projections with relatively narrow areas of intimate association. Whether this type of interaction reflects the distribution of the lymphocyte membrane receptors and the erythrocyte membrane antigen, or whether it is related to the conditions of rosette formation cannot be resolved at present. The temperature of incubation, for example, may be of critical importance. Electron microscopy of rosettes formed for the most part at 4°C with immune guinea pig and mouse lymphocytes and sheep red cells (SRBC),26,27 and of human thymus-derived lymphocytes with SRBC,28 has also shown predominantly narrow zones of contact. In contrast, rosettes formed at 37°C by complement reactive lymphocytes,29 by Rh(D) isoimmunized lymphocytes,30 and by B lymphocytes in an indirect immunocytoadherence test31 have conspicuous broad zones of association, although these systems differ in membrane-membrane spacing. Further studies of the topography of plasma membranes are necessary to elucidate the significance of these interactions.

The cytotoxicity data with idiotypic antisera (Table 3) suggest that a larger number of lymphocytes may be bearing the specific surface antibody than those detected by the rosette test. It is possible that fewer surface receptors are needed for cytotoxicity than for the formation of rosettes of more than four erythrocytes. It is known that there are variable numbers of surface receptors on cells in a given lymphocyte population.25 It is possible that cells with few receptors either do not form rosettes or can attach only one to four erythrocytes. Only lymphocytes with greater than four erythrocytes were considered as RFC in view of the uncertain significance of the adherence of fewer cells, especially, in the presence of glutaraldehyde fixative.

The reason for the reduction in RFC counts after nylon-column filtration is uncertain. The removal of adherent monocytes by the column would be expected to result in an increase in the proportion of RFC. However, it has been reported that some B lymphocytes may be retained by nylon columns and that the effluent may be relatively enriched in thymus-derived cells.32

Since cold agglutinins elute from intact cells at 37°C, it is highly unlikely that the rosette formation was due to binding of serum antibodies to the cells, for care was taken to isolate and wash lymphocytes at 37°C in order to avoid adsorption of cold agglutinins on these cells which may contain I and i antigens.33,34 Furthermore, passive adsorption was not detected when the blood of a healthy person was incubated with serum from two of the cold agglutinin patients prior to the isolation of the lymphocytes. These results are supported by the failure to detect any adsorbed proteins in lymphocytotoxicity assays with idiotypic antisera.39

In man, specific lymphocyte surface receptors have been demonstrated by previous workers. These include RFC with thyroglobulin coated sheep erythrocytes in immune thyroiditis,35 with Rh(D) positive erythrocytes in isoimmunized persons,36 and with gamma globulin-coated erythrocytes in rheumatoid arthritis.37 By lymphocytotoxicity assays lymphocyte IgM receptors have been described in Waldenström’s macroglobulinemia40 which have the same idiotypic specificity as the serum macroglobulins. Rheumatoid-factor-like activity has been described on the lymphocytes of a patient with macroglobulinemia and of certain patients with chronic lymphocytic leukemia38; however, these are
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difficult to distinguish from the receptors for aggregated gamma globulins on normal B cells. The cold agglutinin syndrome falls into the category of lymphoproliferative disorders, and the I or i reactive receptors represent one clearly defined activity on a significant proportion of the peripheral blood lymphocytes. Other activities will undoubtedly be delineated on the lymphocytes of patients with other lymphoproliferative disorders.

ACKNOWLEDGMENT

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