Detection of Ferritin in the Plasma of Guinea Pigs in Experimental Shock

By AARON JANOFF, PH.D., BENJAMIN W. ZWEIFACH, PH.D., ARNOLD L. NAGLER, PH.D., AND ZOLTAN OVARY, M.D.

IT HAS BEEN proposed that the vasotoxic aspects of irreversible shock are due to the release of an iron-bearing protein, ferritin, into the circulation, and that the vasoinhibitory properties of ferritin, in turn, are due to the action of iron associated with this protein. These impressions were gained, in the main, in experiments involving hemorrhagic shock.

Recently, work in our laboratory has shown that increased levels of plasma-bound iron, although consistently present in hemorrhagic shock in rabbits and dogs, are not consistently encountered in several forms of normovolemic shock in these same species. In the majority of instances, plasma-bound iron levels were observed to decrease in animals in shock from nonhemorrhagic procedures, including shock induced by Escherichia coli endotoxin. On the basis of this observation, it was concluded that elevation in plasma-bound iron, although perhaps an important factor in hemorrhagic shock, is not necessarily directly involved in the pathogenesis of all forms of shock.

In our previous experiments, no attempt was made to compare the levels of ferritin present in normovolemic shock with those present during hemorrhagic shock. Assays of total plasma-bound iron would not detect small amounts of ferritin-bound iron released into the circulation when transferrin-bound iron levels are decreasing. Evidence to date, therefore, does not eliminate the possibility that elevated levels of plasma ferritin represent an important factor common to the pathogenesis of all forms of shock.

We therefore undertook to detect, separately, circulating ferritin and total plasma-bound iron during hemorrhagic and endotoxic shock in guinea pigs. The data to be presented will show that, as in the dog and rabbit, hemorrhage is consistently accompanied in the guinea pig by an elevation in plasma-bound iron, whereas fatal endotoxemia produced by extracts of Gram-negative bacteria is, for the most part, not accompanied by an elevation in plasma-bound iron. Positive tests for ferritin were obtained, as a general rule, only in those animals showing relatively large increases in plasma-bound iron. None of the endotoxic animals that showed a decrease in plasma-bound iron gave positive tests for ferritin. On the basis of these data, it appears that neither increased plasma ferritin nor increased plasma-bound iron is an important feature of lethal endotoxic shock in guinea pigs.

Methods

Hemorrhagic Shock

Female, albino guinea pigs of the Hartley strain weighing 500 Gm. or more were used in these studies. The animals received intravenous injections of sodium pentobarbital (20 mg./Kg.), and sodium heparinate (400 units/Kg.) and were allowed to bleed into a sterile reservoir through a carotid cannula. Arterial blood pressure was recorded with a Hg manometer and was controlled by adjusting the height of the reservoir over the level of the heart. Pressure was maintained at 35 mm. Hg until spontaneous reinfusion of 30 to 35 per cent of the extracorporeal blood had occurred (three to five hours), or death had intervened. Control levels of plasma-bound iron and ferritin were determined on blood samples obtained prior to hemorrhage.
In those animals that survived the hemorrhagic procedure, terminal blood samples were collected one to two hours after blood replacement had been completed. In animals that did not survive, terminal blood samples were obtained from the post-caval vein immediately after death.

**Endotoxic Shock**

Polyethylene cannulas were placed in the carotid arteries of guinea pigs anesthetized as before. Each animal was then treated as follows: Immediately after removing a control blood sample, a saline suspension of either *E. coli* endotoxin (Difco B5) or *Salmonella enteritidis* endotoxin (Difco 092722) was injected through the cannula. The quantity of endotoxin employed in this experiment was 0.4 mg./100 Gm. body weight, which proved to be an LD₅₀ dose. The neck wounds were sutured and the animals allowed to recover from the anesthetic. A second blood sample was obtained either six hours after endotoxin administration or at death.

**Immunological Assay for Ferritin by Passive Cutaneous Anaphylaxis (PCA) Procedures**

Guinea pigs of either sex, weighing 250 Gm., were used as test animals. Crystalline ferritin was prepared from guinea-pig liver and spleen, and antiserum against guinea-pig ferritin was obtained from immunized rabbits as described before. This antiserum preparation contained 152 μg. antibody nitrogen/ml. Although PCA techniques have been described that would be sensitive enough to demonstrate physiological titers of circulating ferritin in the guinea pig, these methods were not applicable for detection of ferritin in shocked animals. The release of endogenous epinephrine and the restriction of skin circulation during shock interfere with the PCA test. It was therefore necessary to resort to an indirect method, the PCA neutralization procedure.

Intradermal injections of rabbit antiserum and intravenous injections of ferritin dissolved in 1 per cent Evans blue were given according to standard PCA procedures with the following modifications: Prior to intradermal injection, rabbit antiserum was diluted 1:50 in the aliquots of guinea-pig plasma obtained before and after the shock procedures described above. Separate, contralateral skin sites were injected with these control-plasma and shock-plasma dilutions of antiserum, a total of four test guinea pigs being used for each individual sample. Three to four hours after skin preparations, 100 μg. of guinea-pig ferritin in dye solution was injected intravenously into each test animal. This dose of ferritin had been determined, in preliminary trials, to be optimal for provoking PCA reactions in skin sites prepared with 1:50 dilutions of antiserum.

**Results**

**Titration of Antiserum for Threshold PCA Reaction**

Rabbit antiserum against guinea-pig ferritin was mixed with either saline or normal guinea-pig plasma in dilutions ranging from 1:25 to 1:200. Intradermal injections of 0.1 ml. of diluted antisera were followed, after three hours, by intravenous administration of 100 μg. ferritin in 1 per cent Evans blue. Animals were killed 30 minutes later and the diameters of the resulting PCA reactions measured.

The results of a typical experiment are recorded in table 1. With a 1:25 dilution of rabbit antiserum in guinea-pig plasma, marked PCA reactions were provoked. The 1:50 dilution of antiserum in plasma still produced mild PCA reactions; however, with a 1:100 dilution in plasma the antiserum no longer produced detectable skin lesions. Consequently, a 1:50 dilution of antiserum was used routinely to produce threshold PCA reactions.

It should be noted that dilutions of antiserum greater than 1:50 provoked PCA reactions when these dilutions were carried out in saline rather than in plasma. Inhibition of antiferritin antibody by normal plasma may be due to competition between the nonspecific gamma-globulins of the latter and antiferritin...
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Table 1
Prototype Experiment: Titration of Antiserum for Production of Threshold PCA Reaction*

<table>
<thead>
<tr>
<th>Dilution of antiserum</th>
<th>Concentration of ferritin (μg/ml.)</th>
<th>PCA response (diameter in mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Guinea pig No. 1</td>
</tr>
<tr>
<td>Plasma</td>
<td>1:25</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1:200</td>
<td>1</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1:200</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*All animals received 100 μg. ferritin intravenously in 1 ml. of 1 per cent Evans blue, three hours after intradermal antiserum.

Table 2
Titration of Ferritin Required to Inhibit Threshold PCA Reaction

<table>
<thead>
<tr>
<th>Concentration of ferritin (μg/ml.)</th>
<th>PCA response (diameter in mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>trace</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>0.1</td>
<td>18</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

*Contained in intradermal dose of antiserum.  †Average of four determinations.

Inhibition of Threshold PCA Reaction with Ferritin

Guinea-pig ferritin was added to 1:50 dilutions of antiserum (prepared with normal plasma) in concentrations ranging between 0.1 μg. and 10 μg. of ferritin protein/ml. PCA reactions at sites prepared with these antisera were compared with control reactions given by 1:50 antisera without ferritin. As shown in table 2, 1.0 μg./ml. represents that concentration of ferritin in plasma which is just sufficient to produce clearly detectable inhibition of the threshold PCA response given by 1:50 antiserum. Concentrations of ferritin of 2 μg./ml. or more completely abolished the reaction.

Effect of Residual Plasma Endotoxin on PCA Reaction

There was a possibility that the PCA neutralization test carried out in plasma obtained from animals shocked with massive doses of endotoxin might be interfered with by a delayed hypersensitivity reaction in the guinea-pig skin caused by residual endotoxin in the plasma. Two methods were used to test this. Guinea pigs were injected intradermally with 100 μg. and 500 μg. of E. coli endotoxin and, three hours later, 1 ml. of 1 per cent Evans blue was injected intravenously into each animal. No local dye accumulation was observed at the sites of endotoxin injection.

In another experiment, guinea pigs were injected intradermally with control- and shock-plasma samples obtained from animals given 0.5 mg. S. enteritidis endotoxin/100 Gm. body weight. After three hours, the skin-prepared animals were injected intravenously with Evans blue alone. Although some areas of hyperemia with occasional hemorrhagic centers developed in the skin at injection sites, no extravasation of dye was observed, as occurs typically in the development of a cutaneous anaphylaxis reaction.

Inhibition of Threshold PCA Reaction by Shock Plasma

Using the PCA neutralization technique, tests for ferritin were carried out in shock-plasma samples obtained from guinea pigs subjected to hemorrhagic hypotension or to lethal doses of extracts of Gram-negative bacteria. The results are shown in table 3. Inhibition of threshold PCA reactions by shock plasma (indicating the presence of ferritin) is recorded as the difference between the diameters of contralateral control-plasma and shock-plasma PCA reactions. A decrease of 5 mm. or more was considered a positive neutralization test. A decrease of 15 mm. or more usually signified complete abolition of the control response. Each value represents the average of four determinations. Also included in table 3 are changes in plasma-bound iron concentrations resulting from the shock procedures.
After hemorrhage, plasma-bound iron was elevated in all 12 animals tested. Half of these also gave positive PCA neutralization reactions (table 3, A). After treatment with E. coli endotoxin, plasma-bound iron was elevated in only 4 of 12 animals; of these, one gave a positive test for ferritin. More important is the fact that ferritin was not detected in any of the eight animals showing decreases in plasma-bound iron (table 3, B). In a group of 10 animals treated with S. enteritidis endotoxin, increases in plasma-bound iron occurred in half of the animals, whereas decreases occurred in the remainder. Again, increased ferritin levels were detected only among those animals showing increased iron levels (table 3, C).

Discussion

The presence of a variable, but consistent, elevation in plasma-bound iron in hemorrhagic shock in rabbits and dogs was also observed in bled guinea pigs—and was correlated in the guinea pig with detectable elevation in plasma ferritin in half of the bled animals. These findings appear to indicate that deranged iron metabolism may, after all, play some role in the development of irreversibility in this form of shock. However, if a common, final pathogenetic step leading to peripheral vascular damage occurs in all forms of shock (traumatic, hemorrhagic, and endotoxic), then the absence of consistent elevations in plasma-bound iron in endotoxin-shocked animals is not consonant with the view that release of iron into the circulation represents the common vasculotoxic mechanism of shock states. It has, in fact, been demonstrated that elevated plasma-bound iron is not a constant feature of irreversible shock after infusion of endotoxin in the rabbit. The data presented here extend this observation to include another species, the guinea pig. At the very least, therefore, these findings make it unlikely that elevated plasma-bound iron, per se, plays a direct role in the lethality of endotoxic shock.

An additional purpose of these experiments was to determine whether elevated plasma ferritin is a consistent (and therefore possibly toxic) feature of endotoxic shock despite variable changes in total plasma-bound iron in this form of shock. Two assays are available for the detection of plasma ferritin. The first of these is the rat mesoappendix bio-assay, which has revealed ferritin in concentrations as small as 10 μg./100 cc. in animals shocked by hemorrhage. The second, the PCA neutralization procedure, is capable (according to our determinations) of detect-
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ing ferritin in concentrations of 100 μg/100 cc. Although less sensitive than the mesoappendix bio-assay, the PCA neutralization reaction is the more objective and specific of the two procedures and was therefore used in our experiments.

The principal conclusion of the studies reported here, with regard to ferritin, is limited to its proposed role in the development of lethal endotoxic shock in the guinea pig. Of the 22 animals infused with bacterial endotoxin in these experiments, less than half developed elevated plasma-bound iron. Although circulating ferritin was detected in a number of these animals showing elevated iron, ferritin was not detected in the remaining 60 per cent of endotoxin-treated guinea pigs showing no elevation in plasma-bound iron. Nevertheless, the infusion of endotoxin proved lethal to 70 per cent of the animals in this second group. Clearly, then, neither the appearance of elevated plasma ferritin nor plasma-bound iron can be directly associated with a lethal outcome in endotoxic shock in guinea pigs.

The argument can be advanced that negative PCA neutralization reactions in plasmas obtained from hypoferremic, endotoxin-treated animals cannot entirely rule out the presence, in these animals, of biologically effective amounts of ferritin too small to be detected by the PCA technique. However, at least this technique has clearly demonstrated that there is considerably less ferritin in the circulation of hypoferremic animals than in animals with elevated plasma-bound iron. In lethal endotoxemia, changes in the levels of plasma ferritin thus appear to parallel changes in total plasma-bound iron. Since no apparent correlation exists between levels of plasma-bound iron and lethal outcome in guinea pigs shocked by endotoxin, it would still appear to be a reasonable assumption that there is no correlation between lethality and levels of circulating ferritin in endotoxemic guinea pigs.

Summary

Guinea pigs were subjected to shock by hemorrhage or by infusion of either E. coli or S. enteritidis endotoxin, and changes in levels of plasma-bound iron and ferritin were assayed. Of 12 bled animals, all showed increases in plasma-bound iron and half gave a positive test for ferritin. Of 12 guinea pigs treated with LD₅₀ doses of E. coli endotoxin, only 4 showed an elevation in plasma-bound iron. Of these only one was positive for ferritin. Of 10 guinea pigs treated with LD₅₀ doses of S. enteritidis endotoxin, only five showed elevations in plasma-bound iron, and three of these also showed detectable increases in plasma ferritin. Immunologically detectable increases in plasma ferritin were not found in any of the endotoxin-infused animals showing decreases in plasma-bound iron.

It was concluded: (a) that, in the guinea pig (as in the rabbit), hyperferremia is more frequent after hemorrhage than after the infusion of Gram-negative bacterial endotoxin; (b) that significant increases in ferritin do not appear simultaneously with decreases in total plasma-bound iron in endotoxic guinea pigs; and (c) that hyperferremia and hyperferritinemia do not play a direct role in the lethal progression of endotoxic shock in the guinea pig.

Acknowledgment

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