Effect of Heinz Bodies on Red Cell Deformability

By Alan Lubin and Jane F. Desforges

Decreased deformability of acetylphenylhydrazine-treated erythrocytes, measured by in vitro filtration experiments, was found to parallel Heinz body formation. The decreased deformability seemed unrelated to methemoglobin formation, ATP depletion, red cell size, or membrane effect. Hemolysis of Heinz body erythrocytes occurred during filtration of severely damaged cells, in an all-or-none fashion. In mixtures, separation of untreated cells from Heinz body-containing erythrocytes could be accomplished by filtration. There may be a relationship of these filtration properties to intravascular, in vivo destruction of severely damaged cells in Heinz body anemias.

The role of the spleen in production of Heinz body anemias has been well established. Splenic sequestration, with fragmentation of cells and pitting of Heinz bodies, leading to membrane loss and spherocyte formation, seems a major factor in these anemias. Yet, the liver’s role in Heinz body cell destruction may also be major. The occasional finding of methemalbuminemia, hemoglobinemia, and hemoglobinuria in hemolysis associated with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency suggests the presence of intravascular destruction. Destruction of these cells in liver or peripheral circulation cannot be explained by mechanisms similar to the observed splenic pitting or fragmentation. Instead, grosser mechanical fragility characteristics, with loss of cellular deformability, may be responsible.

Red cell deformability has been measured by cellular filtration characteristics in a variety of red cell disorders. In the present study, filtration properties of drug-treated erythrocytes were investigated in vitro, in order to relate changes in filterability, and hence cell deformability, to known red cell changes during Heinz body formation.

MATERIALS AND METHODS

Erythrocytes containing Heinz bodies were prepared by incubation of freshly obtained, heparinized whole blood from normal physicians and technicians in 2-ml aliquots, each within a 25 ml Erlenmeyer flask containing 15 mg of acetylphenylhydrazine. These, and similar 2-ml samples without acetylphenylhydrazine, were placed in a Dubnoff shaker at 37°C, and flasks were removed from the bath at intervals. Erythrocyte ATP was...
measured in a 1 ml sample of this whole blood, placed directly into 0.6 M perchloric acid, utilizing Sigma kit 366 uv. Another 0.5 ml aliquot of blood was washed thrice with modified Kreb's-Ringer's phosphate buffer (KRP) containing: Na+, 157 meq/liter; Mg2+, 1.9 meq/liter; K+, 4.96 meq/liter; Cl, 121 meq/liter; HPO42-, 40 meq/liter; SO42-, 1.9 meq/liter; albumin, 2.5 g/liter; glucose, 1.0 g/liter. The pH adjustment (7.35) of this solution and addition of glucose and albumin to it were performed daily just before use. Sufficient packed, washed erythrocytes were suspended in a total of 20 ml of the KRP solution to obtain a red cell concentration between 50,000 and 75,000/cu mm. Neither white cells nor platelets was seen in these preparations. Red cell counts and mean corpuscular volume measurements were performed on the Coulter Counter Model B. The red cell suspension was then filtered at room temperature. Forty-seven-millimeters diameter SM Millipore filters of 5 μ pore size, mounted on the lower half of the Swinnex filter holder provided by the Millipore Corp. (Bedford, Mass.), were utilized. The filter holder, in turn, was mounted on a corked 2 liter suction flask. Negative filtration pressure of 35 mm mercury was used throughout the experiments; this was attained by adjustment of a valve and placed in the line leading from the suction flask to wall suction. Once attained, a constant level of pressure was maintained throughout each filtration by closing the valve, after a filter wetted with Ringer's lactate was placed on the holder. Filtration time was measured by stop watch from the onset of pouring the suspension onto the filter until the entire 20 ml had completed passage. A new filter was used for each passage. The filter holder and plastic collection tube were rinsed with water and then 0.85% saline between uses until free flow was assured. Red blood cell count and mean corpuscular volume were measured in the filtrate immediately postfiltration. Per cent passage was calculated as

\[
\text{postfiltration red blood count} \times 100 \div \text{prefiltration red blood count}
\]

A portion of the filtrate was then spun for 1 min at 3400 rpm (Adams Sero-Fuge), and "plasma" hemoglobin of the supernatant determined according to the method of Crosby and Furth. Although initially pH of the red cell suspension was measured before and after each filtration, this was discontinued after a large number of determinations demonstrated stable pH pre- and postfiltration. Experiments were carried out between pH 7.35 and 7.40.

Wet preparations of red cell suspensions before and after filtration were stained with brilliant cresyl blue and examined immediately under the oil immersion lens of a light microscope. Cells containing Heinz bodies were graded 1+ to 4+ according to the following criteria: 1+, small Heinz body or bodies in some cells; 2+, small Heinz body or bodies in all cells; 3+, large Heinz bodies in some cells and no membrane abnormality visible; 4+, multiple large Heinz bodies in all cells and some cells with irregular membrane and crenation.

Methemoglobin was produced by incubation of whole blood at 37°C for 20 min with 0.1 ml of 0.18 M NaNO2. Per cent methemoglobin was measured by the method of Evelyn and Malloy.

Cyanmethemoglobin in intact red cells was prepared by the technique of Beutler and Baluda. Plasma was replaced by an equal volume of 1% NaNO2, and 0.1 ml of 5% neutralized sodium cyanide (prepared by mixing equal parts of 10% sodium cyanide and 12% acetic acid just before use) per ml whole blood was added. After mixing and incubation at room temperature for 20 min, cells were separated by centrifugation from the cyanide-nitrite solution, washed twice with the KRP solution, and resuspended in their own plasma. Incubation of this treated blood, with and without ACP, was carried out as previously described. Whole blood, untreated with cyanide-nitrite, served as an incubation control. Cells were washed with and suspended in KRP solution prior to filtration.

In the experiment involving PMB (as the sodium salt of p-hydroxymercuribenzoate, obtained from Sigma Chemical Co., St. Louis, Mo.), whole blood was incubated at room temperature for 90 min with 5 μM of PMB/ml of red blood cells. Cells were then washed with the KRP solution, suspended in it, and filtered as usual.
RESULTS

Filtration of Normal Erythroctes

Variability of normal cell passage among different filter lots was noted, but remarkable uniformity was present within a given lot. Accordingly, the results reported for passage are from a single filter lot, for both normal and treated cells. In a series of 15 separate filtrations of cells from normal individuals suspended in KRP and filtered without treatment or incubation, per cent passage was 78.1 ± 6.2 (2 SD). Cells appeared discoid and nonfragmented both pre- and postfiltration. Passage time was 7 sec with a range of 6–8 sec.

Filtration of Erythrocytes Containing Heinz Bodies

A summary of five separate filtration experiments involving erythrocytes containing Heinz bodies, and control cells incubated in parallel, is depicted in Fig. 1. Control cells did not form Heinz bodies during the incubation period. Their passage time was 7 sec, and filterability was unaltered throughout the 6-hr incubation. They were discoid and nonfragmented pre- and postfiltration.

ACP-incubate cells uniformly contained 2+ Heinz bodies at 2 hr, 3+ Heinz bodies at 4 hr, and 4+ Heinz bodies at 6 hr in four of the five experiments. Their per cent passage appeared slightly decreased at 2 hr, markedly decreased at 4 hr, with almost total removal of cells by the filter at 6 hr. Passage time was normal (7 sec) at 2 hr but was prolonged beyond 2 min at 4 and 6 hr in all four instances. At 6 hr, incomplete passage of the 20 ml solution was noted in most experiments despite a prolonged passage time.

In the fifth experiment, 3 ml instead of 2 ml of whole blood were incubated with 15 mg of ACP. Only 2+ Heinz body formation was seen at 4 hr, with 3+ Heinz bodies at 6 hr. Filterability was less impaired than in the first four experiments, with only slightly decreased per cent passage at 4 hr.

A similarly less abnormal filterability, despite prolonged incubation with ACP, was noted in two experiments during which cells were not shaken. Here, Heinz bodies attained only the 2+ grade at 6 hr of incubation, consistent with the known enhancement of ACP-induced Heinz body formation by shaking in air.17 Only slight decrease of filterability was seen at 6 hr. In one of the experiments, continued for 8 hr, 3+ Heinz-body formation ensued, coinciding with a decrease in filterability to 39% passage.

Filtered cells seemed to contain as many Heinz bodies as the prefiltration...
Fig. 2. Plasma hemoglobin levels of control and ACP-treated filtrate supernatants. Levels were measured in four of five experiments summarized in Fig. 1.

sample, were not fragmented, and, except in the case of 4+ Heinz body cells at 6 hr, were discoid and not distorted. Four plus cells often had distorted outlines both pre- and postfiltration and many were spheroidal and crenated. At 4 and 6 hr, many free round tiny particles were visible in the wet preparations of the filtrate, similar in appearance to Heinz bodies.

Mean corpuscular volumes, measured by the cell "sizer" of the Coulter Model B, were increased by 3-4 cu µ on incubation in both ACP-treated and control samples, MCV was not decreased postfiltration in any experiments.

"Plasma" hemoglobin concentrations determined on the filtrates of the experiments depicted in Fig. 1 showed little change in the control samples. In contrast, a rise in the supernatant hemoglobin concentration of the ACP-incubated cell filtrates was seen at 4 and 6 hr (Fig. 2).

Erythrocyte ATP levels declined in the incubated whole blood during these experiments (Fig. 3). However, the means of erythrocyte ATP levels of control and ACP-incubated cells were not significantly different ($p$ always $> 0.20$, t test for paired data) at any given time interval.

**Separation of Normal Cells and Those Containing Heinz Bodies by Filtration**

Following a 5-hr incubation period, washed ACP-treated and control cells were mixed in the KRP suspension to attain the relative proportions of approximately 50% Heinz body positive cells in one experiment and 20% Heinz body positive cells in another. Each experiment was performed in triplicate. Average differential counts were derived from the microscopic differentials of positive and negative cells in the suspension before and after each filtration. Three to four plus Heinz bodies were formed at this incubation time in the
ACP-treated cells, so distinction between positive and negative cells was simplified. Per cent passage was calculated as usual and was then averaged.

In the 50% mixture, filterability was markedly reduced to 15.9% passage. However, those cells that passed the filter were almost entirely Heinz body negative (94.9%). In the 20% mixture, filterability was only slightly reduced (66% passage), and removal of Heinz body positive cells, though demonstrable, was less striking than in the 50% mixture (Table 1).

**Filtration of Methemoglobinemic Cells**

In this experiment, done in triplicate, a range of methemoglobin levels from 95.5 to 99.5% was attained. Cells appeared discoid and did not contain Heinz bodies on microscopic examination of cell suspensions. The filterability of methemoglobinemic and normal cells was similar, with average passage of 75% and 78%, respectively.

**Filtration of Cells Containing Cyanmethemoglobin**

Cells were prepared with the cyanide-nitrite mixture to produce cyanmethemoglobin. These cells, prior to incubation, had a normal filterability (74.0% passage). During incubation, no Heinz bodies appeared over the 6 hr period in the cyanmethemoglobinemic, ACP-treated cells. All cells maintained a normal, discoid appearance. Filterability was not reduced in the incubated cyanmethemoglobin cells, with or without ACP (Table 2). Thus, a definite protective effect of cyanmethemoglobin against Heinz body formation and the accompanying reduced filterability was demonstrable.

**Filtration of Cells Treated with PMB**

After 90 min of incubation with PMB, cells were normal, and not spheroidal. Their passage time was not prolonged (7 sec), and passage was 81.3% as compared to 87.3% for the untreated control cells. Thus, PMB, which is known primarily to blockade erythrocyte membrane sulfhydryl groups, had no effect on cell filterability in our filtration system.

**DISCUSSION**

Erythrocyte membrane sulfhydryl depletion leads to electrolyte leak in vitro and splenic destruction in vivo. More severe sulfhydryl depletion can ultimately induce spherocytosis and destruction in liver, as well as spleen.

Table 1. Per Cent Passage and Differential Counts Pre- and Postfiltration of 50% and 20% Mixtures of Heinz Body Positive With Untreated Cells

<table>
<thead>
<tr>
<th></th>
<th>Heinz Body %</th>
<th>Total RBC/cu mm</th>
<th>% Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>50% Mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prefiltration</td>
<td>51.1</td>
<td>48.9</td>
<td>47,830</td>
</tr>
<tr>
<td>Postfiltration</td>
<td>5.1</td>
<td>94.9</td>
<td>7,600</td>
</tr>
<tr>
<td>20% Mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prefiltration</td>
<td>23.1</td>
<td>76.9</td>
<td>81,830</td>
</tr>
<tr>
<td>Postfiltration</td>
<td>9.1</td>
<td>90.9</td>
<td>54,130</td>
</tr>
</tbody>
</table>
Table 2. Per Cent Passage of Incubated Control Cells, Cyanmethemoglobinemic Cells, and ACP-Treated Cyanmethemoglobinemic Cells Over a 6-Hr Incubation Period

<table>
<thead>
<tr>
<th></th>
<th>Control Cells</th>
<th>Cyanmethemoglobinemic Cells</th>
<th>Cyanmethemoglobinemic + ACP Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>74.0</td>
<td>74.5</td>
<td>79.5</td>
</tr>
<tr>
<td>2 hr</td>
<td>79.0</td>
<td>74.5</td>
<td>79.5</td>
</tr>
<tr>
<td>4 hr</td>
<td>74.5</td>
<td>71.8</td>
<td>67.5</td>
</tr>
<tr>
<td>6 hr</td>
<td>84.5</td>
<td>74.0</td>
<td>72.6</td>
</tr>
</tbody>
</table>

Apart from membrane sulfhydryl depletion, and electrolyte leak, the increased erythrocyte destruction in Heinz body anemias may also result from the rigid nature of the Heinz bodies themselves. Electron microscopic studies demonstrate apparent pitting of Heinz bodies by splenic macrophages and suggest erythrocyte fragmentation during passage from cord to sinusoid. Presumably, membrane loss incurred during such processes conditions the resultant spheroidal cell to later premature destruction. However, although such static histologic studies of apparent Heinz body cell rigidity seem convincing, dynamic demonstrations have been lacking.

Erythrocyte "rigidity" presumes a loss of the normal cellular deformability. This may be measured in vitro by observing erythrocyte filtration through 3-8 μ filter pores. Decreased cellular deformability has been demonstrated in several hemolytic states. Cellular ATP loss may also be associated with decreased filterability. Changes in filtration characteristics may be related either to rigidity of membrane or intracellular contents.

Teitel et al. studied filtration of damaged cells. Although employing a 20-40 μ pore size paper filter, and hence not truly measuring erythrocyte deformability, these workers were able to showed marked decreases in filterability of red cells incubated with NEM. Despite their attempt to relate this to GSH depletion, it is apparent from the data presented that profound GSH depletion preceded, rather than accompanied, filtration changes. Partial protection against NEM-induced filtration changes was afforded by incubation in high-K⁺ medium, while more complete protection was provided by conversion of cellular hemoglobin to cyanmethemoglobin prior to incubation. Direct observations of cells for presence or degree of Heinz body formation were not described, however.

In the present experiments, cells incubated with acetylphenylhydrazine developed a decreased filterability that paralleled Heinz body formation as graded visually. Separation of Heinz body positives and negative cells by filtration was striking in the 50% mixtures. Very few Heinz body positive cells could deform sufficiently to traverse the clogged filter, whereas the normal cells could be so deformed. In the mixture containing 20% Heinz body positive cells, presumably the pores became less clogged and passage was less abnormal.

Filter-induced hemolysis of Heinz body cells was indicated by the xanthochromia and increased plasma hemoglobin of the filtrate’s centrifuged supernatant at 4 and 6 hr of incubation. The presence of free particles resembling Heinz bodies, but lack of fragments or decreased MCV in the filtrate, sg-
gested that the hemolysis was an all-or-none phenomenon.

To determine the cause of the rigidity of these Heinz body-containing cells, several factors were examined. Erythrocyte ATP depletion may lead to disc-sphere transformation and decreased cellular deformability. Accelerated ATP depletion in the presence of oxidant agents, which has been reported, did not occur under our experimental conditions. ATP depletion was no greater in the ACP-incubated cells than in the controls, demonstrating that this alone was not responsible for filtration differences between control and drug-treated cells.

Methemoglobin formation is a usual accompaniment of drug-induced Heinz body formation in vitro. However, there was no difference in filterability between methemoglobinemic cells that did not contain Heinz bodies and control cells.

The conversion of hemoglobin to cyanmethemoglobin inhibits Heinz body formation. In our experiments, no Heinz bodies were formed in cyanmethemoglobinemic cells treated with ACP. Although other actions of the drug may have continued unchecked, the cells were normally filterable even after 6 hr incubation. Furthermore, direct attack on membrane thiols by quantities of PMB shown to cause K+ leak in vitro, and to decrease erythrocyte survival due to splenic uptake in vivo, had no effect on filtration.

From these experiments, membrane effects and electrolyte flux seem unlikely as causes of decreased erythrocyte filterability in our filtration system. Instead, the presence of Heinz bodies seems most likely to be responsible for decreased cellular deformability. Filtration changes paralleled Heinz body formation and failed to occur when Heinz body formation was impaired.

Our filtration system is relatively insensitive to milder erythrocyte damage and removes only more severely damaged cells. In this respect, it may be comparable to destruction of cells in areas of the body other than spleen, which is the organ most sensitive to minimally damaged cells. The all-or-none form of mechanical destruction demonstrated in the filter would not require splenic presence. Destruction could occur in liver or even in small peripheral capillaries.

Although caution must be exercised in comparing a highly artificial filtration system to the human circulation, such a system may perhaps serve as a model for destruction of severely damaged erythrocytes in Heinz body anemias of various types. Certainly, it demonstrates in a dynamic fashion the suspected rigidity of cells in these conditions.

ACKNOWLEDGMENT

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REFERENCES

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