Role of Membrane Lipids in Cold Agglutination of Human Erythrocytes

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The membrane lipid fluidity of normal human erythrocytes was modified by enrichment and depletion in cholesterol, and the expression of I and Sp antigens was assayed by quantitative hemagglutination from 4°C to 24°C by use of a continuous flow system. Below 16°C–18°C, cholesterol enrichment increased and cholesterol depletion decreased percent agglutination. As temperatures approached approximately 18°C–20°C, differences in agglutination between modified and unmodified erythrocytes became insignificant despite marked differences in lipid fluidity at that temperature. Thus, fluidity changes alone cannot be responsible for the effect of membrane cholesterol on cold agglutination. In an additional study, the temperature dependence of a relative equilibrium association constant, estimated by probit analysis of percent agglutination at various antisera concentrations, was biphasic with a sharp break at 16°C. Our studies are consistent with the hypothesis that I and Sp antigens preferentially partition into a lipid domain that forms during lateral phase separation of membrane lipid developing at low temperature. A resulting increase in antigen density would then become responsible for augmented agglutination by specific antibody.

Cold Agglutinins are IgM antibodies that agglutinate human erythrocytes optimally at temperatures well below 37°C. The molecular basis of this temperature dependence is unknown. One possible mechanism is a direct influence of temperature on the initial antigen–antibody reaction or on a subsequent step in the agglutination process. Alternatively, temperature might affect primarily the physical state of the membrane lipids and thereby modulate agglutination indirectly, a mechanism supported by the work of Rosse and coworkers showing that solubilized I antigen is as reactive at 37°C as at 4°C. A determinant role for the membrane lipids was proposed by Cooper, who suggested that decreased lipid fluidity resulting from low temperatures may increase the availability of I antigen at the cell surface. Indeed, Shinitzky and coworkers have reported that decreased lipid fluidity enhances the expression of a number of surface receptors, perhaps by vertical displacement of membrane proteins. In this article we examine experimentally the influence of lipid fluidity, as modified by enrichment or depletion of membrane cholesterol, on the temperature-dependent hemagglutination brought about by antisera directed against the I antigen, located on glycoprotein and glycolipid components, and against Sp antigen, present on glycoprotein. The observations to be presented demonstrate that changes in lipid fluidity alone cannot account for the low temperature requirement. Instead, our results support the hypothesis that the role of low temperatures in the cold agglutination phenomenon is to effect a thermotropic change in state of the membrane lipids at approximately 16°C–18°C. A thermotropic change at this temperature has been reported previously through use of numerous techniques.

Materials and Methods

Erythrocyte Agglutination Studies

Venous blood was drawn into acid-citrate-dextrose solution from normal adults (blood type O) and the erythrocytes washed three times in phosphate-buffered saline (PBS) pH 7.4. The cells were either used immediately or modified as described below. Four antisera were used: anti-I(Step), a gift of Dr. Marie Crookston; anti-I(Ma), a gift of Dr. Thalia Papayannopoulou; and anti-I(K) and anti-Sp, gifts of Mr. Laurence Marsh. We define the titer of cold agglutination as the reciprocal of the dilution required to cause 50% ± 5% agglutination of a 1.4% red cell suspension in PBS at 4°C. The titers of the antisera are as follows: I(Step), 5000; I(Ma), 5000; I(K), 300; Sp, 16. Protein concentrations of the antisera at these dilutions in mg/ml are: I(Step), 0.014; I(Ma), 0.012; I(K), 0.190; Sp, 2.85.

An Auto-Analyzer manifold system (Technicon Instruments Corp., Tarrytown, N.Y.) was used to quantify hemagglutination as previously described. Although percent agglutination estimates antigen expression indirectly, we have demonstrated a good correlation of agglutinability with fluorescent antibody staining of individual erythrocytes in a study of I antigen expression. Erythrocytes in a 1.4% suspension in PBS were mixed with antisera, and also with 0.125% bromelin (Technicon Instruments Corp.) for the anti-I studies or with 1% bovine serum albumin (BSA) for the anti-Sp study. The protease bromelin is reported to increase specific agglutination by anti-I antibodies and to inhibit agglutination by anti-Sp antibodies. Incubation at constant temperature was carried out for 15 min in jacketed coils. To ascertain if equilibrium had been attained in this period, we introduced additional mixing coils and noted that percent agglutination was not altered by the prolonged incubation. Temperature was varied by use of a constant temperature (±0.5°C) circulating water bath. Agglutinated cells were removed, nonagglutinated cells lysed, and percent agglutination was assessed by the optical density (OD) of the resulting hemoglobin solution. By introducing the red cell suspension without agglutination...
ing antibodies, a baseline OD corresponding to 0% agglutination was obtained, and 100% agglutination corresponded to the OD obtained with PBS alone. The percent agglutination of each sample was then calculated as:

\[
100 \times \left( 1 - \frac{\text{OD(cells plus antisera) - OD(PBS)}}{\text{OD(cells) - OD(PBS)}} \right)
\]

As antibody activity at high dilution deteriorated over a period of hours at 0°C, duplicate measurements for each cell type at each temperature could not be performed. However, replicate measurements were made in selected cases, and the reproducibility of the assay was found to be ±1% at the 50% hemagglutination level. Relatively large concentrations of antisera were required to obtain measurable amounts of agglutination at the higher temperatures examined. To determine if any of these dilutions were saturating at the lower temperatures, the probit of percent agglutination was plotted against the logarithm of the antiserum concentration. Only those antiserum dilutions that exhibited a linear relationship with percent agglutination were used for comparison of antigen reactivity.

**Modification of Cholesterol Content**

The cholesterol content of erythrocyte membranes was altered by the method of Shinitzky, as previously described. A mixture of 10% heat-inactivated serum in PBS was modified by the addition of solutions of cholesterol (enrichment) or egg lecithin (depletion) in tetrahydrofuran (THF) or THF alone (for control cells). The mixtures were then lyophilized and subsequently reconstituted with distilled water for incubations of erythrocytes at 37°C for up to 24 hr. Control cells did not differ in their reactivity toward the various agglutinins as compared with unincubated erythrocytes. A portion of each preparation of modified cells was used to prepare ghost membranes by the method of Dodge et al. for examination of membrane lipid fluidity. Lipids were extracted from ghost membranes by the method of Folch et al.

**Membrane Lipid Fluidity Studies**

Lipid fluidity of the modified erythrocyte ghost membrane was assessed by steady-state fluorescence polarization measurements using 1,6-diphenyl-1,3,5-hexatriene (DPH) as previously described. The results were expressed as the anisotropy parameter \([r_0/r - 1]^{-1}\), where \(r_0\) is the fluorescence anisotropy of DPH in the sample, and \(r\) is the maximal limiting anisotropy of this probe. The DPH anisotropy parameter varies inversely with lipid fluidity and directly with the cholesterol/phospholipid (C/PL) molar ratio in erythrocyte membranes. An empirical equation:

\[
[r_0/r - 1]^{-1} = 1.11 + 1.53 \times (C/PL)
\]

has been derived in our laboratory to facilitate estimation of the C/PL ratios through use of fluorescence polarization data. This relationship is similar to one reported in a prior study. To validate use of this equation, cholesterol and phospholipid of erythrocyte membranes depleted or enriched in cholesterol were assayed by established techniques periodically during the course of these experiments.

**RESULTS**

**Lipid Fluidity of Erythrocyte Membranes Enriched in or Depleted of Cholesterol**

Data on erythrocyte membranes from three normal donors were combined. C/PL ratios were derived through use of our empirical equation. Control ratios were 0.76 ± 0.09 (SD), as contrasted to 1.30 ± 0.23 (SD) for membranes enriched in cholesterol and 0.54 ± 0.10 (SD) for membranes depleted in cholesterol. DPH \([r_0/r - 1]^{-1}\) values at 24°C for enriched membranes were 36% ± 11% (SD) greater and for depleted membrane were 15% ± 3% (SD) less than control values. Therefore, as expected, cholesterol enrichment decreased and cholesterol depletion increased the fluidity of the membrane. A representative Arrhenius plot of the membrane anisotropy parameter (erythrocytes from donor 1) is shown in Fig. 1. It is noteworthy that the slope (i.e., the apparent flow activation energy) for the cholesterol-enriched membranes is less than that of the control, which in turn is less than that of the depleted membrane, in accord with known effects of cholesterol in bilayer membranes. Thus, the cholesterol-dependent changes in fluidity are relatively greater at the higher end of the temperature range examined.

**Agglutination of Modified Erythrocytes**

Modified and control cells of each of the three donors were tested for agglutination over the temperature range of 4°–24°C. Donor 1 cells were tested with anti-I(Step), donor 2 cells with anti-I(Ma), and donor 3 cells with anti-I(K) and anti-Sp. The temperature-dependent plot of percent agglutination by anti-I(Step) in Fig. 2 demonstrates two characteristics common to all of the agglutination experiments in the
4°C–24°C range: at the lower temperatures (4°C–16°C), cholesterol enrichment enhanced and cholesterol depletion reduced agglutination; and at approximately 20°C, differences in agglutination became insignificant. The data in Table 1 support these conclusions and also demonstrate another property shown by anti-I(K) and anti-Sp: at temperatures greater than approximately 20°C, cholesterol-enriched erythrocytes agglutinated less strongly than cholesterol-depleted erythrocytes.

Statistical analysis (paired, one-tail, Student’s t test) of the differences in agglutinability among all the cell preparations tested (of which Fig. 2 and Table 1 are representative) for all four cold agglutinins showed that cholesterol-enriched erythrocytes were more reactive than control (p < 0.0005, n = 23) in the range of 4°C–16°C. Above 18°C, enriched cells were less reactive than control at borderline significance (p < 0.05, n = 10), while there was no significant difference between control and depleted cells (n = 10).

Temperature Dependence of Agglutination

Percent agglutination of untreated erythrocytes was measured at varying dilutions of anti-I(Step). Probit plots were constructed between percent agglutination and antibody concentration at each temperature, and slopes were calculated by regression analysis. The slope values were used as relative measures of equilibrium association constants36 for the antigen–antibody reaction and were plotted as a function of temperature as shown in Fig. 3. Each point represents the regression-line slope calculated from experiments at four dilutions of the antiserum. The plot is biphasic, with a change in slope at approximately 16°C. A biphasic plot with a change in slope at approximately 16°C was also obtained on plotting the values against 1/θK (van’t Hoff plot).

### Table 1. Summary of Studies on Temperature Dependence of Agglutination by Four Cold Agglutinins*

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Reciprocal Dilution†</th>
<th>Temperature (°C)</th>
<th>Cholesterol Enriched</th>
<th>Control</th>
<th>Cholesterol Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Step)</td>
<td>5,000 4</td>
<td>72 57 51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,000 12</td>
<td>68 57 51</td>
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</tr>
<tr>
<td></td>
<td>5,000 20</td>
<td>47 43 41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,000 24</td>
<td>26 27 29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (Ma)‡</td>
<td>5,000 4</td>
<td>63 54 48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,000 12</td>
<td>59 48 47</td>
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<td></td>
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<tr>
<td></td>
<td>5,000 20</td>
<td>26 24 30</td>
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<tr>
<td></td>
<td>2,500 24</td>
<td>28 28 29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp</td>
<td>16 4</td>
<td>49 46 41</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>16 12</td>
<td>58 53 52</td>
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<td></td>
<td>16 20</td>
<td>58 57 54</td>
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<td></td>
<td>16 23</td>
<td>10 24 24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (K)§</td>
<td>3,600 4</td>
<td>18 14 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>300 24</td>
<td>11 10 16</td>
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</tbody>
</table>

*Full-temperature range curves for anti-I (Step) are shown in Fig. 2. Temperatures were selected for this table to demonstrate the common phenomenon of disappearance of differences in agglutination of modified and unmodified erythrocytes at temperatures approaching approximately 20°C.

†All dilutions are on the linear portion of the curve relating probit of percent agglutination to logarithm of antiserum concentration.

‡Agglutination was unreadable at 24°C with an antiserum dilution of 1:5000.

§Reaction characteristics of this antibody prevented a significant temperature spectrum analysis.

DISCUSSION

In studies presented here, we examined the effects of induced alterations in membrane cholesterol content of intact normal human erythrocytes on the agglutination of these cells by cold agglutinins as a function of temperature (Fig. 2 and Table 1). Enrichment of membranes in cholesterol enhanced, and depletion of membranes in cholesterol reduced, agglutination at temperatures below 18°C. With increasing temperature, agglutination of all erythrocyte preparations decreased, and differences in agglutination between cholesterol-enriched and cholesterol-depleted erythrocytes became insignificant at temperatures approaching approximately 18°C. As it has been reported that an increase in erythrocyte membrane microviscosity can lead to increased antigenic expression at the cell surface,3–9,20 decreased fluidity at low temperatures (Fig. 1) might be partly responsible for the effects of
Cholesterol modification on cold agglutination noted in
lipid fluidity must be sought for our findings. The
membrane lipid may separate into gel-like and fluid-like
domains. However, other possible explanations should
not be neglected. For example, temperature-dependent
conformational changes in the membrane, the antigen,
or the antibody might all play a role.

Finally, it is noteworthy that I-i antigen expression
is enhanced not only by low temperatures and choles-
terol enrichment, but also in a number of hematologic
disorders, including sickle cell disease, megaloblastic
anemias, Rh null disease, abetalipoproteinemia,
and HEMPAS hemolytic anemia. Inasmuch as
membrane abnormalities accompany certain of these
disorders, it is worth considering that one mecha-
nism for the altered expression of the antigen could be
a change in the extent or nature of lipid domains that
separate and coexist below the transition temperature.

Fig. 3. Temperature dependence of the relative equilibrium
association constant as measured by the slope of the regression
line obtained from probit analysis of agglutination. Each data point
represents a regression line slope obtained from a probit plot of
percent agglutination at four separate concentrations of anti-
I(Step) antiserum. Where tested, replicability at any one concen-
tration lay within ±1% agglutination. The lines in this figure,
showing an apparent slope change at 16°C, were drawn by eye. A
polynomial best-fit equation computed for the data points has its
break-point in the figure.

The temperature at which influences of cholesterol content on agglutination became minimal (approximately 18°C) (Fig. 2 and Table 1) corresponds to the thermotropic transition at 16°-20°C observed in erythrocyte membranes by a variety of techniques, including viscosity measurements, light scattering, fluorescence, electron spin resonance, nuclear magnetic resonance, and Raman spectroscopy. Membrane transport and enzyme activities also show changes in activation energy in this temperature range. In a closer analysis of the temperature dependence of agglutination by anti-I(Step), we used probit plots as a measure of the equilibrium association constant for the antigen-antibody reaction at each temperature. When the slope values were plotted against temperature, a distinct break was observed at 16°C (Fig. 3), corresponding again to the thermotropic transition of the erythrocyte membrane. In contrast to these findings for the cold agglutination process, the equilibrium binding constant for IgG anti-Rh,D(D), which is not cold dependent, showed no discontinuity with temperature in the range 2°-40°C.

We propose that our findings can be explained as follows. It is likely that the lipid thermotropic transition of human erythrocyte membranes involves lateral phase separation, unaccompanied by cooperative melting of phospholipid acyl chains, particularly since the transition was not detected by differential scanning calorimetry. Below 16°-20°C, therefore, the membrane lipid may separate into gel-like and fluid-like domains. Preferential partitioning of the I (or Sp) antigen components into one type of domain, most likely the more fluid regions, would increase the local concentration of the antigen. If multivalent attachment of antibody to the cell surface is indeed one of the requirements for cold agglutination, then preferential partitioning of antigen would facilitate antigen-antibody binding and thus would increase the formation of effective cross-bridges between cells. In support of this hypothesis, preferential partitioning of intrinsic membrane proteins and lipid fluorophores into microdomains of lipid bilayers has been reported. Our observations on the effect of modification of membrane cholesterol content on agglutination at low temperatures (Fig. 2 and Table 1) contribute to our hypothesis in the following way. Experimental studies of model bilayers suggest that increases in cholesterol content enhance the extent of gel-like domains at the expense of fluid domains. Hence, in cholesterol-enriched human erythrocytes below the transition temperature, the I and Sp antigen components might be concentrated further by reduction of the area of the fluid domains. Although necessarily speculative, these proposals are in accord with our observations and those of others. However, other possible explanations should not be neglected. For example, temperature-dependent conformational changes in the membrane, the antigen, or the antibody might all play a role.
REFERENCES

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