Granulocytes and Red Cell Lysis

By Richard I. Walker, J. C. Herion and S. W. Smith, Jr.

An unexpected increase in resistance to osmotic lysis of red cells passed through cotton has been observed many times in this laboratory during procedures for obtaining samples of pure lymphocytes for chemical analysis and in vitro culture. This increased resistance has been manifest by incomplete lysis on exposure to hypotonic solution usually sufficient to hemolyze red cells completely.

Stasis in vivo in the spleen and erythroconcentration in vitro produced increased fragility of red cells in hypotonic solutions. Turner, studying red cell lysis by cobra venom, noted, in red cells removed from spleens, complete lysis by traces of venom that did not hemolyze red cells isolated from blood. He correlated lysis with the presence of leukocytes, and by adding leukocytes he effected hemolysis in a venom-red cell system in which lysis did not ordinarily occur. He did not further characterize the phenomenon.

Since granulocytes are removed quantitatively from blood by incubation on cotton, it seemed appropriate to determine if these elements might participate in red cell lysis. The general plan of these studies has been to test the effect of granulocytes and some granulocyte components on the amount of hemolysis produced by exposure to solutions of different osmolarity. This report presents the substance of these studies showing that granulocytes and granulocyte lysosomes do possess hemolytic activity.

METHODS

Procedures to avoid bacterial contamination and growth were employed. Heparinized rabbit and human blood were rid of granulocytes by incubation on cotton columns, washed with sterile cold saline until free of hemoglobin, and suspended in saline to a volume of packed cells of about 50 per cent. Total and differential leukocyte counts done by standard technics on these suspensions showed 1000 to 2000 white cells/mm³ with 99–100 per cent lymphocytes. Red cells so obtained represent “eluate” cells. Blood not placed on a column, but processed otherwise in parallel with the eluate cells by equivalent washing, provided red cells containing granulocytes and lymphocytes in about the number and distribution found in whole blood.

Mixed leukocytes were obtained from human and rabbit blood by dextran sedimentation, as previously described. Large quantities of rabbit granulocytes were obtained from peritoneal exudates induced by the injection of a sterile saline solution of glycogen.
exudate was aspirated into heparinized, iced tubes 12 hours after induction, harvested by centrifugation at 400 g at 4 C., and the cell button washed 3 times with cold saline. The leukocytes were suspended in 5 volumes of saline and total and differential counts were done. Exudate granulocytes were used in studies on the red cells of the same rabbit in most instances.

Granulocyte lysosomes were obtained from blood and peritoneal exudate cells by the method of Cohn and Hirsch, using centrifugation of the ruptured cells in 0.34 M sucrose. Lysosomal cationic proteins were isolated by extraction with 0.2 N HSO₄ and precipitation in 20 per cent ETOH, as described by Zeya and Spitznagel. The quantity of intact lysosomes and basic proteins used in the experiments reported was based on protein count determined by the method of Lowry et al., using egg lysozyme as a standard.

A stock of pH 7.4 buffered saline with the osmolal equivalence of 10 per cent NaCl was prepared according to Davidsohn and Wells, filtered through a 0.45 micron pore-sized Millipore filter (Millipore Filter Corp., Bedford, Mass.), and stored at 4 C. Dilution with sterile distilled water was done before each use. pH 7.0 buffered saline was similarly prepared. pH was measured with a Radiometer pH meter (The London Co., Welwyn-International, Inc., Cleveland). Red cells were added to buffered saline of differing osmolality and the amount of hemolysis was determined at intervals. Penicillin and streptomycin were used in concentrations of 100 units and 50 micrograms per ml. of buffer to obviate problems with bacterial contamination. Free hemoglobin in sample supernates was quantitated by reading against an appropriate blank in a Beckman DU spectrophotometer at 540 mµ in matched, calibrated cuvettes. Free hemoglobin in 0.2 per cent buffered saline at 30 minutes has been taken for 100 per cent lysis. Replicate determinations on the same sample at 30 minutes were done to test the precision of the technic. The standard error of the mean computed for six such determinations at each concentration of saline was greatest, 1.5 per cent, for samples in 0.45 per cent saline. In every instance appropriate controls and spectrophotometric blanks were run in parallel with the experimental samples.

Seeking in vivo effects of the lysosomes, intravenous injections of intact lysosomes from peritoneal exudate leukocytes, 1.0 mg. of protein per Kg., were given at 8:00 A.M., 12:00 noon, and 4:00 P.M. for seven days to each of six healthy adult male New Zealand rabbits. A similar group of controls received injections of equivalent volumes of the injection vehicle, 0.34 M sucrose. By standard technics serial determinations of hemoglobin, volume of packed red cells and reticulocytes were made on ear vein blood.

**Results**

Table 1-A shows the results of two studies using human cells. The washed red cells contained leukocytes in about the number and proportion found in whole blood while the eluate cells contained no granulocytes. Intact granulocytes isolated from blood were added to a portion of the eluate red cell suspension to a concentration of 2500/mm.³. After thorough mixing, the samples were left stand at room temperature for 30 minutes and then 0.02 ml. of each was pipetted into 5 ml. of buffer of different osmolalities and free hemoglobin determined after incubating at 37 C. for 30 minutes. (This general technic was used also to obtain the data in Table 1-B, except intact lysosomes were used in place of intact granulocytes.) Hemolysis in the eluate cells lags behind that in the washed red cells. The addition of granulocytes erases or partially erases this lag. In ten other experiments done the same way, using either human or rabbit cells, similar findings were obtained. An obvious criticism of comparing the amount of hemolysis of washed red cells with that of eluate cells is that passage through the column might remove easily hemolyzed cells, thus accounting for the apparent lag in lysis of the eluate cells. Removal of easily lysed cells on column passage, however, cannot account for the increase in the amount of hemolysis obtained when granulocytes are added to eluate red cells.
To magnify the hemolytic effect to further evaluate these observations, granulocytes were added directly to the buffer solutions rather than to the red cell suspension to effect a higher concentration of granulocytes in the buffer by avoiding the dilution attendant to pipetting a small volume of the red cell suspensions into a large volume of buffer. Length of incubation was also extended to increase the exposure time of the red cells in the test system. The data in Table 2 are from a study testing the effect of rabbit peritoneal exudate granulocytes on the lysis of the red cells of the same rabbit. Figure 1, plotted from the data in Table 2, shows osmotic fragility curves representative of a number of such determinations. It is apparent that more lysis has occurred in the presence of granulocytes. After long incubation in pH 7.4 buffer in the presence of granulocytes, greater lysis in 0.85 per cent solution than in the solutions of immediately lower osmolarity has been observed consistently. The rate of hemolysis in the presence of granulocytes continues with time to exceed that in eluate cells without granulocytes, as shown by the data presented in Table 2. This also has been a consistent finding.

Another study using rabbit red cells and peritoneal exudate granulocytes showed that the amount of hemolysis produced (43-65 per cent) is proportional to granulocyte concentration, at least in the range tested (0-2.5×10⁶/ml.).

The addition of glucose to the buffer solution to a concentration of 500 mg per cent reduces the total amount of hemolysis in test systems, but the amount of lysis of eluate cells in the presence of granulocytes continues to exceed that of the eluate cells alone. In two such studies, for example, the average per cent hemolysis at 25 hours in 0.85 per cent buffer in the presence of granulocytes (24 per cent) was nearly triple that without (8.5 per cent). After 25 hours of in-
Table 2.—Effect of Granulocytes on the Lysis of Rabbit Eluate Red Cells During Prolonged Incubation

<table>
<thead>
<tr>
<th>pH Buffer</th>
<th>Sample</th>
<th>Hour</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td></td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>0.85</td>
<td>E₁</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>E₂</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>0.75</td>
<td>E₁</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>E₂</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>0.65</td>
<td>E₁</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>E₂</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>0.55</td>
<td>E₁</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>E₂</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>0.50</td>
<td>E₁</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>E₂</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>0.45</td>
<td>E₁</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>E₂</td>
<td>76</td>
<td>78</td>
</tr>
</tbody>
</table>

E₁: Eluate cells incubated in buffer.
E₂: Eluate cells incubated in buffer containing 1.6 × 10⁶ granulocytes/ml.

Granulocyte-conditioned lysis proceeds therefore, even in the presence of quantities of glucose adequate for cell sustenance. After 23 hours of incubation in pH 7.4 buffer, the pH of samples containing granulocytes and glucose had decreased to 6.98–7.04 with a mean of 7.0, while pH of samples containing no granulocytes remained at 7.4.

In separate studies no decrease in pH was demonstrated in solutions containing granulocytes but no glucose in either pH 7.0 or pH 7.4 buffers. After 21 hours of incubation the pH of all samples in the pH 7.4 buffer lay between 7.45 and 7.52, while the pH of all samples in the pH 7.0 buffer lay between 6.98 and 7.05. Granulocytes increased lysis in both buffers. Figure 2 shows that in pH 7.0 buffer a normally-shaped fragility curve is obtained in the presence of granulocytes, in contrast to that obtained in pH 7.4 buffer (see Fig. 1).

Similar studies evaluating the effect of lysosomes were done. Table 1-B shows the results of two studies using human lysosomes and red cells in pH 7.4 buffer. Intact lysosomes from blood granulocytes were added to a portion of the eluate cells. After thorough mixing, the samples were let stand at room temperature for 30 minutes, and then 0.02 ml. of each was pipetted into 5 ml. of buffered saline of different osmolarities. Free hemoglobin was determined after incubating at 37 C. for 30 minutes. The addition of lysosomes erases the lag in hemolysis observed between eluate cells and washed cells. Similar results were obtained in 6 other studies using rabbit red cells and lysosomes from peritoneal exudate granulocytes. The results of studies employing longer incubations confirm these observations. Decreasing buffer pH to 7.0 produces in the system with lysosomes an effect similar to that obtained in the system using intact granulocytes. The data in Table 3 show the hemolytic effect of
increasing concentrations of lysosomes. No significant difference between the effect of intact and disrupted lysosomes was found. The relationship between hemolysis and lysosome concentration is not linear throughout the range tested, the amount of lysis increasing sharply between 51 and 68 µg/ml. In Figure 3 it is apparent that rate of hemolysis of eluate cells in systems containing lysosomes continues with time to exceed that of eluate cells in systems without lysosomes in a manner analogous to those with and without granulocytes (Table 2).

Lysosomal protein precipitated by 20 per cent ethanol from an acid extract of rabbit lysosomes, after purification by ethanol washing, dialysis against distilled water and lyophilization, was dissolved in .01 N HCl. The protein was electrophoretically heterogenous, there being several bands separable on cellulose acetate. This fraction has been shown to be free of lysosomal enzymes.13 Figure 4 shows the effect of increasing concentrations of this protein fraction on eluate cell lysis. The bottom curve in this figure shows the effect in the control of the pH decrease caused by the addition of an equal volume of .01 N HCl. The cationic protein possessed equally potent hemolytic activity in isosmotic pH 7.4 glyceryl-glycine buffer.

The addition of sucrose to pH 7.4 buffered saline to a concentration of 0.3 molar does not inhibit the hemolytic activity of granulocytes, as is evident from the data in Table 4. pH change was comparable with and without granulocytes. Since no inhibition of the hemolytic activity of intact granulocytes was observed, tests for inhibition of the effect of lysosomes or lysosomal protein were not done.

The polyanion heparin in concentrations from 2.5–70 units/ml substantially inhibited the hemolytic activity of granulocytes; no pH change occurred as a
Table 3.—Effect of Rabbit Granulocytes Lysosomes on the Lysis of Rabbit Eluate Red Cells

<table>
<thead>
<tr>
<th>Intact lysosomes µg. protein per ml.</th>
<th>Concentration of pH 7.4 buffer in %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>% Hemolysis at 4 Hours</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.6</td>
</tr>
<tr>
<td>17</td>
<td>7.6</td>
</tr>
<tr>
<td>34</td>
<td>12.5</td>
</tr>
<tr>
<td>51</td>
<td>20.0</td>
</tr>
<tr>
<td>68</td>
<td>79.4</td>
</tr>
</tbody>
</table>

consequence of adding these quantities of heparin. Results of a representative study are graphed in Figure 5. Heparin produced like inhibition of the hemolytic activity of intact or disrupted lysosomes and the lysosomal protein fraction. The inhibitory effect remained evident during 28 hours of incubation, the longest time tested.

The hemolytic activity of intact granulocytes and the basic protein fraction of lysosomes in the concentrations used in these studies was not manifest during incubation at 4 C. The hemolytic effect of intact lysosomes was only partially inhibited by low temperature, as shown in Table 5. This inhibition was not apparent at the higher concentrations of lysosomes. Heating lysosomes at 78 C. to denature protein only partially decreased the hemolytic activity, suggesting that some lysosomal constituent other than protein also participates in the lysis of red cells.

Lysosomal membrane material was therefore examined and found to possess potent hemolytic activity at 37 C. that was greatly reduced but not eradicated at 4 C. This material was obtained in two ways. First, the residue resulting from extraction of lysosomes with 0.2 N H₂SO₄ was reextracted twice with 0.2N H₂SO₄ and then washed twice with distilled water. This residue contained significant protein, as determined by the Lowry method, and exhibited strong hemolytic activity at 37 C. Incubation at 4 C. diminished the hemolytic effect. Second, lysosomes suspended in 0.34 molar sucrose were disrupted by freezing-thawing four times with acetone-dry ice, and the membranes obtained by centrifugation washed with sucrose. These also demonstrated hemolytic activity at 4 and 37 C. This material, too, contained protein as determined by the Lowry method.

Chromatographic analysis of this membrane material revealed phosphatidyl ethanolamine, lecithin, sphingomyelin and lysolethithin.

Intravenous injection into rabbits of intact lysosomes, 1 mg./Kg., three times daily for nine days, did not produce significant changes in hemoglobin or volume of packed red cells. There was after three days a slight increase in the reticulocyte count in the animals receiving lysosomes, but the changes were not very striking.

Discussion

These studies demonstrate that human or rabbit granulocytes lyse red cells in vitro. This hemolytic activity appears to be associated with the leukocyte lysosomes, since cellular debris remaining after removal of lysosomes does not,
in comparable quantities, cause hemolysis. At least two components of the lysosomes, the cationic protein fraction and the membrane residue, produce lysis of red cells. Other constituents of the lysosomes may have some hemolytic activity as well, since this possibility was not specifically excluded in these studies.

The hemolytic activity of granulocytes is not the result of glucose depletion or pH change and is more prominent at pH 7.4 than pH 7.0 in isosmotic buffered saline. The failure of sucrose to inhibit granulocyte-induced lysis implies that there are produced defects in the red cell membranes large enough to allow the entry of sucrose and water. Low temperature (4 C.) incubation completely inhibited the hemolytic activity of intact granulocytes in the concentrations used but did not inhibit completely lysis caused by lysosomes or lysosomal membranes. This suggests that granulocyte dissolution and lysosomal disruption may be required for the action of the hemolytic agents to occur.

These observations lead to the provocative thesis that granulocytes in vivo may well participate in red cell destruction. The intimate association of red cells and leukocytes during splenic sequestration make this hypothesis especially

Table 4.—Effect of Sucrose on the Hemolytic Activity of Rabbit Granulocytes in pH 7.4 Buffered Saline

<table>
<thead>
<tr>
<th>% Buffer in 0.1 M Sucrose</th>
<th>% Hemolysis at 25 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>E&lt;sub&gt;1&lt;/sub&gt;</td>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>0.85*</td>
<td>44</td>
</tr>
<tr>
<td>0.85</td>
<td>48</td>
</tr>
<tr>
<td>0.75</td>
<td>44</td>
</tr>
<tr>
<td>0.65</td>
<td>44</td>
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<tr>
<td>0.55</td>
<td>44</td>
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<tr>
<td>0.50</td>
<td>40</td>
</tr>
<tr>
<td>0.45</td>
<td>39</td>
</tr>
<tr>
<td>0.40</td>
<td>36</td>
</tr>
<tr>
<td>0.0</td>
<td>33</td>
</tr>
</tbody>
</table>

E<sub>1</sub>: Eluate cells
E<sub>2</sub>: Eluate cells + 3.3 X 10<sup>6</sup> peritoneal exudate granulocytes/ml.
* Buffer without sucrose
attractive. The findings of Ham and Castle\(^4\) and Weisman et al.,\(^14\) who showed increased osmotic fragility of red cells trapped in the spleen, may be partly explained on this basis.

Although intravenous injection of intact granulocyte lysosomes produced little evidence of increased red cell destruction or formation, this does not exclude a significant role for these elements in red cell destruction in vivo, since the effect is concentration-dependent and particles injected intravenously might well be rapidly phagocytized and separated from the red cells.

The injection of sensitized red cells into dogs results in red cell-leukocyte-platelet agglutinates\(^15\) and a prompt leukopenia.\(^16\) Leukocytes may be actively participating in the destruction of these sensitized red cells.

Heparin therapy in Coombs' positive hemolytic anemia was first tried by Owren,\(^17\) and others\(^18-20\) have since reported that heparin decreases the rate of red cell destruction in such anemias. Storti et al.\(^18\) found heparin to have an anticomplement effect and stated that it also has antilysolecithin activity. Heparin appears to have antiantibody activity as well.\(^19,21\) Our observation that heparin in vitro blocks the hemolytic activity of granulocytes, granulocyte lysosomes, and lysosomal cationic protein suggests another mechanism whereby

\begin{table}[h]
\centering
\caption{Effect of Heat Inactivation of Lysosomes and Low Temperature Incubation on Hemolytic Activity}
\begin{tabular}{lll}
\hline
Lysosomal protein & 4 C. & 37 C. \\
mg. per ml. & 428 & 218 * \\
\hline
0.00 & 1.9 & -- \\
0.025 & 14.3 & 30.2 \\
0.05 & 31.1 & 95.2 \\
0.10 & 53.6 & 91.2 \\
0.20 & 84.7 & 94.7 \\
0.40 & 97 & 91.4 \\
\hline
\end{tabular}
\* Lysosomes heated 78 C. X 10 minutes.
\end{table}
heparin might decrease the rate of blood destruction in patients with Coombs' positive hemolytic anemia.

Intravenous injection of bacterial endotoxin into rabbits has been shown to produce a hemolytic anemia, although endotoxin in vitro produced no red cell lysis in 30-minute incubations. The hemolytic anemia produced by endotoxin would appear to be the result of other than a direct action of endotoxin on red cells and could in part be a consequence of its effect on leukocytes. It is known to produce a rapid egress of granulocytes from the circulation and lability of some lysosomal membranes.

**Summary**

Intact granulocytes from man and rabbit hemolyze red cells in vitro. The hemolytic activity is related to the lysosomes. A highly-basic protein fraction from the lysosomes and lysosomal membrane material produce hemolysis in isosmotic buffered saline and increase the osmotic fragility of red cells. Sucrose does not inhibit this hemolytic activity but low temperature incubation and heparin do.

**SUMMARIO IN INTERLINGUA**

Intacte granulocytos ab humanos e conilios es capace a effectuar in vitro hemolyse de erythrocytos. Le activitate hemolytic es relationate con le lysosomas. Un altemente basic fraction proteinic ab le lysosomas e material de membrana lysosomal produce hemolyse in solution salin a tamponage iso-osmotic e augmenta le fragilitate osmotic de erythrocytos. Sucrosa non inhibi iste activitate hemolytic, sed illo es inhibite per incubation a basse temperaturas e per heparina.

**ACKNOWLEDGMENT**

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**REFERENCES**


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