Hemorrhagic shock of germfree rats

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Germfree and ordinary laboratory rats of the same strain, half of each sort treated with oral aureomycin for 3 days prior to shock, were subjected to hemorrhagic shock. At the level and duration of hypotension selected, the procedure was highly lethal for all four groups. The single survivor was not a germfree rat. No evidence was found for protection against fatal outcome by either germfree status or treatment with aureomycin, and the gross pathological changes seen at autopsy following death from shock were essentially similar in all groups.

It is concluded that bacteria and bacterial products are not essential for the development of irreversibility in hemorrhagic shock as customarily produced in rats.

CIRCUMSTANTIAL but impressively cumulative evidence has appeared linking endotoxins released from bacteria in the bowel to the appearance of irreversibility in hemorrhagic shock (1–9). Animals in sublethal shock are extremely sensitive to endotoxins; a substance with endotoxin-like properties appears to be present in the plasma of animals in irreversible shock; and animals made resistant to endotoxin are also resistant to the effects of prolonged hypotension (2). Furthermore, a period of pretreatment with antibiotics has been reported to postpone the appearance of irreversibility during hypotension.

Germfree animals provide opportunity for critical tests of the role of bacterial endotoxins in the induction of irreversible shock and of the nature of the protective effect of antibiotics.

In the experiments reported here, approximately equal groups of germfree and ordinary laboratory rats, both with and without pretreatment with aureomycin, were subjected to severe hemorrhagic hypotension. Fatal hemorrhagic shock was easily induced in all groups, without apparent differences in susceptibility.

METHODS

Both the germfree and ordinary laboratory rats (males, 300–500 gm) were obtained from the Lobund Institute at the University of Notre Dame. All animals were given water and diet ad libitum. The diet, L-c56 (10), was sterilized by steam under pressure. The germfree rats were housed since birth in individual wire mesh cages in a standard Reyniers RSU-400 Rearing Unit (10). The microbiologic examinations of the germfree animals and environment consisted of weekly sampling of food, feces, cage debris and anal contents cultured on blood agar plates, Sabouraud's plates, thioglycollate broth and trypsinase soy broth. There was no growth in any of the cultures. These animals, then, are termed 'germfree,' i.e. free from bacteria and fungi by our tests.

The ordinary animals were fed a commercial rat chow and kept singly in cages in the open laboratory until the last 3 days before shock, during which they were kept in a tank in all respects like the germfree tanks except for lack of sterilization of the environment and food. The temperature in the tanks, which was not regularly recorded, ranged from 30° to 32°C.

In each experiment, four animals were bled simultaneously, one pair each in germfree and nonsterile tanks. Forty-eight, twenty-four and five hours prior to bleeding and immediately after reinfusion, one member of each pair received by stomach tube 15 mg/100 gm body weight of aureomycin hydrochloride freshly suspended in water, 50 mg/ml. This was the dose and route reported to increase resistance to hemorrhagic shock in rats (7). The animals were caged in a manner to prevent any cross transfer of feces, etc. No dummy gavage was given to the untreated animals.

The germfree tanks and animals were routinely checked for sterility using standard media, and cultures were taken from blood, feces and cecal contents of each germfree rat at autopsy. Fecal samples were collected from the ordinary animals at the time of gavage and the numbers and kinds of bacteria present were semiquantitatively estimated by dilutions on standard differential media.

Shock was induced by bleeding anesthetized (sodium pentobarbital, i.p., 40 mg/kg body weight) rats through a carotid cannula into a reservoir system constructed to

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allow maintenance of the mean arterial blood pressure within 1 mm Hg of a predetermined level and measurement of the volume of blood shed; it fit entirely within the germfree tanks. All rats were maintained at 45 mm Hg for 4 hours, following which the remaining shed blood was forcibly reinfused into the carotid artery over a period of approximately 15 minutes. Heparin (1 mg/100 gm body weight) was given intra-arterially prior to bleeding, and an equivalent amount of protamine sulfate was administered by the same route following reinfusion of the blood. Clean, but not sterile, technique was used in the surgical preparation of the ordinary animals.

All rats were observed for 24 hours. Those which died were promptly autopsied. One rat, alive at the end of this time, was counted as a permanent survivor, sacrificed and autopsied.

**RESULTS**

The results in 21 animals distributed in four different groups are shown in table 1. Deaths have been listed in three columns. Some rats died before the 4 hours had elapsed. Such an event was heralded by irregular, spasmodic respirations and rapid uptake of the shed blood. In a few instances, a brief rapid outpouring of blood occurred just before the final uptake. Two rats in one group died suddenly during reinfusion. The remaining deaths occurred at varying times following reinfusion, as noted in the table.

The initial mean blood pressures ranged from 130 to 160 mm Hg in both the ordinary and germfree animals. The blood pressures shortly after reinfusion were variable, ranging from 50 to 130 mm Hg. No records were kept of the respiratory or pulse rates.

The procedure was highly lethal (20/21) regardless of germfree status or antibiotic therapy. It is of interest that the single survivor was not germfree. The mortality figures are shown again in table 2, combined with respect to the two factors taken singly—treatment with aureomycin and germfree status. In this comparison, there appears to be a greater tendency for the ordinary rats to succumb earlier than their germfree counterparts. Final 24-hour mortality rates, however, were unaffected by germfree status or by treatment with aureomycin.

The maximum outputs and times at which maximum outputs were reached (table 1) showed wide variation within each group. In general, a low, early maximum output (compared to the group average) was followed by an early death, though there were exceptions. Although maximum outputs averaged higher in aureomycin-treated than in untreated rats, the difference was not significant at the 5% level (F test). The times of maximum outputs were reached within each group. In general, a low, early maximum output (compared to the group average) was followed by an early death, though there were exceptions. Exceptions to the two factors taken singly—treatment with aureomycin and germfree status. In this comparison, there appears to be a greater tendency for the ordinary rats to succumb earlier than their germfree counterparts. Final 24-hour mortality rates, however, were unaffected by germfree status or by treatment with aureomycin.

At autopsy, all animals dying late showed marked hemorrhages in the small intestine, particularly in the distal portion. These changes were less marked in animals dying acutely, but in no instance was the bowel completely free from hemorrhage. No microscopic examinations were made. The surviving animal showed grossly normal viscera when sacrificed with sodium pentobarbital. Only germfree rats showed hemorrhages into the wall and lumen of the cecum. This organ is usually considerably enlarged in germfree rats and guinea pigs. Zweifach and his associates have also just recently repeated some preliminary experiments of hemorrhagic shock in ordinary and germfree rats which had been cecectomized 1 or more weeks earlier; the behavior of both groups was similar and changed little.

**TABLE 2. Hemorrhagic Shock of Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mortalities</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>During</td>
</tr>
<tr>
<td></td>
<td>reinfusion</td>
<td>reinfusion</td>
</tr>
<tr>
<td>Aureomycin</td>
<td>b/11</td>
<td>2/11</td>
</tr>
<tr>
<td>No aureomycin</td>
<td>5/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Germfree</td>
<td>3/11</td>
<td>2/11</td>
</tr>
<tr>
<td>Ordinary</td>
<td>8/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>
HEMORRHAGIC SHOCK OF GERMFREE RATS

from that of ordinary and germfree rats with their ceca present (Zweifach, personal communication).

The results of bacteriological examination of the feces of the ordinary animals are shown in table 3. Both aureomycin-treated and untreated animals showed a general drop in bacterial count during the 3 days' residence in the tank. The decrement was not large, and no difference appeared between the treated and untreated.

DISCUSSION

Irreversibility of hemorrhagic shock is defined as a state of refractoriness in which replacement of lost blood fails to prevent or at most delays circulatory failure and death. The development of this state is not marked by any sudden change in any commonly measured physiologic variable and can be determined with certainty only by the ultimate death or survival of the animal following transfusion. Prediction of death may be made with increasing confidence as hypotension is allowed to persist until the amount of blood spontaneously autotransfused becomes an appreciable fraction of the total shed blood, but in the experience of the authors individual variation among rats in this respect is very wide.

Several sorts of evidence point toward a parallelism in tolerance to hypotension and to endotoxin, and even toward the appearance in the circulation of a substance with endotoxin-like properties. Bacterial endotoxin administered intravenously in minute amounts converts early and presumably reversible hemorrhagic shock into irreversible shock in rats and rabbits (1, 2). Similarly, a plasma fraction from the blood of dogs adjudged to be in irreversible shock has been found to convert reversible shock due to relatively brief hypotension in a recipient dog or rabbit into irreversible shock (3). Chemical identification of this circulating factor has not yet been made, but the fraction has been found to substitute for the challenge dose in the generalized Schwartzman reaction (5). Further, Fine and his associates (11) found that a chemical isolation procedure of plasma calculated to yield the lipopolysaccharide fraction and endotoxin, if present, yielded a preparation which was toxic when it was applied to the plasma of irreversibly shocked dogs, but nontoxic when applied to the plasma of normal dogs. Further, the toxicity of the plasma from the irreversibly shocked dogs was limited to this fraction.

Repeated injections of small doses of endotoxin, a procedure which induces resistance to a normally lethal dose of endotoxin, also induces resistance to shock from hemorrhage (2, 11). Furthermore, repeated injections of plasma from rabbits in shock are as effective as endotoxin for inducing resistance to hemorrhagic shock (4). Conversely, rats which have been exposed to repeated sublethal exposures to drum shock, thereby developing a tolerance to ordinarily lethal amounts of drumming, show resistance to normally fatal doses of endotoxin as well (2).

If bacteria or bacterial products in the bowel are involved in the development of irreversibility, any procedure which might suppress bacteria could be expected to increase resistance to the development of reversibility during hemorrhage. Broad-spectrum antibiotics, when given over a period of days prior to a challenging hemorrhage, are protective, provided the antibiotics are effective against bacteria normally found in the bowel (6, 8). These agents are of such diverse chemical nature that the likely common action is one against bacteria.

However, rats raised in a totally bacteria-free environment have not shown any significant difference in behavior in hemorrhagic shock from ordinary rats (12).

The experiments described here confirm the similarity in behavior of germfree and ordinary rats to hemorrhagic shock, and fail to confirm an antibiotic effect, either in ordinary or germfree animals. These observations are difficult to reconcile with the endotoxin hypothesis, unless substances with actions similar to those of endotoxins are liberated from the animals' own tissues during hypotension. Such substances have been produced, for example, by chemical treatment of bacteria.

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Our data are open to criticism in one important respect. About half the rats died before the designated period of hypotension had elapsed. Such deaths are commonly said to be due to 'acute cardiorespiratory failure' rather than to hemorrhagic shock. Thus it may be objected that in these animals irreversible hemorrhagic shock was never induced. Yet the occurrence of death before transfusion does not necessarily preclude a mechanism similar to that in deaths occurring later. In most such acute deaths, automatic transfusion of all shed blood occurred before pulse and respiration ceased. By definition, then, irreversibility had supervened since replacement of all lost blood was not life-saving. Furthermore, the hemorrhages in the bowel seen at autopsy seemed similar to those seen in the rats dying later, though they were less extensive.

However, even discounting those dying early, all the germfree animals which survived past reinfusion died of a delayed death following a degree and duration of hypotension which regularly caused death in ordinary

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**TABLE 3. Fecal Bacterial Counts**

<table>
<thead>
<tr>
<th></th>
<th>Coliform</th>
<th>Cocci</th>
<th>Gram Pos. Rods</th>
<th>Fecal Strep.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aureomycin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1 (control)</td>
<td>$7 \times 10^6$</td>
<td>$4 \times 10^8$</td>
<td>$7 \times 10^3$</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>$1 \times 10^6$</td>
<td>$4 \times 10^8$</td>
<td>$1 \times 10^7$</td>
<td>$7 \times 10^6$</td>
</tr>
<tr>
<td>3</td>
<td>$2 \times 10^6$</td>
<td>$7 \times 10^8$</td>
<td>$7 \times 10^3$</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td><strong>No Aureomycin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1 (control)</td>
<td>$2 \times 10^6$</td>
<td>$7 \times 10^8$</td>
<td>$7 \times 10^3$</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>2</td>
<td>$7 \times 10^6$</td>
<td>$7 \times 10^8$</td>
<td>$7 \times 10^3$</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>3</td>
<td>$4 \times 10^6$</td>
<td>$3 \times 10^7$</td>
<td>$2 \times 10^7$</td>
<td>$7 \times 10^6$</td>
</tr>
</tbody>
</table>

* Figures represent geometric means of counts per gram feces of all animals of a given group on a given day.
rats in tanks and about a 50% mortality in ordinary rats in the open laboratory (unpublished data). This at least establishes that germfree rats can die of irreversible hemorrhagic shock in the total absence of bacteria following an insult of hypotension which regularly produces death of rats in the ordinary laboratory environment.

It has been objected that the germfree animals used in these experiments are not necessarily endotoxin-free, as dead bacterial bodies are introduced with the food and water (11). While the total mass of bacterial material so introduced must be very small compared to that present normally in the gut of an ordinary animal, it has been suggested that germfree animals may be unusually sensitive to bacterial endotoxin because of their limited experience in handling it (12). The amount of endotoxin in prepared diets has not yet been measured. Others in the Department of Germfree Research, Walter Reed Army Institute of Research, have just shown that germfree mice are not more sensitive than ordinary mice to an endotoxin prepared from a strain of E. coli isolated from the ordinary control mice.

In the reported work demonstrating a protective effect of antibiotics, the drugs have been effective only when given over a period of several days prior to shock (6, 8). It has been proposed that the action of antibiotics may be to release small amounts of endotoxin from killed bacteria, producing a resistant state in the same manner as by injection of small doses of exogenous endotoxin for some days prior to bleeding (11). In this view, aureomycin could not be expected to act protectively in the germfree rat. However, in our experiments, no effect was seen either in germfree or in ordinary rats.

Inability to show protective antibiotic effect in ordinary dogs has been reported from other laboratories (14), and some have attributed such failures to the prevalence of antibiotic-resistant strains of bacteria in the animal colony (15). It is noteworthy in this respect that the total and differential counts of the feces of the treated and untreated ordinary rats showed that this dose of aureomycin did not depress the flora of the bowel, and it may be that the strains of organisms present in the gut of these animals were largely resistant. Evidence that the drug did reach the bowel was provided by the prompt appearance of black stools in the treated rats.

In summary, the failure to find increased resistance among germfree compared to ordinary laboratory rats on one hand and the failure to find a protective antibiotic effect on the other raises strong doubts concerning the requirement for bacteria or their products in the pathogenesis of irreversible hemorrhagic shock.

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REFERENCES