Normal Human Lymphocytes Treated in Vitro With the Sulfhydryl Compound AET: Relationship to the Lymphocytes of Paroxysmal Nocturnal Hemoglobinuria

By G. Sirchia and S. Ferrone

Because in vitro treatment with the sulfhydryl compound AET is known to alter normal red cells in such a way that they become highly susceptible to the lytic action of complement, an investigation was carried out to evaluate whether the same substance could modify normal lymphocytes in a similar way. The lymphocytes from paroxysmal nocturnal hemoglobinuria were also studied as a comparison. AET lymphocytes appeared to be more sensitive than the same untreated cells to the lytic action of antibody and C, as indicated both by the percentage of stained cells and by the titers displayed by the antisera used in a dye exclusion cytotoxicity test. Two HL-A monospecific antisera that gave false negative results with untreated lymphocytes gave clear-cut positive results with the same cells treated with AET. AET-lymphocytes could be employed for the detection of leukocyte antigens and antibodies.

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a disorder characterized by a membrane defect of erythrocytes, leukocytes, and platelets. The defect is such that PNH cells are more susceptible than normal to the lytic action of complement (C), especially when this is activated by antibodies.

In recent years it has been shown that some sulfhydryl compounds, such as AET (2-aminoethylisothiouronium bromide), can alter normal red cells so that they become similar in some respects to PNH red cells; in particular, they share with PNH red cells a high susceptibility to lysis by C. It is possible that the same chemicals could modify normal leukocytes in a similar way; in fact, Essig et al. have briefly reported the formation of a “PNH-like lesion” in normal white blood cells treated in vitro with the sulfhydryl compound reduced glutathione. These treated cells are said to resemble PNH leukocytes in their susceptibility to lysis in slightly acidified normal serum, especially in the presence of antibody.

The aim of the present investigation was to determine whether treatment of normal leukocytes with AET could modify their susceptibility to lysis by antibody and/or C. For comparison, PNH leukocytes were also studied.

Materials and Methods

Four normal subjects and two patients suffering from chronic lymphocytic leukemia were employed as a source of lymphocytes for treatment with AET. PNH cells were obtained from three patients currently under study.
For treatment with AET, isolated lymphocytes instead of mixed leukocytes were used, since in preliminary experiments it had become apparent that AET-treated granulocytes were stained nonspecifically with trypan blue. Blood was drawn into heparin; erythrocytes were removed by dextran sedimentation and lymphocytes were isolated by filtration of the mixed leukocyte suspensions through nylon columns. One volume of packed lymphocytes contaminated with red cells was mixed with four volumes of a 2 per cent solution of AET in distilled water previously adjusted to pH 8.0 with 5 N NaOH. The mixture was left standing in a water bath at 37°C for 30 minutes. At the end of the incubation period, the cells were washed six times with large volumes of saline and then used for testing. When lymphocytes were resuspended with saline after the first centrifugation, gelatinous filaments were sometimes seen; they could be easily removed with a thin Pasteur pipette. Treatment caused a cell loss of approximately 50 per cent. Observed under the microscope, AET-lymphocytes appeared nonagglutinated; they were viable, as assessed by trypan blue exclusion. AET-lymphocytes were used within 1 hour of preparation; however, they remained viable for at least 48 hours if stored in compatible serum at 4°C. The above procedure for lymphocyte treatment was chosen because it proved satisfactory during preliminary investigations.

Sera containing lymphocytotoxic antibodies were obtained from polytransfused patients. Monospecific antisera of HL-A1, HL-A2, HL-A3, HL-A8, and HL-A9 specificity were from Hyland, rabbit C from Hyland and from selected rabbits. Human C was a pool of fresh sera from three healthy male blood donors of group AB. For the lymphocytotoxicity test the trypan blue exclusion method was used, as described by Engelfriet and Britten;12 human C was used in the test. A reaction was recorded as positive if at least 15 per cent of the cells were stained. Controls usually showed less than 10 per cent stained cells.

For the acid lysis test, lymphocytes were suspended in human C acidified to pH 6.5 by 0.25 N HCl to obtain a concentration of 4000 cells/μl. Ten volumes of this suspension were incubated for 1 hour at 37°C; 1 volume of a 2 per cent solution of trypan blue in distilled water was then added, the incubation at 37°C continued for 30 minutes and the percentage of colored cells evaluated. For the sucrose lysis test, 1 volume of a lymphocyte suspension in human C containing 50,000 cells/μl was added to 9 volumes of the phosphate buffer–human C mixture suggested by Jenkins et al. Incubation at 37°C, addition of the trypan blue solution, and readings were performed as above.

Titration of the antibody was carried out by bringing about a reaction between a constant volume of lymphocyte suspension in C and the antiserum serially diluted in Hanks solution.

The concentration of C required for cell lysis was evaluated by bringing about a reaction between a constant volume of a suspension of lymphocytes in the antiserum (inactivated by heating at 56°C for 30 minutes) and serial dilutions of C in Hanks solution.

RESULTS

AET-treated lymphocytes, incubated for 1 hour at 37°C in human C, remained viable as assessed by trypan blue exclusion. On the contrary they stained nonspecifically when incubated in rabbit C. Therefore, the latter, which is widely used in cytotoxicity reactions,14 could not be used in our lymphocytotoxicity tests.

Both normal and AET-lymphocytes incubated in acidified normal serum or in a medium of low ionic strength did not stain with trypan blue. All the lymphocytotoxic antisera used appeared to react more strongly with AET-treated lymphocytes than with the same untreated cells, as indicated both by the percentage of damaged cells and by the titer displayed by the antiserum (Table 1). Two antisera (respectively of HL-A2 and HL-A8 specificity) that did not react visibly with normal lymphocytes of either the examined subjects
NORMAL AET-TREATED AND PNH LYMPHOCYTES

Table 1.—Reaction of a Polyspecific Cytotoxic Antiserum With Normal and AET-treated Lymphocytes From the Same Subject*

<table>
<thead>
<tr>
<th>Serum Dilutions</th>
<th>1:1</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal lymphocytes</td>
<td>95</td>
<td>60</td>
<td>40</td>
<td>30</td>
<td>0 †</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AET-treated lymphocytes</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers indicate the percentage of stained cells. (Typical experiment chosen among the seven performed.)
† 0 = negative reaction.

gave a clear-cut positive reaction with the same cells treated with AET (Table 2). These lymphocytes, in fact, carried the antigens HL-A2 and HL-A8 respectively as shown through the employment of other test sera in Ceppellini’s laboratory in Turin and in our laboratory. This latter result is noteworthy, since the monospecific antisera used were selected to work in the presence of rabbit C and react poorly or not at all with normal lymphocytes when human C is used.

The reaction of AET-lymphocytes with cytotoxic antisera was C-dependent. Cells suspended in compatible normal serum inactivated by heating at 56°C for 30 minutes did not react with heat-inactivated lymphocytotoxic antisera. AET-lymphocytes reacted visbly with the antibody at C concentrations not sufficient to give a positive result with untreated cells (Table 3).

Insofar as PNH lymphocytes are concerned, they did not lyse in the acid lysis and sucrose lysis tests. They did however react readily with the cytotoxic antisera used, including the two above-mentioned HL-A antisera that had given false negative results with normal lymphocytes (Table 4). In the lymphocytotoxicity test, both human and rabbit C could be used since the latter was not cytotoxic for PNH cells.

**DISCUSSION**

The above results indicate that treatment with AET renders human lymphocytes more than normally reactive in the lymphocytotoxicity test. This is possibly due to a greater than normal sensitivity of AET lymphocytes to C, as is the case for AET-treated normal red cells. This abnormal sensitivity could also explain why false negative results were not observed in the cytotoxicity test. In this reaction C plays a crucial role: any factor lowering the ratio of C to antigen and/or antibody (excessive number of cells, anticomplementary activity of the reaction mixture, and so forth) can prevent lysis of the lymphocytes; an increase in this ratio through a rise in C sensitivity of

Table 2.—Reaction of an HL-A2 Antiserum With Normal and AET-treated Lymphocytes From the Same Subject*

<table>
<thead>
<tr>
<th>Serum Dilutions</th>
<th>1:1</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal lymphocytes</td>
<td>0 †</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AET-treated lymphocytes</td>
<td>70</td>
<td>30</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers indicate the percentage of stained cells. (Typical experiment chosen among the five performed.)
† 0 = negative reaction.
the cells makes false negative results less likely to occur. It is interesting to note that similar results and conclusions have recently been arrived at by Mittal et al.\textsuperscript{16} for lymphocytes treated in vitro with ficin.

In contrast to AET-red cells, AET-lymphocytes did not lyse when incubated in human serum at pH 6.5 or in a medium of low ionic strength. Essig et al.,\textsuperscript{4} on the contrary, apparently found that normal leukocytes, treated in vitro with the sulphydryl compound reduced glutathione, lyse when incubated in serum acidified to pH 6.3, with or without antibody. It is noteworthy that the authors\textsuperscript{4} have observed that PNH leukocytes also lyse in the acid lysis test; this has not been found to be so by Aster and Enright.\textsuperscript{3} In our hands PNH lymphocytes did not lyse in the acid lysis test. Their reactivity in the lymphocytotoxicity test however appeared to be stronger than normal. This possibility must be further checked, since Aster and Enright\textsuperscript{3} have found that granulocytes but not lymphocytes are more sensitive than normal to antibody and C in PNH.

The unique susceptibility of PNH red cells to lysis by C has proved remarkably valuable for the detection of C-fixing red cell antibodies. Preliminary results indicate that for the same purpose AET-red cells can also be used. Recently Aster and Enright\textsuperscript{3} found that PNH platelets and normal platelets treated with papain are highly susceptible to lysis by antibody and C and suggested that they could prove useful for studies of certain platelet-antiplatelet systems.

Mittal et al.\textsuperscript{16} suggested that ficin-treated lymphocytes be used to improve and accelerate their lymphocytotoxicity test. Similarly AET-lymphocytes could offer interesting practical applications. At present, for the detection of leukocyte antigens and antibodies the lymphocytotoxicity test is widely used. This technique, though satisfactory, is not completely free of drawbacks. For instance, rabbit serum, the most widely employed source of C, must be properly selected since some rabbits contain in their serum a naturally occurring antibody cytotoxic against human lymphocytes.\textsuperscript{15,17,18} More important, some cells, though absorbing the antibody, do not show visible signs of the reaction, and

### Table 3.—Lysis of Normal and AET-treated Lymphocytes From the Same Subject at Different Concentrations of Human Complement*\textsuperscript{17}

<table>
<thead>
<tr>
<th>Complement (vol):</th>
<th>10</th>
<th>8</th>
<th>6</th>
<th>4</th>
<th>2</th>
<th>1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks (vol):</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Normal lymphocytes</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>60</td>
<td>01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AET-treated lymphocytes</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers indicate the percentage of stained cells. (Typical experiment chosen among the three performed.)
† 0 = negative reaction.

### Table 4.—Reaction of an HL-A8 Antiserum With PNH and Normal Lymphocytes*\textsuperscript{16}

<table>
<thead>
<tr>
<th>Serum dilutions:</th>
<th>1:1</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal HL-A8 positive lymphocytes (three subjects)</td>
<td>0 †</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PNH lymphocytes (patient P. L.)</td>
<td>80</td>
<td>65</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers indicate the percentage of stained cells. (Typical experiment employing PNH-lymphocytes from one patient.)
† 0 = negative reaction.
therefore can give rise to false negative results. Our data suggest that these two drawbacks can be eliminated if lymphocytes are treated with AET, since rabbit C is no longer required in the reaction and false negative results were not observed. The enhanced reactivity in the lymphocytotoxicity test could be an additional reason to introduce AET-lymphocytes into leukocyte serology.

REFERENCES


Normal Human Lymphocytes Treated in Vitro With the Sulfhydryl Compound AET: Relationship to the Lymphocytes of Paroxysmal Nocturnal Hemoglobinuria

G. SIRCHIA and S. FERRONE