Observations on Heinz Bodies in Normal and Splenectomized Rabbits

by Harvey Rothberg, Leon A. Corallo and William H. Crosby

The ability of the spleen to remove and destroy abnormal red blood cells has been known for many years. However, only recently has it been appreciated that in certain situations the spleen can remove abnormal intraerythrocytic inclusions without destroying the red cells which contain them. It has been demonstrated that intact subjects, in contrast with those who have been splenectomized, rapidly lose the iron-staining granules from transfused Cr\(^{51}\)-labeled siderocytes while most of the tagged cells continue circulating.\(^1\) It has been proposed that the normal spleen can assist red cells to rid themselves of inclusion bodies; this has been called the spleen’s “pitting function,” by analogy with the removal of a cherry stone without crushing the fruit.\(^2\)

Loss of the “pitting function” might explain the well known occurrence of Howell-Jolly bodies, siderocytes, Heinz bodies\(^3\)\(^4\) or bartonella organisms in the red cells after splenectomy. Congenital absence of this function may well have been involved in the appearance of Heinz bodies—without anemia—in the two full-term infants with splenic agenesis described by Gasser and Willi.\(^5\) Normal operation of the mechanism would account for the extrusion of nuclei of transfused avian erythrocytes in the mammalian spleen, so clearly portrayed by Vorhaus and Turner.\(^6\)

It seemed worthwhile to study this phenomenon further, and to learn to what extent the spleen could effect the removal of other types of inclusion bodies. It was decided to study Heinz bodies, which can be conveniently produced in vitro in large quantities. Early in the course of this work, we were encouraged by the report of Selwyn,\(^4\) who also postulated that the spleen might remove Heinz bodies from intact red cells.

Materials and Methods

Adult rabbits were used. Splenectomy was performed two months prior to the experiment. Twenty to 50 ml. of blood were removed by cardiac puncture and mixed with equal volumes of pH 7.6 M \(15\) phosphate buffer. Freshly prepared aqueous phenylhydrazine solution was then added to give a final concentration of 0.5 mg. per ml., and the mixture was incubated in a water bath at 37 C. for 50 minutes. Oxygenation was accomplished by blowing several hundred ml. of sterile air through the mixture at 10 or 15 minute intervals. At the end of 50 minutes, at least 99.8 per cent of the incubated erythrocytes contained 10 to 40 (usually 15 to 30) well defined Heinz bodies of small to medium size. The treated cells were then tagged by the addition of 20 \(\mu\)c of Cr\(^{51}\) (as sodium chromate) and reincubated for 35 minutes, after which 50 mg. of ascorbic acid were added. The cells were then washed three times and finally reconstituted in normal saline, and injected into the marginal ear vein of the animal from which they had been obtained.

From the Department of Hematology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

Submitted Jan. 9, 1959; accepted for publication June 1, 1959.

1180
Samples were obtained from an ear vein, and hematocrits, reticulocyte counts, radioactivity and “Heinz body cell” counts were determined.

Heinz bodies were conveniently stained by mixing in a capillary tube one drop of blood with one or two drops of 0.5 per cent methyl violet in saline. After five minutes cover-slip preparations were made, allowed to dry and mounted on slides. No counterstain or fixative was used. The resultant dry preparations could be kept indefinitely and examined at leisure.

Removal of Heinz bodies from the circulation was studied chiefly by noting the rate of disappearance of Heinz body cells from the blood. The number of such cells among at least 3000 erythrocytes was determined and expressed as a percentage of the concentration of Heinz body cells present 10 or 15 minutes after infusion of the treated blood.

It was recognized that a relatively high concentration of phenylhydrazine had been used, resulting in a large number of Heinz bodies per cell and a severely damaged erythrocyte population. This was done to achieve uniformity of the inoculum, since preliminary experiments had indicated that a lower phenylhydrazine concentration resulted in appearance of Heinz bodies in only a portion of exposed cells. Production of the inclusions in essentially 100 per cent of treated and reinfused cells facilitated the counting of significant numbers of surviving transfused Heinz body cells, and also avoided the possibility of differences in Cr51 labeling of Heinz body cells and erythrocytes of normal appearance. However, it seemed possible that the red cells incubated in 0.05 per cent phenylhydrazine might have been so grossly damaged that the intraerythrocytic inclusions could not be removed without destruction of the entire cell. Alternatively, there might be so many inclusions present that only a few could be removed by the time the cell was destroyed. In an effort to approach this problem, the number of discernible Heinz bodies per affected cell was determined in the serial samples from a normal and a splenectomized animal.

**Results**

Serial observations on the first four rabbits studied are summarized in table 1. It should be noted that the anemia was mild even though from 28 to 40 per cent of the animals’ circulating red blood cell mass was destroyed within a few days. The Cr51 half-life of the phenylhydrazine-treated red cell was 20 to 38 hours, in contrast to the normal rabbit red cell half-life of about 19 days.7 The notable feature of these data was the parallel decline of the Cr51 radioactivity and of the circulating Heinz body cell count (fig. 1). The near-coincidence of the two curves indicates that removal of the Heinz bodies from the circulation was accomplished by removal of the red cells containing them, rather than by a “pitting mechanism.”

In table 2 are results of experiments in which the number of inclusions in each surviving cell was counted at various intervals after reinfusion of treated erythrocytes. It is evident that the surviving Heinz body cells did not lose even a part of their Heinz body content as they aged in the circulation. Differences in Heinz body counts in the two animals may be attributed to longer incubation of the cells of rabbit no. 2 rather than to any intrinsic difference between the two animals.

The spleen as site of removal of damaged red cells.—The presence of a high concentration of Heinz bodies in the spleens of phenylhydrazine-treated rabbits has been reported.4 In one of our experiments, a rabbit was sacrificed three days after infusion with autologous phenylhydrazine-treated erythrocytes, when the concentration of Heinz body cells circulating in the peripheral blood was only 0.17 per cent, but their frequency in a smear of the
Table 1.—Disappearance of Phenylhydrazine-Treated Erythrocytes from the Circulation of Normal and Splenectomized Rabbits

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hours after transfusion</th>
<th>Reticulocytes</th>
<th>Heinz body cell survival</th>
<th>RBC survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% of 3000 cells</td>
<td>% of baseline</td>
<td>% of baseline Cr²⁺ activity</td>
</tr>
<tr>
<td>Intact rabbit</td>
<td>1/4</td>
<td>37</td>
<td>27.6</td>
<td>100.0</td>
</tr>
<tr>
<td>(no. 1)</td>
<td>1</td>
<td>36</td>
<td>25.6</td>
<td>92.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35</td>
<td>19.5</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>33</td>
<td>15.8</td>
<td>57.2</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>28</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>112</td>
<td>29</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Splenectomized</td>
<td>1/4</td>
<td>30</td>
<td>1.4</td>
<td>40.2</td>
</tr>
<tr>
<td>(no. 2)</td>
<td>5</td>
<td>32</td>
<td>1.8</td>
<td>39.2</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>31</td>
<td>3.0</td>
<td>34.0</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>27</td>
<td>5.6</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>27</td>
<td>11.2</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>25</td>
<td>2.9</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>142</td>
<td>32</td>
<td>5.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Splenecto-</td>
<td>1/6</td>
<td>28</td>
<td>4.4</td>
<td>28.4</td>
</tr>
<tr>
<td>tomitized (no. 3)</td>
<td>1</td>
<td>28</td>
<td>2.8</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>24</td>
<td>2.7</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>24</td>
<td>3.4</td>
<td>21.7</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>26</td>
<td>6.7</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>27</td>
<td>2.7</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>27</td>
<td>6.4</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>28</td>
<td>5.1</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>169</td>
<td>31</td>
<td>4.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Splenec pulp was 37.9 per cent, or 223 times their concentration in the peripheral blood. A large number of extracorpuscular Heinz bodies was seen in the spleen preparations, but this might well have been an artefact of the smear technic. Fixed sections were not examined.

Concentration of damaged erythrocytes in the spleen was vividly demonstrated by comparison of the color of spleen homogenates or of the cut surface of spleens from rabbits given phenylhydrazine-treated erythrocytes and from controls. Normal spleen was the usual red color; the treated animal's spleen was a deep brown, due presumably to the methemoglobin content of the Heinz body cells.

Discussion

The existence of irregular refractile inclusion bodies in the red cells of animals given phenylhydrazine and similar drugs was first reported in detail...
HEINZ BODIES IN NORMAL AND SPLENECTOMIZED RABBITS

Fig. 1.—Disappearance of autologous phenylhydrazine-treated erythrocytes from the circulation of a normal rabbit (no. 2). The near-coincidence of the two curves indicates that removal of the Heinz bodies from the circulation is accomplished by removal of the red cells containing them.

by Heinz in 1890.9 Since then there have been numerous clinical and experimental studies on Heinz bodies in toxic anemias and occasionally in premature infants9 or splenectomized subjects. Most of this work has been summarized.10–12

Clues to the pathogenesis of Heinz body formation have been provided by biochemical studies on red cells of patients liable to the hemolytic anemia produced by primaquine and related aniline derivatives, including phenylhydrazine.13 Red cells from such subjects differ from normal erythrocytes in at least four ways14: (1) their reduced glutathione content is lower15; (2) their reduced glutathione is more rapidly destroyed during incubation with acetylphenylhydrazine16; (3) on exposure to drug they exhibit increased Heinz body formation both in vivo and in vitro17; (4) they are genetically deficient in glucose-6-phosphate dehydrogenase,18 which is believed to be the primary enzymatic abnormality in such subjects.19,20 It would be of interest to determine whether immaturity of a part of this metabolic system is responsible for the Heinz body anemia of premature infants.

Chemically, Heinz bodies are generally thought to be particles of denatured hemoglobin. They are readily produced only under aerobic conditions.17,21 Beaven and White21 found that in the presence of phenylhydrazine and similar compounds, oxyhemoglobin is degraded in vitro to an insoluble "green hemoglobin" (containing heme and other iron pigments bound to denatured globin) whereas the phenylhydrazine is oxidized to benzene and nitrogen. Some of the oxyhemoglobin is oxidized to methemoglobin prior to the denaturation process. Similar heme pigments and denatured globin were demonstrated in the Heinz bodies of a patient with acetylphenylhydrazine poisoning.21

It is evident that the intrinsic metabolism of normal erythrocytes provides them with considerable resistance to the action of the agents which produce
Heinz bodies. The overpowering of these defenses permits oxidation and degeneration of hemoglobin and involvement to some extent of the stromal fabric of the red cells. It may be that any degree of damage of this sort represents an injury which is incompatible with survival of the affected cell. Certainly under the conditions of these experiments, the alterations induced in the phenylhydrazine-damaged red cell were so extensive that normal survival was not possible. The cells studied each contained 10 to 40 discernible Heinz bodies; it seems likely that the major portion of their hemoglobin content was denatured. Furthermore, the denaturation process was probably irreversible, since neither size nor number of the Heinz bodies was diminished on successive days after the infusion. Thus there was no evidence for operation of a "pitting mechanism" for removal of Heinz bodies from the damaged cells. It may yet be possible to demonstrate such a mechanism by varying the conditions of the experiment. For example, one might use a lower concentration of phenylhydrazine, or use another animal, such as the mouse,22 in which the tendency is to form single large inclusion bodies rather than multiple small ones as in the rabbit.

Despite the demonstrated role of the spleen in removing phenylhydrazine-damaged erythrocytes from the circulation, there is a striking similarity in the survival times of Heinz body cells in normal and in splenectomized rabbits (table 1). The Cr51 half-lives for the intact animals were 20 and 36 hours; for the splenectomized, they were 34 and 38 hours. The question naturally arises, where then are the red cells destroyed in the splenectomized animal? The apparent inseparability of the Cr51 and Heinz body tags in the phenylhydrazine-treated erythrocytes (fig. 1) makes it possible to study the removal of these damaged red cells by following the radioactivity alone, which is easily measured. This was done in a few rabbits by sacrificing them three or four days after infusion of autologous Cr51-labeled, phenylhydrazine-damaged erythrocytes, and determining the radioactivity of homogenates of various body tissues. Only preliminary studies of this sort could be carried out; but indications are that in the absence of the spleen the major burden of abnormal red cell destruction is shared by the liver and the bone marrow, with lungs, kidney, lymph node, fatty marrow (in an old rabbit) and skeletal muscle contributing little to the task.
HEINZ BODIES IN NORMAL AND SPLENECTOMIZED RABBITS

Such technics utilizing drug-damaged autologous erythrocytes may provide a valuable tool for the quantitative study of the reticuloendothelial system, both under normal conditions and in pathologic states.

SUMMARY
The disappearance from the circulation of Heinz bodies, produced by incubation of blood with phenylhydrazine, was studied in normal and splenectomized rabbits. In the intact animal, red cells damaged by this procedure are removed largely by the spleen. In both intact and splenectomized rabbits, the Heinz bodies are withdrawn from the circulation by removal in toto of the red cells containing them.

SUMMARIO IN INTERLINGUA
Le disparition, ab le circulation, de corpores de Heinz que habeva essite producite per incubation de sanguine con phenylhydrazina esseva studiate in conilios intacte e in conilios splenectomisate. In animales intacte, erythrocytos ledeite per ille processo esseva eliminate in grande mesura per le splen. Tanto in animales intacte como etiam in animales splenectomisate, le corpores de Heinz es eliminate ab le circulation per le elimination total del erythrocytos que contine los.

REFERENCES
16. —: The glutathione instability of drug-sensitive red cells: a new method


Observations on Heinz Bodies in Normal and Splenectomized Rabbits

HARVEY ROTHBERG, LEON A. CORALLO and WILLIAM H. CROSBY