Hypoferremia in Mice and Its Application to the Bioassay of Endotoxin

PHILLIP J. BAKER and J. B. WILSON

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin

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ABSTRACT

Baker, Phillip J. (University of Wisconsin, Madison), and J. B. Wilson. Hypoferremia in mice and its application to the bioassay of endotoxin. J. Bacteriol. 90:903–910. 1965.—The ability of endotoxin to induce hypoferremia in mice was used for the bioassay of endotoxin. A marked depression in the serum-iron levels of mice occurred 12 hr after the intraperitoneal injection of 0.01 to 100 μg of Escherichia coli endotoxin; similar results were obtained with 1.0 to 100 μg of Brucella abortus endotoxin. This biological response to endotoxin appeared to be specific, reproducible, and dose-dependent. As heat-killed cells of B. abortus and E. coli were also able to induce hypoferremia, this bioassay could be employed for the determination of the endotoxin content of killed-cell preparations. Treatment of endotoxin by acid hydrolysis, acetylation, or pyridine-formic acid greatly diminished the hypoferremic response as well as its lethality for mice. Pretreatment of mice with Thorotrast had little effect upon the ability of endotoxin to induce hypoferremia; however, a stimulation of the activity of the reticuloendothelial system (RES) by treatment of mice with triolein markedly reduced the ability of endotoxin to induce hypoferremia. The relationship between the hypoferremic response to endotoxin and alterations in the activity of the RES are discussed.

These investigations are a continuation of work concerning factors related to virulence in Brucella abortus (Wilson and Dasinger, 1960). In particular, we were interested in determining whether strains of B. abortus of high virulence have a greater endotoxin content than do strains of low virulence. This necessarily requires a reliable and sensitive assay for endotoxin, as well as one which is suitable for use with a large number of samples.

It has been our experience (Baker, 1962) and the experience of others (Ribi et al., 1964) that it is difficult to extract endotoxin quantitatively from intact-cell preparations, even if the extraction process is repeated several times. Therefore, it would be desirable to use a system of bioassay in which killed-cell preparations as well as purified endotoxin preparations could be employed. Thus, one could determine the endotoxin content of killed-cell preparations directly and eliminate the need for quantitative extraction.

In prior experiments (Wilson, Kolbye, and Baker, 1964), mouse LD₅₀ values were determined with heat-killed and acetone-killed cells of B. abortus 2308 (high virulence) and B. abortus 11

1 Present address: Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

(low virulence). Although no differences in endotoxin content were noted, we felt that a more sensitive method of bioassay might reveal small differences in endotoxin content. An evaluation of commonly used methods for the bioassay of endotoxin has been prepared by Braude (1964), but few of the listed procedures were suitable for our purposes since we desired to use mice, which could be obtained in large numbers and at a low cost. We attempted to use the mouse urinary nitrogen excretion assay for endotoxin as described by Berry and Smythe (1961); however, we had difficulty obtaining a uniform response to adrenocorticotropic hormone (ACTH) in several strains of mice (Wilson et al., 1964).

Kampschmidt and Upchurch (1962) had reported that a single injection of 0.0001 μg of Escherichia coli endotoxin produced significant hypoferremia in rats. A maximal decrease in serum or plasma iron occurred within 8 to 16 hr after the administration of endotoxin, and the degree of hypoferremia appeared to be dose-dependent. It occurred to us that these findings might be applied in an extremely sensitive system for the bioassay of endotoxin with mice as the experimental animal. In the studies to be described, the specificity of the response for endotoxin, the range
of sensitivity of the proposed system of bioassay, and the relationship between the hypoferremic response to endotoxin and its lethal effects in mice were investigated.

**Materials and Methods**

* Cultures. Two smooth strains of *B. abortus*, 11 (low virulence) and 2308 (high virulence), were used in these studies. These were obtained from the Bureau of Animal Industry, U.S. Department of Agriculture, and have been maintained and preserved for about 20 years in this laboratory, in the culture in a lyophilized condition, without change in virulence or changes in cultural characteristics. The virulence of the two strains, as assessed in guinea pigs, shows ID₅₀ values of 224 viable cells for strain 2308, and 10⁶ viable cells for strain 11 (Dasinger, 1960; Baker, 1962). The acriflavine test of Braun (1947) and the crystal violet Test of Whole and Wilson (1951) were used as tests for colonial morphology.

* Cultures of *E. coli* H-52, *E. coli* Gratia, *E. coli* B, and *Bacillus cereus* G were obtained through the courtesy of W. B. Sarles, Department of Bacteriology, University of Wisconsin. Cultures of *E. coli* Rolf and *E. coli* ICR were isolated by conventional methods from the Rolfsmeyer Swiss Webster and the A. R. Schmidt ICR strains of mice, respectively.

* Trypticase Soy Broth and Trypticase Soy Agar (BBL) were used exclusively for the cultivation and maintenance of all cultures.

* Experimental animals. Swiss Webster female mice, weighing 22 to 25 g, obtained from the Rolfsmeyer Farm and Mouse Farm, Madison, Wis., were used exclusively. The mice were fed Purina Laboratory Chow and were given water ad libitum.

* The temperature of the animal rooms was maintained at 25 C.

* Cultivation of cells for the extraction of endotoxin. As the source of cells, 48-hr broth cultures of *B. abortus* and 12-hr broth cultures of *E. coli* were used. The cells were cultivated on a rotary shaker at 37 C. The cells were harvested by centrifugation at 10,000 × g for 20 min at 4 C. The harvested cells were washed three times with cold saline and then were suspended in distilled water for the extraction of endotoxin.

* Heat-treated cell preparations. Freshly harvested and washed viable cell suspensions were adjusted turbidimetrically and by direct microscopic count to contain 10⁹ cells per milliliter. This cell suspension was kept at 65 C for 1 hr, after which time it was cooled to room temperature, diluted, and stored at 4 C until used. Viability tests were made with Trypticase Soy Agar to determine the effectiveness of the heat-killing process.

* Preparation of Bovin (TCA) endotoxins. These endotoxins were prepared by the method of Boivin and Mesrobeanu (1935) with minor modifications (Baker, 1965).

* Preparation of Westphal (PW) endotoxins. These endotoxins were prepared from strains of *B. abortus* by extraction with hot phenol-water as described in detail by Redfearn (1960). Redfearn reported that only fractions 3 and 5, obtained by this method, had biological activity, and these were the only fractions considered in our studies. The extraction procedure (Redfearn, 1960) is given below.

About 5 g (dry weight) of cells of *B. abortus* were suspended in 170 ml of distilled water. The cell suspension was heated to 66 C in a water bath. A 90% (w/v) phenol reagent was prepared, and 190 ml of the phenol reagent was heated to 66 C and then added to the cell suspension. This resulted in a phenol-water ratio of 1:1 (w/w). The mixture was agitated vigorously with the aid of a power stirrer for 15 min. The mixture was then dispersed among eight 50-ml Lusteroid centrifuge tubes and rapidly cooled to 5 C by immersion in an ice bath. The mixture was centrifuged at 13,000 × g for 15 min at 4 C, causing the formation of four layers in the tube. The top layer consisted of the phenol-saturated water layer. The interface precipitate separated the top layer from the water-saturated phenol layer, and the sedimented cell residue, at the bottom of the tube, constituted the fourth layer.

The upper phenol-saturated water layer (aqueous phase) was drawn off carefully with the aid of a capillary pipette attached to a vacuum line. An amount of 120 ml of phenol-saturated water was dispensed equally among the eight centrifuge tubes, and the centrifugation step was repeated. The aqueous phase was drawn off and added to the previous aqueous-phase sample. The pooled aqueous phases were dialyzed against several changes of distilled water and were concentrated to about 50 ml by pervaporation at room temperature.

The lower water-saturated phenol layer (phenol phase) and the precipitate formed at the interface were separated by filtration through Whatman no. 42 filter paper under slight negative pressure. To this filtrate, the phenol phase was added 3 volumes of cold methanol reagent (methanol-water). The methanol reagent was prepared by adding 1 part of methanol saturated with sodium acetate to 99 parts of methanol. The solution was mixed for 5 min and was then held at 4 C for 1 hr to allow for maximal precipitation. The precipitated material was collected by centrifugation at 9,000 × g for 15 min at 4 C and kept. The supernatant liquid was discarded.

The precipitate was suspended in 80 ml of distilled water and agitated overnight with the aid of a magnetic stirrer. The suspension was centrifuged at 10,000 × g for 30 min at 4 C, and the supernatant liquid was collected. The precipitate was resuspended in 80 ml of distilled water, and the mixture was agitated for 1 hr. After centrifugation, the process was repeated an additional time. The three supernatant liquids were pooled and dialyzed against several changes of distilled water. After dialysis, the solution was pervaporated at room temperature to a final volume of 50 ml. Then
procedure was and removed, the commercially available solution was slowly and standby phase. X material was dissolved X 10,000 complete precipitation. mixture by centrifugation 2 volumes of Escherichia serum-iron level of mice of various doses of Escherichia coli 055:B5-PW endotoxin. 2 volumes of cold methanol SA were added, and the mixture was allowed to stand for 2 hr to complete precipitation. The precipitate was recovered by centrifugation at 10,000 × g for 15 min and was dissolved in 20 ml of distilled water. The resulting material was lyophilized (fraction 5).

To the dialyzed and pervaporated aqueous phase were added 5 volumes of methanol SA, slowly and with stirring. The mixture was allowed to stand for 1 hr and was then centrifuged at 10,000 × g for 15 min at 4 C. The gel-like precipitate was dissolved in 25 ml of distilled water, and the solution was clarified by centrifugation at 10,000 × g for 30 min. The supernatant liquid was removed, and the product was reprecipitated by the addition of 4 volumes of methanol SA. The procedure was repeated an additional time after which the final clear solution was lyophilized (fraction 3).

Commercially available endotoxin. Two commercially available endotoxin preparations (Difco) were also used in these studies. These were Boivin and Westphal endotoxin preparations obtained from E. coli 055:B5. The preparations will be referred to as E. coli 055:B5-TCA (Difco Catalog No. 3923) and E. coli 055:B5-PW (Difco Catalog No. 3120), respectively.

Determination of serum-iron levels in mice. Serum-iron levels in mice were determined according to the 2,2',2"-terpyridine method described in detail by Schade et al. (1954). The results are expressed either as the reduction in the serum-iron level or as the serum-iron level in micrograms of iron per 100 ml of serum.

Collection of mouse serum. Pooled blood samples from five mice were used to obtain a sufficient amount of serum (1.0 ml) to be used for a single serum-iron determination. The blood samples...
TABLE 1. Serum iron levels (micrograms per 100 ml) of mice 6 and 12 hr after receiving various test materials*

<table>
<thead>
<tr>
<th>Test material and dose</th>
<th>6 hr</th>
<th>12 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated mice..........</td>
<td>310</td>
<td>306</td>
</tr>
<tr>
<td>Pyrogen-free saline (0.2 ml)</td>
<td>312</td>
<td>304</td>
</tr>
<tr>
<td>Crystalline egg albumin (10 μg)</td>
<td>302</td>
<td>319</td>
</tr>
<tr>
<td>Glucose (10 μg).........</td>
<td>319</td>
<td>304</td>
</tr>
<tr>
<td>Bacillus cereus G (100 μg)</td>
<td>273</td>
<td>342</td>
</tr>
<tr>
<td>Brucella abortus 11, TCA endotoxin (10 μg)</td>
<td>223</td>
<td>197</td>
</tr>
<tr>
<td>Heat-killed B. abortus 11 (100 μg)</td>
<td>289</td>
<td>166</td>
</tr>
<tr>
<td>Heat-killed E. coli ICR (100 μg)</td>
<td>244</td>
<td>113</td>
</tr>
<tr>
<td>Heat-killed E. coli B. (100 μg)</td>
<td>193</td>
<td>141</td>
</tr>
</tbody>
</table>

* A single sample of pooled serum, obtained from five mice given a stated dose of test material, was used to determine the serum-iron level.

**FIG. 5. Effect of acid hydrolysis of Escherichia coli 055:B5-PW endotoxin with 0.1 N acetic acid on its ability to induce hypoferremia in mice.**

**TABLE 2. Lethal effects for mice of Escherichia coli Rolf-TCA endotoxin before and after treatment with acetic anhydride or pyridine-formic acid**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Untreated endotoxin</th>
<th>Acetylated endotoxin</th>
<th>Pyridine-formic acid-treated endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>2/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>400</td>
<td>5/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>1,000</td>
<td>5/5</td>
<td>0/5</td>
<td>2/5</td>
</tr>
</tbody>
</table>

* The figures listed represent number of deaths/total number of mice used.

**TABLE 3. Reduction (micrograms per 100 ml) in the serum-iron levels of mice after receiving 100 μg of Escherichia coli Rolf-TCA endotoxin before and after treatment with acetic anhydride or pyridine-formic acid**

<table>
<thead>
<tr>
<th>Untreated endotoxin</th>
<th>Acetylated endotoxin</th>
<th>Pyridine-formic acid-treated endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>210 (3)</td>
<td>137 (5)</td>
<td>213 (5)</td>
</tr>
<tr>
<td>201 (3)</td>
<td>106 (5)</td>
<td>210 (5)</td>
</tr>
<tr>
<td>197 (4)</td>
<td>115 (5)</td>
<td>157 (5)</td>
</tr>
<tr>
<td>225 (3)</td>
<td>130 (5)</td>
<td>197 (5)</td>
</tr>
<tr>
<td>—</td>
<td>111 (5)</td>
<td>177 (5)</td>
</tr>
</tbody>
</table>

**Mean 208 119 190**

* A single sample of pooled serum, obtained from the number of mice given in parentheses, was used to obtain the serum-iron level. This value was then subtracted from the mean serum-iron level of the saline control group (308 μg per 100 ml) to determine the reduction in the serum-iron level.

**RESULTS**

Time after the intraperitoneal administration of endotoxin at which there is a maximal depression of the serum-iron level of mice. A maximal depression of the serum-iron level occurred 12 hr after the intraperitoneal administration of 10 μg of E. coli O55:B5-PW endotoxin (Fig. 1). After 12 hr, the serum iron appeared to increase progressively. Since 12 hr postinjection was the time at which a maximal depression in the serum-iron level occurred, blood samples in all subsequent experiments were collected at this time. Similar results were obtained with B. abortus endotoxin (fraction 5).

Relationship between reduction in serum iron and dose of endotoxin preparations. The relationship between the reduction in the serum-iron level and the logarithm of the dose of E. coli O55:B5-PW endotoxin is linear within the range of 0.001 to 10 μg of endotoxin (Fig. 2). By use of Westphal endotoxin preparations (fraction 5) obtained from B. abortus 2308 and 11, a reasonably linear dose-response curve was obtained within the range of 1.0 to 100 μg (Fig. 3).

**TABLE 3. Reduction (micrograms per 100 ml) in the serum-iron levels of mice after receiving 100 μg of Escherichia coli Rolf-TCA endotoxin before and after treatment with acetic anhydride or pyridine-formic acid**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Untreated endotoxin</th>
<th>Acetylated endotoxin</th>
<th>Pyridine-formic acid-treated endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>210</td>
<td>137 (5)</td>
<td>213 (5)</td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>106 (5)</td>
<td>210 (5)</td>
<td></td>
</tr>
<tr>
<td>197</td>
<td>115 (5)</td>
<td>157 (5)</td>
<td></td>
</tr>
<tr>
<td>225</td>
<td>130 (5)</td>
<td>197 (5)</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>111 (5)</td>
<td>177 (5)</td>
<td></td>
</tr>
</tbody>
</table>

**Mean 208 119 190**

* A single sample of pooled serum, obtained from the number of mice given in parentheses, was used to obtain the serum-iron level. This value was then subtracted from the mean serum-iron level of the saline control group (308 μg per 100 ml) to determine the reduction in the serum-iron level.

Since 7 of 20 of the mice given the untreated endotoxin died, the survivors were redistributed at random into groups of two or four mice to obtain pooled serum samples for analysis.
Relationship between reduction in serum-iron level and dose of heat-killed cells. The results of a typical dose-response experiment conducted with heat-killed cells of the cultures of *E. coli* used in these studies are presented in Fig. 4. There appeared to be differences in the activities of these heat-killed cell preparations which suggest differences in endotoxin content, assuming no significant differences in average cell size between the strains; however, the data also suggest qualitative differences in the biological effect of the endotoxins of the strains. Heat-killed cells of *E. coli* B produced the greatest hypoferremia of the strains tested at 10⁸ cells; at 10⁹ cells, they were the least effective. Differences in biological activity were also shown between the endotoxins of *E. coli* and *B. abortus* on the basis of the slopes of the dose-response curves (Fig. 2 and 3). The principal significance of these dose-response curves, obtained with endotoxins and with heat-killed cells, is that the degree of hypoferremia is dose-related and linear within a given range; however, the endotoxins of various strains or species of bacteria may differ in biological activity.

**Specificity of the induction of hypoferremia.** To determine the specificity of the induction of hypoferremia, endotoxins, materials containing endotoxin, and materials thought to be free from endotoxin activity were used (Table 1). Only endotoxin or materials containing endotoxin, namely, heat-killed cells of *B. abortus* or *E. coli*, produced a hypoferremia at 12 hr postinjection. The fact that no hypoferremia occurred with heat-killed vegetative cells of *Bacillus cereus* G indicates that the hypoferremia is not dependent upon merely the particulate nature of some of the test materials employed, but probably is due to a more specific effect of endotoxin.

**Effect of acid hydrolysis on the ability of endotoxin to induce hypoferremia.** The effect of acid hydrolysis upon the ability of *E. coli* 055:B5-PW endotoxin to induce hypoferremia in mice is shown in Fig. 5. Loss in the ability of endotoxin to induce hypoferremia began within 15 min of acid hydrolysis, after which time the activity of the endotoxin preparation progressively diminished until little if any ability to induce hypoferremia remained after 60 min of acid hydrolysis.

**Effect of acetylation and pyridine-formic acid treatment upon the lethal effects of endotoxin and its ability to induce hypoferremia.** The effects of acetylation and pyridine-formic acid treatment on the lethal effects of *E. coli* Rolf (TCA) endotoxin are summarized in Table 2. Whereas the mouse *LD₅₀* of the untreated parent endotoxin preparation was between 200 and 400 μg, the *LD₅₀* of the acetylated and pyridine-formic acid-treated preparations appeared to be greater than 1,000 μg. The fact that a few mortalities were observed with the pyridine-formic acid preparation suggested that this preparation was partially detoxified, whereas the acetylated preparation could be considered to be completely detoxified.

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**Table 4. Effect of pretreatment of mice with Thorotrast on the ability of Escherichia coli O55:B6-PW endotoxin to induce hypoferremia**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Serum-iron levels</th>
<th>Mean serum-iron level</th>
<th>Mean reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment</td>
<td>253, 331, 300, 298, 300</td>
<td>308</td>
<td>—</td>
</tr>
<tr>
<td>plus saline</td>
<td>227†</td>
<td>227</td>
<td>81</td>
</tr>
<tr>
<td>Saline plus 0.1 μg of endotoxin</td>
<td>228, 225, 192</td>
<td>215</td>
<td>93</td>
</tr>
<tr>
<td>Thorotrast plus saline</td>
<td>137, 110, 142</td>
<td>129</td>
<td>179</td>
</tr>
</tbody>
</table>

*The values listed represent the actual serum-iron levels obtained after a stated treatment. The mean reduction in serum-iron level was obtained by subtracting the mean serum-iron level for each experimental group from the mean serum-iron level obtained for the group that received no pretreatment.
†This value was obtained from the dose-response curve experiment (Fig. 2).*

**Table 5. Effect of pretreatment of mice with triolein on the ability of Escherichia coli O55:B6-PW endotoxin to induce hypoferremia**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Serum-iron levels</th>
<th>Mean serum-iron level</th>
<th>Mean reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment plus saline</td>
<td>253, 331, 300, 298, 300</td>
<td>308</td>
<td>—</td>
</tr>
<tr>
<td>Saline plus 0.1 μg of endotoxin</td>
<td>300, 311, 325, 338</td>
<td>319</td>
<td>—</td>
</tr>
<tr>
<td>Triolein plus saline</td>
<td>285, 346, 237</td>
<td>289</td>
<td>19</td>
</tr>
</tbody>
</table>

*The values listed represent the actual serum-iron levels obtained after a stated treatment. The mean reduction in serum-iron level was obtained by subtracting the mean serum-iron level for each experimental group from the mean serum-iron level obtained in the group that received no pretreatment.
†This value was obtained from the dose-response experiment (Fig. 2).*
When the above three preparations were tested for their ability to induce hypoferremia (Table 3), the parent endotoxin preparation had a high hypoferremic activity, whereas the acetylated endotoxin preparation showed a loss in hypoferremic activity. The fact that a substantial amount of hypoferremic activity was demonstrable in endotoxin preparations, which appeared to be partially or almost completely devoid of lethal activity after the above treatments, probably can be explained on the basis of the relative sensitivities of the two systems of bioassay.

Effect of pretreatment of mice with Thorotrast on the ability of endotoxin to induce hypoferremia. To determine the effect of pretreatment with Thorotrast, 0.2 ml of Thorotrast was given 6 hr before either pyrogen-free saline or 0.1 μg of E. coli O55:B5-PW endotoxin (Table 4). The hypoferremia obtained when endotoxin was given alone (81 μg per 100 ml) and when Thorotrast was given alone (93 μg per 100 ml), when added together, was nearly equal in magnitude to the hypoferremia obtained when both materials were given in combination (179 μg per 100 ml).

Effect of pretreatment of mice with triolein on the ability of endotoxin to induce hypoferremia. Triolein itself had little effect on the serum-iron level (Table 5); however, in mice that had been given 0.1 ml of a 20% (v/v) suspension of triolein intravenously and 24 hr later were given 0.1 μg of E. coli O55:B5-PW endotoxin, the effect of endotoxin on the serum-iron level was somewhat reduced.

Discussion

Kampschmidt and Schultz (1961) and Kampschmidt and Upchurch (1962) reported the development of hypoferremia in rats 12 to 16 hr after the administration of 0.0001 to 0.1 μg of E. coli endotoxin. Since this biological response to endotoxin appeared to be dose-related and could be elicited with relatively small amounts of endotoxin, we felt that this response might be used in an extremely sensitive system for the bioassay of endotoxin in mice. Because virtually all of the nonheme iron found in plasma is bound to transferrin (Bothwell and Finch, 1962), serum-iron levels rather than plasma-iron levels were determined in our studies.

We found that hypoferremia was maximal 12 hr after the intraperitoneal injection of 10 μg of E. coli O55:B5-PW endotoxin. Similar results were obtained with 100 μg of B. abortus Fraction 5. Therefore, serum-iron levels were determined at 12 hr postinjection in our studies. Our results with mice are in general agreement with the findings of Kampschmidt and Schultz (1961) and Kampschmidt and Upchurch (1962) with rats.

There was a reasonable linear relationship between the logarithm of the dose of endotoxin and the degree of hypoferremia induced in mice. With E. coli O55:B5-PW endotoxin, the relationship was linear within the range of 0.001 to 10 μg. Fraction 5 endotoxin preparations of B. abortus 2308 and 11 gave similar dose-response curves within the range of 1.0 to 100 μg. Thus, the response obtained with E. coli endotoxin exceeded that obtained with B. abortus endotoxin preparations.

A reasonable linear relationship between the logarithm of the number of heat-killed cells of E. coli and the degree of hypoferremia produced was also observed within the range of 10⁴ to 10⁹ heat-killed cells. Since heat-killed cells as well as purified endotoxin preparations were capable of inducing hypoferremia, this response could be employed for the determination of the endotoxin content of killed-cell preparations.

In preliminary experiments, only endotoxin or materials containing endotoxin induced hypoferremia. Heat-killed vegetative cells of Bacillus cereus G did not induce hypoferremia, although a modest hypoferremia, which was well within experimental error, occurred at 6 hr postinjection. Acid hydrolysis progressively reduced the ability of endotoxin to induce hypoferremia, in a manner similar to the loss of other biological properties of endotoxin under the same conditions (Ribi et al., 1961). In our studies and in those of Ribi et al. (1961), nearly complete inactivation of endotoxin was noted after 60 to 90 min of hydrolysis. These data indicate that, in respect to the ability of endotoxin to induce hypoferremia as with other biological properties of endotoxin, an endotoxin complex of a particular size is essential for biological activity.

When E. coli Rol-TCA endotoxin was detoxified by the pyridine-formic acid method (Nowotny, 1963) and by acetylation (Freedman, Sultzar, and Kleinberg, 1961), there was a loss in the ability of the detoxified preparations to induce hypoferremia. Although these data suggest a relationship between the ability of endotoxin to induce hypoferremia and toxicity, the two systems of bioassay differ greatly in sensitivity. Undoubtedly, amounts of endotoxin that were sublethal but were sufficient to induce hypoferremia still remained in the detoxified preparations.

Biozzi, Benacerraf, and Halpern (1955) and Benacerraf and Sebestyen (1957) reported that, soon after the administration of endotoxin to mice or rabbits, there is a depression of the phagocytic activity of the reticuloendothelial system (RES). A maximal depression occurred 12 hr after the administration of endotoxin, after which time the activity of the RES increased until, at 24 to 36 hr
postinjection, normal activity was restored. With regard to time sequence, these observations coincide remarkably well with our observations and those reported by Kampschmidt and Schultz (1961) concerning the hypoferremia induced by endotoxin. It appears that the hypoferremia might be related to the transient depression of the activity of the RES after the administration of endotoxin, since the cells of the RES play an important role in iron metabolism (Bothwell and Finch, 1962; Vannotti, 1957). One would expect that alterations in the activity of the RES would lead to corresponding changes in the serum-iron level. Although the mechanism of the hypoferremic response to endotoxin is unknown, Kampschmidt and Arredondo (1963) noted an accumulation of iron in the cells of the RES at the time of hypoferremia.

When mice were given Thorotrast to suppress RES activity (RES blockade), a marked hypoferremia occurred. Although the mode of action of Thorotrast is not known, it does possess some of the toxic properties of endotoxin, namely, the ability to produce fever and to prepare animals for the Shwartzman reaction (Petersdorf and Shulman, 1964). Whether Thorotrast acts solely by producing RES blockade or whether it possesses additional pharmacological effects is open to question. The fact that Thorotrast per se is capable of inducing hypoferremia and that the combined effect of Thorotrast and endotoxin is additive suggests that, in respect to their ability to induce hypoferremia, they may have a common mode of action; however, the possibility that some Thorotrast preparations might be contaminated with endotoxin still remains to be investigated.

Cooper and Stuart (1961) noted that, 24 hr after the intravenous administration of triolein to mice, there occurred a marked increase in the phagocytic activity of the RES, and the LD₅₀ for triolein-treated mice was 175 μg of E. coli endotoxin as opposed to 325 μg for nontreated mice. The authors suggested that endotoxin activity is dependent upon its rate of uptake by cells of the RES. In our experiments, triolein treatment nearly eliminated rather than increased hypoferremic response to endotoxin. Our data on this point are difficult to reconcile with those of Cooper and Stuart (1961); perhaps the great difference in amount of endotoxin accounts for the difference in results.

Acknowledgments

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