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Enhanced Phagocytosis and Bactericidal Activity of Hepatic Reticuloendothelial System During Endotoxin Tolerance

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The effects of tolerance to *Escherichia coli* endotoxin on the phagocytic and bactericidal activity of the hepatic reticuloendothelial system against viable *E. coli* were examined using ex vivo perfused rat livers. Livers were isolated from control and endotoxin-tolerant rats and perfused with a medium containing 5% homologous serum from either control or tolerant rats. After the addition of the *E. coli* (2×10^7 cells per ml) to the perfusate, the hepatic clearance of the bacteria was followed for 30 min. The highest activation of the hepatic reticuloendothelial system was observed when serum from tolerant animals was added to the perfusate. Under these conditions phagocytosis was 47% (12% in controls), and 37 to 38% of the bacteria were killed (5% in controls). This activation was less when livers obtained from tolerant rats were perfused with serum from controls or with saline only. The data suggests that, during endotoxin tolerance, humoral factors play an important role in the activation of the hepatic reticulendothelial system, although a direct stimulation of Kupffer cells also occurs. The enhancement of phagocytosis by tolerant serum did not require the presence of homologous antibodies and involved the activation of the alternative complement pathway, since it was lost after removal of factor B activity. On the other hand, stimulation of intracellular killing required both complement and specific antibodies. The data suggest a role of endotoxin in the activation of humoral and cellular mechanisms involved in the host resistance to gram-negative bacterial infection.

It is generally accepted that circulating endotoxins (lipopolysaccharides, LPS) play an important role in the pathogenesis of infections with gram-negative bacteria (1, 17, 18) and that pretreatment with bacterial LPS can alter the host response to infection by modifying the mechanism of nonspecific resistance (8, 30).

Repeated exposure of experimental animals to sublethal doses of endotoxin induces tolerance to many of the toxic effects of LPS. This complex phenomenon, although widely studied, has not yet been fully explained. Evidences were originally provided indicating an enhanced phagocytic activity of the reticuloendothelial system (RES), with accelerated clearance of endotoxin, as the basis of tolerance (2). This concept was later questioned when tolerance was shown to persist even after RES blockade and to be induced in the absence of increased RES activity (15, 19). Furthermore, the fact that tolerance could be passively transferred with serum also to RES-blockaded recipients (12, 15) suggested a role of humoral factors in resistance to endotoxin. In this respect, an immunological mechanism of tolerance involving antibodies against the toxic antigen of endotoxin has been postulated (13, 19, 24), but controversies still remain (41).

Most of the studies on tolerance have considered mainly the diminution of toxic properties of LPS (33). In contrast, less is known about the relationship between LPS tolerance and resistance to infections. In humans, tolerance to endotoxin has been found to occur during convalescence from such infections as typhoid, paratyphoid fever, tularemia, and malaria (17, 26, 29, 37). It has been suggested that the development of endotoxin tolerance can contribute to host recovery from infections by gram-negative bacteria (29), but the experimental evidence is controversial. Tolerance was found to protect mice from a mixed infection with *Staphylococcus aureus* and *Proteus vulgaris* (34), but it did not protect humans against a subsequent challenge with live *Salmonella typhi* (18).

The purpose of the present paper was to investigate whether the state of endotoxin tolerance was associated with alterations of the phagocytic and bactericidal activity of hepatic RES toward gram-negative bacteria. To exclude extra-hepatic factors, we used the isolated perfused rat liver system. This model affords the addi-

tional advantage of separating the effects of cellular and humoral factors in the clearance of viable bacteria by the liver (5, 38).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Morini Laboratories, Italy) weighing 280 to 320 g were used as liver and blood donors. They were fed commercial rat food and water ad libitum and were not starved before sacrifice.

Endotoxin. *Escherichia coli* endotoxin (O55:B5, Boivin type) was purchased from Difco Laboratories. The same lot was used for all experiments, and the LPS was dissolved in sterile, pyrogen-free saline just before use.

Tolerance schedule. Rats were rendered tolerant by daily intraperitoneal injections of increasing doses of LPS in 0.25 ml of saline: day 1, 0.01 mg; day 2, 0.025 mg; day 3, 0.1 mg; day 4, 0.2 mg; day 5, 0.6 mg; days 6 and 7, no treatment; day 8, 1 mg; day 9, 1 mg (31). Control rats received saline only. The development of tolerance was demonstrated by the survival of LPS-pretreated rats challenged with 5 mg of LPS 48 h after completion of the tolerance schedule. This dose killed all the saline-treated animals. After completion of the tolerance-inducing regimen, the animals were permitted a 48-h rest period before use.

Bacteria. *E. coli* (ATCC 25922, Difco) was used as a test organism. Cultures were grown overnight in 50 ml of brain heart infusion (Difco) at 37°C in a Dubnoff shaker. Bacteria were then harvested, washed twice, and suspended in phosphate-buffered saline (pH 7.4) at a final concentration of 2×10^9 cells per ml.

Blood serum. Homologous serum was obtained from control and LPS-tolerant rats by sterile cardiac puncture. The serum was pooled in aliquots and stored at −20°C until required. When determinations of immune hemolytic titers were performed (32), pools of fresh sera from control or tolerant animals were used.

Liver perfusion procedure. Livers were isolated from controls or endotoxin-tolerant animals and perfused according to the procedure of Miller et al. (27). The perfusing medium consisted of 5% serum from either control or tolerant rats in 100 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 200 mg of glucose and 3 g of bovine serum albumin (Sigma). Four sets of experiments, each of four perfusions, were run as follows: control liver perfused with medium containing control serum (control liver–control serum); livers from tolerant animals perfused with medium containing control serum (tolerant liver–control serum); livers from control animals perfused with medium containing tolerant serum (control liver–tolerant serum); and livers from tolerant animals perfused with medium containing tolerant serum (tolerant liver–tolerant serum).

In other experiments, control livers were perfused with medium containing 5% tolerant serum which had received one of the following treatments: (i) heating at 56°C for 30 min to remove the complement activity (control liver–heated tolerant serum; four perfusions); (ii) absorption, overnight at 4°C, with heat-killed and washed homologous bacteria to remove specific antibodies (38) (control liver–absorbed tolerant serum; four perfusions); (iii) absorption as above, followed by heating at 50°C for 20 min to inactivate factor B activity (control liver–absorbed and factor B-depleted tolerant serum; three perfusions). The effects of LPS tolerance on the activity of cellular elements per se were studied by perfusing livers from five control and five tolerant animals with serum-free perfusate consisting of Krebs-Ringer bicarbonate-albumin buffer.

After an initial 15-min equilibration period, 1 ml of the bacterial suspension was added to the perfusate to yield a zero-time bacterial concentration of 2×10^7 cells per ml. Perfusions were then conducted for 30 min at a rate of 3 ml/min per g of liver. During this period, no appreciable multiplication of microorganisms occurred. The phagocytic function of the hepatic RES was measured by following the clearance of viable bacteria from the perfusate. Samples of perfusing medium (0.5 ml) were taken at 0, 10, 20, and 30 min. After perfusion, the liver was washed with 50 ml of sterile saline, and the effluent was kept at 4°C. Then the liver was weighed, and a sample (approximately 3 g) was excised and homogenized for 2 min at 21,000 rpm in sterile saline, 10% (wt/vol), using a blender homogenizer (Thomas, Philadelphia). No differences were found in the weight of livers from control or endotoxin-treated rats. Samples of medium, liver homogenate, and effluent wash were sonicated at 21 kc/s at 4°C for 20 s; the bacterial concentration was then determined by plating triplicate samples on Trypticase soy agar. The phagocytic index (K) was calculated as described by Benacerraf et al. (3). For each liver perfusion, the bactericidal activity of the serum perfusate was measured by following the survival of the test inoculum incubated in the perfusate only; all other experimental conditions were comparable. The amount of bacteria phagocytized by the liver during perfusion experiments was calculated by subtracting both the number of bacteria recovered in the perfusate at 30 min and the number of organisms washed out of the liver at the end of perfusion from the total amount of bacteria inoculated into the perfusate at zero time. The value was also corrected for the bactericidal activity of perfusate. The amount of phagocytized bacteria that could not be recovered in the liver at the end of perfusion was considered to represent the amount of bacteria killed by Kupffer cells after phagocytosis. Both phagocytosis and killing are expressed as percentage of initial inoculum. All the data are mean values ± standard error.

RESULTS

The hepatic clearance rates of viable *E. coli* from the perfusate are shown in Fig. 1. In all groups the kinetics followed an exponential curve. In the control liver–control serum group, the disappearance rate was low with a calculated phagocytic index (given as $K \times 10^3$) of 2.3 ± 0.7, whereas, in the tolerant liver–control serum set, bacteria were removed at a faster rate (K = 6.6 ± 0.4; $P < 0.01$). Addition of serum from tolerant rats caused even greater increases in the clearance rate by liver preparations from either control (K = 10.0 ± 0.6) or tolerant rats (K = 9.9 ±

0.4; both $P < 0.001$ versus controls). Since the bactericidal activity of the perfusion medium per se for *E. coli*, tested in parallel with each perfusion experiment, was very low (0 to 6%; Table 1), the disappearance of bacteria from the medium during liver perfusions can be considered a consequence of the phagocytic activity of the liver.

At the end of the experimental period, in the control liver-control serum system, 12% phagocytosis resulted, and 5% of the bacteria were killed (Fig. 2). When livers from tolerant rats were perfused with control serum, the total amount of phagocytized bacteria was 33% ($P < 0.01$) and killing was 19% ($P < 0.05$). Addition of serum from tolerant animals to the perfusion buffer further increased phagocytosis (46% for both control and tolerant livers; $P < 0.01$) and increased lethality of *E. coli* (38% for controls and 37% for tolerant liver preparations; $P < 0.01$).

Heat treatment of tolerant serum destroyed its enhancing effect on RES activity in that both phagocytosis and killing values were similar to controls (Fig. 1 and 2). In contrast, removal of specific antibodies from tolerant serum did not affect phagocytosis (46%), but decreased the intracellular killing of viable bacteria to 5% (Fig. 1 and 2).

When tolerant serum, absorbed and depleted of factor B activity, was present in the perfusate, phagocytosis by control liver was 14% and no killing of bacteria occurred (Fig. 1 and 2). It was also found that the immune hemolytic titer of tolerant serum was the same as that of control serum (492 50% hemolytic complement units per ml).

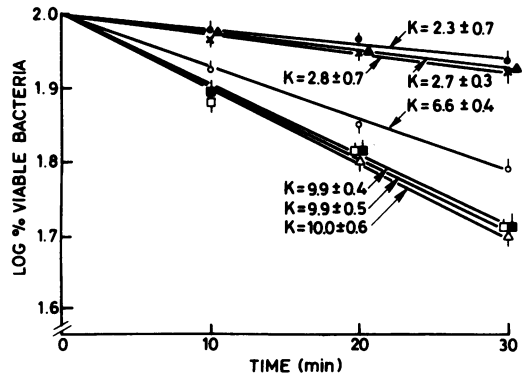

FIG. 1. *Kinetics of disappearance of E. coli from medium during isolated rat liver perfusion. Each curve gives mean and standard error of four experiments.* (●) *Control liver-control serum;* (○) *tolerant liver-control serum;* (△) *control liver-tolerant serum;* (■) *tolerant liver-tolerant serum;* (×) *control liver-preheated tolerant serum;* (□) *control liver-absorbed tolerant serum;* (▲) *control liver-absorbed and factor B-depleted tolerant serum. Phagocytic index* ($K \times 10^3$) *is also given.*

TABLE 1. *Survival of E. coli during 30-min in vitro incubation in 5% rat serum diluted in buffered Krebs-Ringer bicarbonate solution with albumin*

Incubation of bacteria in:	No. of expts	Percent of survived bacteria
Buffered saline-albumin	5	100
Control serum	8	98 ± 1[a]
Tolerant serum	8	96 ± 2
Tolerant serum, heated: 56°C, 30 min	4	100
Tolerant serum, absorbed with homologous cells	4	100
Tolerant serum, absorbed and heated: 50°C, 20 min	3	100

[a] Data are mean ± standard error.

Bacterial phagocytosis and killing were also studied during liver perfusions of control or tolerant animals with a serum-free perfusate to evaluate the functional capacity of activated macrophages per se. In the absence of humoral factors, control liver preparations phagocytized 15% of the bacteria but did not kill bacteria (Table 2). Under similar experimental conditions, livers from endotoxin-tolerant rats phagocytized 38% ($P < 0.05$) and killed 20% of the bacterial inoculum.

DISCUSSION

The present study has demonstrated that the induction of endotoxin tolerance increased the clearance of viable *E. coli* by the isolated perfused rat liver (Fig. 1). These results agree with those of other studies (4, 15), which showed an accelerated clearance of colloidal particles in animals made tolerant to endotoxin. In addition, we found that in the tolerant state (tolerant liver, tolerant serum, or both) the increased phagocytosis was accompanied by an increased destruction of the engulfed bacteria (Fig. 2). Intracellular killing of the microorganisms is important in the outcome of an infection, since increased phagocytosis without killing may be detrimental (8, 23, 36).

In the perfused rat liver model, humoral factors were found to play a major role in the enhancement of the hepatic RES activity during tolerance to endotoxin. The highest rates of phagocytosis and killing of bacteria were noted when serum from tolerant rats was present in the perfusate, irrespective of the status of the liver (Fig. 2). These data are consistent with those of Rowley (35, 36), who found that after a single injection of LPS, serum factors were rate-

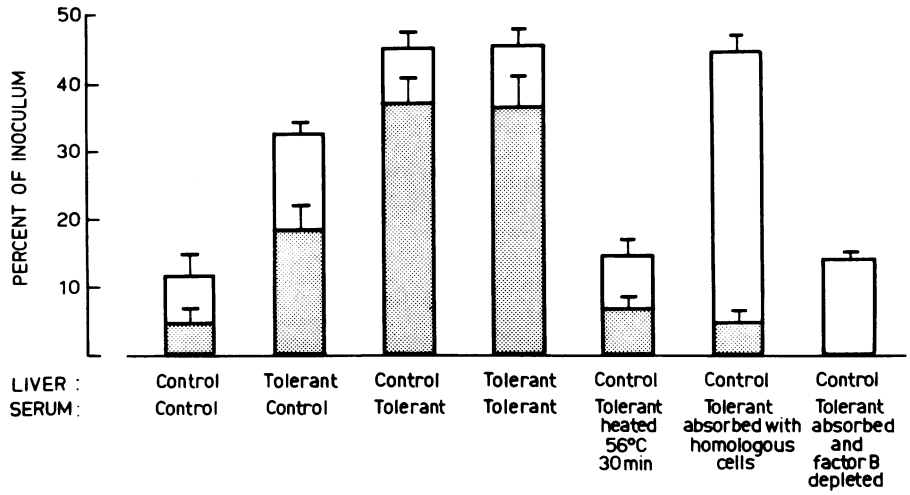

FIG. 2. *Phagocytosis and killing of viable E. coli by isolated rat liver 30 min after bacterial inoculation into the perfusate. The entire bar represents the amount of bacteria phagocytized by the liver; the shaded portion of each bar represents the amount of bacteria killed by the liver after phagocytosis. Values are means ± standard error.*

limiting in the clearance of bacteria. Our results are also in agreement with previous observations (15, 34) indicating that humoral factors are primarily responsible for tolerance and that the enhanced RES activity is secondary.

Perfusion of the tolerant liver with either control serum or with serum-free buffer indicates that an activation of hepatic macrophages per se can occur independently of serum factors (Fig. 1, Table 2). For example, in serum-free perfusions, the tolerant liver cleared and destroyed greater numbers of bacteria than did control liver. Our findings suggest that endotoxin tolerance may play an important role in the host resistance to infection by gram-negative bacteria.

The mechanisms underlying the ability of tolerant serum to promote the clearance and destruction of bacteria are unknown. An increased bactericidal activity against *E. coli* has been found in undiluted bovine serum after induction of LPS tolerance (21). This bactericidal effect was not seen in our experiments, since we used diluted serum to minimize the extrahepatic killing of bacteria (Table 1).

Both complement and homologous antibodies are known to promote clearance and destruction of bacteria by macrophages (22, 25, 36, 39). We demonstrated an absolute requirement of complement, but not of antibodies, for the LPS-induced stimulation of bacterial phagocytosis by hepatic RES. This is in agreement with the known ability of LPS to stimulate both the classical and the alternative complement pathways, even in the absence of specific antibodies (14, 28).

TABLE 2. *Phagocytosis and killing of E. coli by isolated rat liver perfused with serum-free perfusate, 30 min after bacterial inoculum*

Livers (no. of expts)	Phagocytic index ($K \times 10^3$)	Phagocytosis (%)	Killing (%)
Control (5)	4.3 ± 0.9[a]	15 ± 3	0
Tolerant (5)	8.2 ± 0.7[b]	38 ± 3[b]	20 ± 2

[a] Mean ± standard error.
[b] $P < 0.05$ versus controls.

We also observed that the immune hemolytic titers of both control and tolerant sera were similar and that tolerant absorbed serum lost the capacity to enhance phagocytosis when depleted of factor B activity. These data suggest that in the tolerant state the increased RES phagocytic activity is mediated by complement activation via the alternative pathway. This interpretation is supported by previous observations indicating that the alternative complement pathway is involved in the opsonization of some microorganisms and fungi, although variability in their opsonic requirements has been found (9–11, 20).

The presence of homologous antibodies in the tolerant serum was not necessary to stimulate phagocytosis by isolated perfused rat liver (Fig. 2). This agrees with the view that, in general, endotoxin tolerance is an antibody-independent phenomenon (16, 40, 42). However, the enhance-

ment of intracellular killing of bacteria required the presence of antibodies in addition to that of complement. This finding agrees with that of Leijh et al. (25), who demonstrated that intracellular killing of bacteria by human monocytes requires the interaction of specific cell membrane receptors with the F_c fragment of immunoglobulins and with the C_{3b} component generated via the alternative complement pathway.

Immunization with the endotoxic glycolipid or rough mutants of gram-negative bacteria has been attempted to induce protection against gram-negative bacteremia (6, 7). Although the induction of endotoxin tolerance might differ from such immunization with respect to humoral and cellular mechanisms, time dependence, and specificity, our results lend support to these attempts to stimulate host resistance against gram-negative infections.

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