Effect of Cell Shape on Extravascular Hemolysis

By Patricia Cosgrove and Michael P. Sheetz

Although it has been inferred that alteration of erythrocyte shape causes cell clearance, no direct measure of the effect of varying shape on clearance has been made. In this study, the protein oxidant, diamide, was employed to fix rat erythrocytes in altered shapes produced by the addition of different amphipaths or by changes in membrane structure. Clearance of cells in vivo as measured by $^{51}$Cr uptake in the spleen and liver correlated with the extent of shape alteration, either crenation or cupping. Whereas only 5%-10% of discoid cells were cleared by the spleen after 1 hr, there was 50%-80% clearance of severe echinocytes (crenated cells) and severe stomatocytes (cupped cells) primarily by the liver. This was not an effect of the crenating or cupping agents, since they were washed away prior to injection, and treatment of cells with the agents alone did not cause clearance. Further, if cells were crenated by ATP depletion through incubation without glucose for 12-16 hr, then conversion of their shape to a discoid form reduced clearance. To determine if serum components might be involved in the clearance, shape-altered cells were incubated in vitro in autologous plasma. With severe stomatocytes but not severe echinocytes, the binding of IgG was increased by 3-8-fold over controls, whereas no differences in C3 binding were observed. It is concluded that in diamide-fixed cells, the alteration of shape from a biconcave disc will increase in vivo clearance and, in the case of stomatocytes, the binding of IgG.

Because diamide treatment alone altered the deformability properties of the discs, we suggest that the clearance of shape-altered cells results from their altered membrane surface properties rather than mechanical factors.

THE CLEARANCE OF erythrocytes from the blood by extravascular hemolysis occurs by a variety of mechanisms involving IgG opsonization, C3 opsonization, membrane oxidation, and possibly alteration of cell shape. It is the question of the role of cell shape in clearance that we will address. Erythrocyte shape changes are believed to contribute to in vivo hemolysis in the hereditary spherocytic anemias. In hereditary spherocytosis, for example, the morphology of the cells becomes progressively more spherocytic with longer in vitro incubations. The absence of spherocytes in freshly drawn blood, except in severe cases, is taken as an indication that the spherocytes are cleared from the circulation. When abnormally shaped erythrocytes are found in peripheral blood, as in acanthocytosis or irreversibly sickled cells, they are signposts of either dysfunction in the clearance organs or severe hemolysis. Furthermore, hemolysis of aged erythrocytes is believed to be associated with their membrane loss and sphering, and the spherical morphology can result from severe crenation or cupping. All of these findings and others support the hypothesis that shape alteration per se can cause extravascular hemolysis.

These arguments, however, are indirect and no direct causal relationship between the alteration of shape and in vivo clearance has been found. The problem has been that most agents that preserve or “fix” erythrocyte shape also fix the erythrocyte cytoplasm. Recently, however, Haest and coworkers demonstrated that erythrocyte membranes could be selectively “fixed” with the oxidant, diamide. Cells that were crenated by a crenating agent and “fixed” by diamide remained crenated when the agent was washed away, whereas induced echinocytes not treated with diamide returned to the disc form. Further, diamide treatment of discs did not result in their immediate clearance from the circulation. In this study diamide fixation was employed to measure the effect of shape alteration on erythrocyte clearance in a rat model.

**MATERIALS AND METHODS**

**Materials**

Male Sprague-Dawley rats (200-400 g) were purchased from Charles River Breeding Labs, Wilmington, Mass. Diamide (D 3260), 2,4-dinitrophenol (D 7004), chlorpromazine (C 8138), and tetracaine (T 7508) were purchased from Sigma Co. St. Louis, Mo. The source of chromium-51 as sodium chromate was New England Nuclear, Boston, Mass.

$^{51}$Chromium Labeling of Erythrocytes

Rat blood was drawn by cardiac puncture into a syringe containing heparin as an anticoagulant. The blood was centrifuged (1100 g for 10 min), the supernatant and buffy coat were aspirated, and the pellet was resuspended in phosphate-buffered saline (PBS; 140 mM NaCl, 10 mM Na$_2$HPO$_4$, pH 7.4). This procedure was repeated 3 times. The final pellet was resuspended in PBS to a hematocrit of 20%. Chromium $^{51}$ (Cr) was added and the mixture was incubated at 37°C for 15 min. Following incubation, the cells were washed 3 times to remove unbound $^{51}$Cr, then resuspended in PBS to 20% hematocrit.

**Shape-Change-Inducing Treatments**

2,4-Dinitrophenol (DNP), chlorpromazine, and tetracaine treatments were achieved by mixing equal volumes of $^{51}$Cr-labeled cells with the respective drugs.
(hematocrit 20%) and chemical stocks giving a final hematocrit of 10% and a final concentration of the treating agents as indicated in the text.

Erythrocytes were depleted of ATP by incubating a 20% cell suspension in PBS and 1 mg/ml penicillin and streptomycin at 37°C for 12–22 hr. Erythrocytes were induced to become stomatocytes by incubating a 10% cell suspension in a PBS buffer containing 5 mM DNP, 10 mM glucose, 10 mM myo-inositol, 10 mM cytidine, and 1 mg/ml penicillin and streptomycin at 37°C for 16 hr, then washing the cells in PBS. Because extensive incubation with chromium causes cell lysis, ATP-depleted cells and stomatocytes prepared from echinocytes were 51Cr-labeled after incubation.

**Diamide Treatment**

An appropriate amount of 100 mM diamide was added to a 10% suspension of treated erythrocytes to give a final concentration of 1 mM diamide. The cells were incubated at 37°C for 45 min, washed 3 times with PBS, and resuspended to a 50% hematocrit in PBS.

**Determination of Crenating or Cupping Index**

A crenating index or cupping index was designed to represent the degree of shape change in treated cells. Portions of treated erythrocytes were fixed in 2% glutaraldehyde and observed under the microscope. The echinocytes were scored according to Weed's four gradations, and each category was assigned a value 1–4, 4 being the most severely crenated. The number of cells in each category was multiplied by the value for that category. The sum of these products represents the crenating index.

The cupping index was calculated in a similar manner. Each cupped cell was given a value of two and each spherostomatocyte was given a value of four.

**Analysis of Clearance**

One-hundred microliters of 51Cr-labeled, treated cells were injected into the jugular vein of an ether anesthetized rat. Blood samples were drawn by retroocular puncture at various time points to determine rate of clearance of labeled erythrocytes from the blood stream. Tests were done with a Coulter counter and determined that the hematocrits were uniform. After 1 hr, the rat was sacrificed and the spleen, liver, kidneys, and heart were removed to determine the location of cleared erythrocytes. No systematic differences in organ sizes were noted.

Aliquots of blood samples and the organs were counted on a Beckman gamma 7000 counter. The percentage of labeled erythrocytes remaining in the blood was determined by the ratio of counts/ml of sample to calculated counts/ml at time of injection. The percentage of 51Cr counts injected found in an organ was calculated directly.

**Gel Electrophoresis**

The effect of shape-change-inducing treatments on membrane composition was determined by separating the membrane proteins of treated cells on SDS polyacrylamide gels. Membrane ghosts of erythrocytes were prepared by diluting a 50% erythrocyte suspension 1:100 in a lysis buffer (5 mM phosphate, pH 7.4) and centrifuging (35,000 g for 10 min) at 4°C. This procedure was repeated twice to obtain a fluffy white pellet.

**Determination of IgG Binding**

Treated cells (hematocrit 50%) were incubated with an equal volume of fresh, homologous heparinized plasma or PBS for 30 min at 37°C to allow the IgG in the serum to bind to the treated cells. The cells were washed 3 times and resuspended in PBS. Aliquots of these cell suspensions were incubated with iodinated Staph-A protein for 30 min at 37°C. The cells were washed 3 times to remove unbound 125I-Staph-A protein, resuspended in PBS, and counted. Results were expressed as a ratio of counts bound to plasma-treated versus PBS-treated cells.

**Determination of C3 Binding**

The gel transfer method for polyacrylamide electrophoresis of Towbin et al. was used to assay for complement component 3 (C3) bound to treated erythrocyte membranes. Treated cells (hematocrit 50%) were incubated with an equal volume of fresh homologous serum for 30 min at 37°C to allow C3 in serum to bind to the treated cells. Reduced ghosts (10⁹ membranes) from these erythrocytes were run on SDS polyacrylamide gels. The separated proteins were electrophoresed from the gel to nitrocellulose paper. The nitrocellulose paper with ghost proteins was incubated in a buffer containing rabbit anti-rat C3, was rinsed, then was incubated with 125I-Staph-A protein. (The 125I-Staph A protein bound to the Fc portion of the rabbit IgG.) The nitrocellulose was rinsed well, dried, and exposed to x-ray film. As determined by analyzing dilutions of whole serum, the limit of sensitivity under these conditions was approximately 10 ng of C3.

**RESULTS**

**Diamide as a Shape-Stabilizing Agent**

Several concentrations of diamide were tested for ability to stabilize echinocytes. Maintenance of cell shape was not observed at 0.1 mM or 0.5 mM, whereas 1.0, 5.0, and 10.0 mM were all found to be effective with no significant difference in the degree of shape stabilization. Incubation of a 10% suspension of shape-altered erythrocytes in PBS with 1 mM diamide at 37°C for 45 min resulted in reproducible cell shape maintenance.

In order to test the effect of diamide on erythrocytes over time, control and diamide-treated cells were incubated in PBS with 10 mM glucose for up to 4 hr. Control cells remained as discs throughout the incubation period. Diamide-treated cells became progressively more crenated, with the most dramatic change occurring between 1 hr, when 66% of the diamide-treated cells were discs, and 2 hr, when 22% were discs. Therefore, although diamide could be expected to maintain cell shape for a period, it is evident that this lock on shape is not permanent. To avoid the problem of changing cell shape, a 1-hr in vivo incubation period was chosen.

It was necessary to ascertain that echinocytes or stomatocytes "fixed" with diamide remained as such after being injected into the blood stream. Five-hundred microliters of 51Cr-labeled treated cells were injected into a rat. After 5 min, aliquots of blood were removed by retroocular puncture and were fixed. On the basis of cpm/ml, it was possible to predict the percentage of treated cells that should be found in these samples. With 4 mM DNP-treated cells, microscopic observation revealed that 0.4% ± 0.3% (count-
EFFECT OF CELL SHAPE IN HEMOLYSIS

Table 1. Time Dependence of Clearance from Blood

<table>
<thead>
<tr>
<th>Time</th>
<th>Untreated</th>
<th>Control</th>
<th>Diamide*</th>
<th>DNP†</th>
<th>Chlorpromazine‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>10–20 min</td>
<td>102 ± 16 (16)§</td>
<td>72 ± 12 (20)</td>
<td>69 ± 5 (7)</td>
<td>53 ± 9 (4)</td>
<td></td>
</tr>
<tr>
<td>1 hr</td>
<td>121 ± 11 (16)</td>
<td>61 ± 13 (18)</td>
<td>50 ± 11 (7)</td>
<td>45 ± 8 (4)</td>
<td></td>
</tr>
<tr>
<td>4 hr</td>
<td>94 ± 6 (2)</td>
<td>43 ± 12 (2)</td>
<td>31 ± 4 (2)</td>
<td>11 ± 2 (2)</td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td>79 ± 9 (2)</td>
<td>17 ± 2 (4)</td>
<td>20 ± 1 (2)</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

*1 mM diamide.
†6 mM DNP.
‡0.4 mM chlorpromazine.
§Values are percentage of total injected cpm remaining in the blood stream ± SD, values in parentheses represent number of experiments.

In Vivo Clearance

Studies were then done to determine the effect of diamide treatment on erythrocyte longevity in the bloodstream (Table 1). The initial cpm/ml in the blood stream was calculated by determining the total blood volume from the body weight (4.95 ml blood/100 g body weight)\(^5\) and dividing total counts injected by this volume. The cpm/ml of blood injected is greater than the calculated cpm/ml with the untreated erythrocytes, indicating a possible problem of incomplete mixing of injected cells with the total blood volume. The data do show a more rapid clearing of diamide-treated cells than untreated cells from the bloodstream, particularly after 1 hr when the shape changes might be expected to occur. The addition of a cupping or crenating agent to the cells causes an even more rapid clearance from the bloodstream.

Clearance of erythrocytes from the blood is reflected in the percentage of the total \(^{51}\)Cr injected that was found in the organs (Table 2). The liver and spleen are thought to be the major organs of clearance. Kidneys clear lysed cells and the heart is included as a control organ. There are very few labeled untreated cells in the spleen (1%), kidney (1%), or heart (1%), and slightly more in the liver (8%). These liver cells presumably represent the volume of blood residing in the liver and the filtering of cells damaged by handling or by labeling with \(^{51}\)Cr. Diamide treatment causes a 6% increase in cells trapped in the spleen, while liver, kidney, and heart clearance levels remain approximately the same as the control. In the case of the diamide-treated cells, there is an apparent loss of counts from the blood (Table 1) without their appearance in the spleen or liver (Table 2). This difference could represent an increase in the blood pool size or clearance of diamide cells in other regions of the animal. To check for possible clearance of diamide cells by the first capillary bed encountered, the lungs were excised and counted, but no clearance was observed. Rather, it seems more likely that the difference represents a change in blood pool size and to an extent a statistical variation in the blood counts. Because of the reproducibility and the direct interpretation of the clearance as determined by the organ counts, the percentage of counts found in the various organs was primarily used as a measure of clearance.

Effect of Shape-Inducing Treatments on Clearance of Erythrocytes

The effects of two crenating treatments, 2,4-dinitrophenol (DNP) and ATP depletion, were studied. Figure 1 illustrates cell populations representative of the DNP-crenated and of ATP-depleted erythrocytes.
An increase in DNP concentration causes an increase in the number of echinocytes and a shift to more severely crenated cells (as defined by Weed).5

The echinocytes formed by treatment with DNP or by ATP depletion and held in shape by diamide were cleared from the blood by the organs more rapidly than diamide-treated erythrocytes after 1 hr (Table 1). Examination of individual organs reveals an increase in splenic clearance of 1%-7% by all concentrations of DNP tested and of 16% by ATP-depleted erythrocytes. Liver clearance, however, was dramatically increased. ATP-depleted cells plus diamide had 64% more clearance in the liver than diamide alone; 6 mM DNP and diamide a 24% increase; 4 mM DNP and diamide a 16% increase, and little change noted with 2 mM DNP and diamide. The association between CI (crenation index) and liver clearance suggests that the echinocyte is being cleared from the blood by the liver specifically as a consequence of the changed shape (Fig. 2).

Stomatocytes were induced by tetracaine, by chlorpromazine, and by washing cells that had been incubated overnight with 5 mM DNP, 10 mM glucose, 10 mM cytidine, and 10 mM myo-inositol. As with echinocytes, increasing drug concentrations caused a larger percentage of the cells to be cupped and caused a greater severity in the degree of cupping. A cell population representative of 0.4 mM chlorpromazine is shown in Fig. 1.

Stomatocytes held in shape by diamide were cleared from the blood stream more rapidly than discs treated with diamide (Table 1). There is a corresponding increase in the percentage of counts found in the organs (Table 2). With 6 mM and 4 mM tetracaine there were increases in liver clearance of 68% and 17%, respectively, over diamide levels. The lower concentration of tetracaine (2 mM) caused no change in clearance. Both 0.4 mM chlorpromazine and washed incubated cells resulted in a substantial elevation of clearance by the spleen as well as the liver. Chlorpromazine at 0.2 mM did not change the level of clearance from that of diamide. Again, the association between the severity of shape change as represented by CI and the amount of clearance by the organs suggests that changed shape causes clearance by the liver and perhaps also by the spleen in the case of chlorpromazine (Figs. 3 and 4).

**Drug Effects and Membrane Changes**

Several experiments were done to eliminate the possibility that clearance of echinocytes or stomatocytes is due to a direct drug effect or to a drug-dependent change in the erythrocyte membrane.
Erythrocytes were incubated with \(^3\)H-DNP or \(^3\)H-chlorpromazine plus diamide for 45 min at 37°C. The cells were then washed with PBS and counted. Less than 1% of the labeled DNP remained with the echinocytes. Approximately 2% of the \(^3\)H-chlorpromazine seemed to be bound to the stomatocytes.

Further evidence for noninvolvement of the shape-inducing agent itself in clearance comes from the fact that altered shape needed to be preserved for clearance to occur. Erythrocytes were treated with several concentrations of DNP and then with 1 mM diamide for 30 min at 37°C, which was not sufficient to maintain the crenated shape. The cells then became discocytes after the DNP was washed away. The percentage of counts found in the spleen, liver, and heart were the same for all DNP concentrations plus diamide and for diamide alone. This suggests that DNP itself does not cause a chemical alteration in the membrane that results in clearance.

**Reversibility of Shape Change**

Not only are there no effects of the shape-inducing agent itself, but also the changed morphology is reversible, as was seen above with DNP and is seen in Table 3 with ATP depletion. Erythrocytes were incubated for 12 hr at 37°C in PBS with added penicillin and streptomycin. When these echinocytes were incubated with 10 mM adenosine for 1 hr, 59% of the cells reverted to discs. There is a corresponding reduction in clearance of cells by the spleen and liver. If the echinocytic shape was reversed with tetracaine and diamide treatment, there was also a decrease in the amount of clearance (Table 3). Shape reversal in these experiments was not uniform, and a range of shapes from cupped to crenated cells was observed in each sample. There was, however, an increase in the percentage of cupped cells and a decrease in clearance with increasing tetracaine concentration. This suggests that shape change, not alterations in membrane or metabolic processes, triggers the clearing mechanism.

**Protein Crosslinking**

Polyacrylamide gel electrophoretic analysis of erythrocyte ghosts revealed that diamide caused S-S crosslinking of membrane proteins as in Palek et al.\(^{16}\). Electrophoresis of membrane proteins from erythrocytes treated with shape-change-inducing agents and diamide resulted in the same pattern of separation as that of diamide alone. Reduction with \(\beta\)-mercaptoethanol of ghosts from diamide-treated erythrocytes resulted in protein separation identical to that of ghosts from untreated erythrocytes.

**IgG and C3 Binding**

Echinocytes and stomatocytes were assayed for an increase in IgG and complement component 3 (C3) binding (see Materials and Methods for assays). Cells treated with the highest concentrations of DNP showed approximately the same amount of IgG binding as control, 4 mM tetracaine-treated cells almost 4 times control, and 0.4 mM chlorpromazine 10 times control levels of IgG binding (see Table 4). With the cupping agents, there was a correlation between the extent of clearance and the amount of IgG binding. All transfer analyses revealed no C3 binding with any of the treatments tested.

### Table 3. Effect of Shape Recovery on Clearance

<table>
<thead>
<tr>
<th></th>
<th>Spleen</th>
<th>Liver</th>
<th>Morphology*</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP depleted + diamide (5)†</td>
<td>34 ± 11</td>
<td>46 ± 13</td>
<td>16/0/84</td>
<td></td>
</tr>
<tr>
<td>ATP depleted + 10 mM adenosine (3)</td>
<td>18 ± 1</td>
<td>16 ± 5</td>
<td>59/34/7</td>
<td></td>
</tr>
<tr>
<td>ATP depleted + 2 mM tetracaine + diamide (2)</td>
<td>21 ± 10</td>
<td>59 ± 14</td>
<td>0/48/52</td>
<td></td>
</tr>
<tr>
<td>ATP depleted + 3 mM tetracaine + diamide (2)</td>
<td>9 ± 0.8</td>
<td>50 ± 10</td>
<td>3/74/23</td>
<td></td>
</tr>
<tr>
<td>ATP depleted + 4 mM tetracaine + diamide (2)</td>
<td>5 ± 0.2</td>
<td>32 ± 3</td>
<td>4/97/0†</td>
<td></td>
</tr>
<tr>
<td>Control + diamide (2)</td>
<td>7 ± 2</td>
<td>6 ± 2</td>
<td>98/0/2</td>
<td></td>
</tr>
</tbody>
</table>

*Values represent percent discocytes / percent stomatocytes / percent echinocytes.
†Number of rats.
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densometric scans of the gel transfer autoradiographs. Six samples each in three experiments.

Changes in clearance of labeled erythrocytes occurred in the diamide-treated cells at early times, and the diamide-treated cells had altered shapes. Several methods of altering shape were employed to control for amphipath or system-dependent effects. Of particular interest are the reversibility experiments with either washing of the drug from DNP-treated cells or the addition of a cupping agent to ATP-depleted cells. ATP-depletion and crenation have been extensively studied and has been shown to produce hepatic clearance.

Reversal of the cell shape change with tetracaine is sufficient to decrease the extent of clearance. Tetracaine would be expected to reverse only the shape change and not the other effects of ATP depletion. Further, 4 mM tetracaine increases the clearance of normal cells. Thus, tetracaine increases or decreases erythrocyte clearance depending on whether it alters the shape from or towards a biconcave disc, respectively. This and other observations can best be explained by a direct effect of shape on erythrocyte longevity.

Earlier studies of erythrocytes damaged with phenylhydrazine, IgG, or heat noted splenic clearance with mild damage, and with more severe damage, hepatic clearance. With an increase in the crenation or cupping index, there was only a correlative increase in hepatic clearance. The exception was chlorpromazine, which was different from the other agents in that it has been shown to displace calmodulin from membranes. The unexpected pattern of clearance with chlorpromazine may reflect an alternative mechanism of clearance for shape-altered cells or may be the result of the much greater IgG binding to chlorpromazine-treated cells.

In many respects the rat’s erythroclastic system is analogous to that of the human. The histologic structure of the spleen and its relative size is similar. Both IgG-opsonized and heat-denatured erythrocytes are primarily cleared by the spleen in rats and in humans. Although the spleen is thought to be the major organ for clearance of aged cells in both animals, in neither case is there a change in erythrocyte lifespan after splenectomy. The major difference between human and rat erythrocytes is that rat erythrocytes are much more fragile in vitro and can undergo spontaneous lysis.

Numerous mechanisms have been proposed for the clearance of damaged erythrocytes. In one case, the shape changes are believed to lead to erythrocyte sphering and decreased deformability, which results primarily in splenic trapping. The observations presented here do not support that theory, since deformability is already decreased by diamide, and shape changes result in hepatic, not splenic, clearance. An alternative model to the mechanical mechanism of clearance is the alteration of surface receptors such as the IgG receptor. Studies by Kay indicate that surface IgG binding increases with erythrocyte aging. These data show a correlation between clearance and an increase in IgG binding for cupping but not crenating agents. IgG binding per se leads to splenic clearance and would explain why there is splenic clearance with chlorpromazine cupped cells, which binds IgG most strongly. The correlations between hepatic clearance, the crenation and cupping indexes are not explained by IgG binding.

Because the liver is presumed to sequester erythrocytes on the basis of their altered surface properties, it is logical to suggest that the shape change alters surface properties, which in turn produce clearance. The exact surface property that is correlated with clearance is not known. Maintenance of the biconcave disc morphology appears from these studies to be important not only for O2 exchange, but also for erythrocyte survival.
REFERENCES


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