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Suppressive Effect of Bacterial Endotoxin on the Expression of Cell-Mediated Anti-*Listeria* Immunity

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Intravenous injection of bacterial endotoxin into mice at any time during ongoing infection with *Listeria monocytogenes* resulted in a markedly increased multiplication of this organism in the liver and spleen. Experiments designed to investigate the basis of this infection-enhancing effect revealed that endotoxin was also capable of inhibiting the expression of adoptive T-cell-mediated anti-*Listeria* immunity if given to normal recipient mice up to 48 h before they were infused with protective T-cells. On the other hand, endotoxin had only a marginal effect on the expression of adoptive immunity if given to donor mice before their spleen cells were harvested for adoptive transfer. Taken together, these results indicate that endotoxin probably interferes with the antibacterial function of macrophages rather than with mediator lymphocytes. The additional finding that the infection-enhancing action of endotoxin could be greatly reduced by making mice "tolerant" to endotoxin suggests that the acquisition of tolerance to this effect of endotoxin may be an important adaptive mechanism in acquired resistance to infection with gram-negative bacteria.

Parenteral injection of bacterial endotoxins can either enhance or diminish microbial infection depending on the timing of injection (3). The infection-enhancing property of endotoxin has been observed with a variety of organisms, including trypanosomes (24, 25), viruses (6, 7), fungi (2, 5, 10), gram-negative bacteria such as *Escherichia* (22) and *Pseudomonas* (16), gram-positive bacteria (4, 15, 16, 23), and mycobacteria (9, 26, 27).

Evidence for the infection-enhancing action of endotoxin has been based almost exclusively on measurements of increased mortality (2, 5-7, 9, 10, 15, 22-24, 26, 27). The amount of endotoxin required to enhance infection varied between 1 μg and 7.5 mg according to the choice of experimental animal and the infectious agent under study (16, 23). Most investigators have found that the timing of endotoxin injection relative to initiation of infection is important, in that the reduced level of resistance after endotoxin treatment generally enhances already ongoing infections.

For the most part, the mechanism by which endotoxin causes enhancement of infection remains undetermined, although the large number of disturbing effects that this compound is known to have on mechanisms of homeostasis leaves many possible explanations from which to choose (17).

The purpose of this paper is to show that injection of bacterial endotoxin strikingly increases the level of infection with *Listeria monocytogenes* in mice, as measured by increased bacterial multiplication in the liver and spleen. It also shows that endotoxin has a suppressive effect on the expression of adoptive T-cell-mediated anti-*Listeria* immunity, which is dose and time dependent. Additional evidence supports the view that endotoxin interferes with the macrophage component, rather than the lymphocyte component, of the antibacterial response.

MATERIALS AND METHODS

Animals. Adult (6 to 8 weeks old) male and female B6D2F1 (C57Bl/6 × DBA/2) mice were used. The animals were housed, fed, and watered according to standard procedures. All mice were obtained from the Trudeau Institute Animal Breeding Facility.

Bacteria. *L. monocytogenes* strain EGD was passaged regularly in mice to maintain its virulence. For experimental use, a log-phase culture (approximately 2×10^8 bacteria per ml) was grown in Trypticase soy broth and frozen at −70°C in 1-ml portions. For each experiment, a portion was thawed and diluted in 0.85% NaCl solution for intravenous injection. The standard immunizing dose was 5×10^3 cells per ml, and the challenge dose was 10^5 cells per ml, injected in a volume of 0.2 ml. Bacterial growth was followed in livers and spleens at the times indicated below by homogenizing whole organs in saline and plating 10-fold serial dilutions of the homogenates on Trypticase soy agar.

Endotoxin. Bacterial endotoxin (Boivin) derived from *Salmonella enteritidis* (Difco; lot 648750) was suspended in Dulbecco phosphate-buffered saline at

pH 7.2 at a concentration of 1.0 mg/ml, divided into 1-ml amounts, and stored at −20°C. A fresh vial was used for each experiment. Endotoxin was diluted appropriately in phosphate-buffered saline and injected intravenously in a volume of 0.2 ml.

Adoptive immunization. Spleen cell suspensions from 6-day *Listeria*-infected donor mice were prepared by a method previously described (14). To reduce the number of contaminating *Listeria* infused with spleen cells, donor animals were treated subcutaneously 24 h before spleen cell transfer with 10,000 U of penicillin G (Lilly) and were given drinking water containing 500 mg of ampicillin (Wyeth) per liter. To further reduce contaminating *Listeria*, spleen cells were harvested and prepared over a period of approximately 1 h in phosphate-buffered saline containing 1% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (25 μg/ml). The cell suspension was filtered through several layers of sterile gauze and washed twice with antibiotic-free medium by centrifugation (340 × *g*) and resuspension. Recipient mice were injected intravenously with one spleen equivalent of cells (1.7×10^8 cells) 1 h before they were challenged intravenously with *Listeria*.

Making mice "tolerant" to endotoxin. Mice were made tolerant to endotoxin by injecting them intraperitoneally with 10, 50, and 100 μg of endotoxin on days 1, 2, and 3, respectively, and with 100 and 10 μg on days 5 and 7, respectively. Mice were used in experiments on day 9.

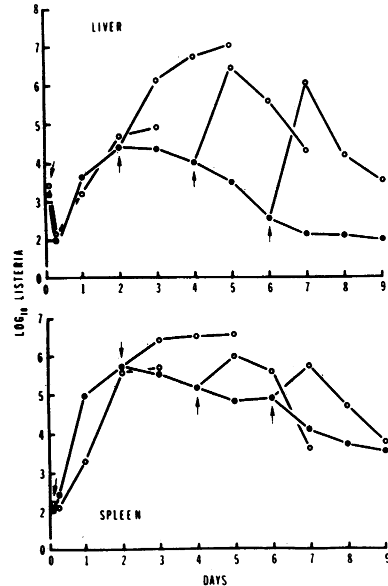

FIG. 1. *Infection-enhancing effect of 10 μg of endotoxin injected intravenously at different times (arrows) of a primary Listeria infection. Endotoxin administration was followed by increased bacterial multiplication (O) in the liver and spleen. Means of five mice per time point.*

RESULTS

Enhancing effect on primary infections. It was found (Fig. 1) that a 10-μg dose of endotoxin injected intravenously into mice on day 2, 4, or 6 during infection resulted in a striking increase in bacterial multiplication in the liver and spleen. The infection-enhancing effect was most striking when endotoxin was given on day 2 of infection, and many of these mice succumbed to overwhelming listeriosis. After day 2 of infection, a 24-h period of increased bacterial multiplication was followed by a progressive decrease in bacterial numbers, indicating that the effect of a 10-μg dose of endotoxin is reversible after the host begins to acquire and express high levels of adaptive immunity.

Only a minimal infection-enhancing effect was measured in the livers when endotoxin was given at the time of initiating infection. The reason why there was less bacterial growth in the spleens of these mice during the first 24 h is not known.

Suppressive effect on expression of adoptive immunity. It is known that, although the cells responsible for the eventual destruction of *Listeria* are activated macrophages (13), the activation of macrophages is mediated by an acquired population of specifically sensitized T-cells, as evidenced by the capacity of an infusion of the T-cells from immune donors to adoptively immunize normal recipients against a lethal challenge infection (11, 14, 20). Therefore, endotoxin could mediate its infection-enhancing effect by suppressing either the macrophage or the lymphoid component of the anti-*Listeria* response. To determine whether macrophage function was suppressed, experiments were designed to investigate whether endotoxin treatment of normal recipient mice would prevent their macrophages from expressing adoptive T-cell-mediated immunity passively transferred with spleen cells from immune donors.

It was found that 25 μg of endotoxin injected intravenously into normal recipient mice 4 h before they were infused with spleen cells from immune donors prevented them from expressing adoptive immunity to a lethal *Listeria* challenge injection. Figure 2 shows that, whereas control recipients of immune spleen cells were easily capable of eliminating *Listeria* from their livers and spleens, the livers and spleens of endotoxin-treated recipients supported log linear bacterial growth, which eventually resulted in death from overwhelming infection.

Figure 3 shows, in addition, that the level of endotoxin-induced suppression of adoptive immunity was dose dependent, in that the larger the dose of endotoxin, the greater the level of

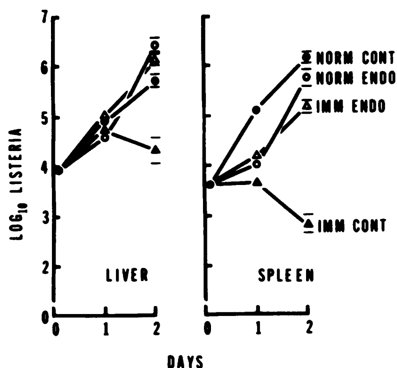

FIG. 2. *Suppression of expression of adoptive immunization by the injection of 25 μg of endotoxin into recipient mice 4 h before the infusion of immune cells and an intravenous Listeria challenge of 10^5 cells (IMM ENDO). Endotoxin greatly suppressed the protective effect of immune spleen cells (IMM CONT) and resulted in bacterial growth comparable to that measured in the livers and spleens of normal controls (NORM CONT) and endotoxin-treated controls (NORM ENDO). Means of five mice per time point ± 2 standard errors.*

suppression. This is best illustrated by the results obtained with the spleen. Moreover, as little as 0.5 μg given intravenously to recipient mice caused a suppressive effect on the expression of adoptive immunity.

The suppressive action of endotoxin on adoptive immunity also depended on the time that this compound was injected relative to the time that immune spleen cells were infused and the mice were challenged with *Listeria*. Figure 4 shows that the suppressive effect of 25 μg of endotoxin progressively declined with time. The effect almost completely disappeared 3 days after treatment.

Effect of endotoxin on protective capacity of donor spleen cells. To determine whether the function of mediator T-cells is also affected by endotoxin, prospective immune donors of spleen cells were injected intravenously with 5 μg of endotoxin 24 h before their cells were harvested for adoptive immunization. This relatively small, although suppressive, dose of endotoxin was chosen to decrease the amount injected with donor spleen cells. It was found (Fig. 5) that intravenous endotoxin treatment had little effect on the capacity of splenic T-cells from immune donors to protect normal recipients against lethal challenge infection. The apparent small suppressive effect in the liver was probably caused by a carry-over of endotoxin with donor macrophages.

Reduced endotoxin effect in recipients which have been made tolerant. It is known that animals can be protected from the toxic effects of endotoxin by an appropriate schedule of increasing doses of this compound, which makes them tolerant (12). It was considered possible that a similar schedule given to prospective recipients of immune spleen cells would prevent endotoxin from suppressing their ability to express adoptive immunity.

Figure 6 shows that recipient mice pretreated with increasing doses of endotoxin over a 7-day period were tolerant to the effect of an otherwise infection-promoting dose of endotoxin (25 μg) given at the time of adoptive immunization 2 days later. These animals were essentially as capable as adoptively immunized controls at expressing resistance to lethal challenge.

Effect of endotoxin on early antimicrobial events in the liver during primary infection. It is known that an intravenous inoculum of *Listeria* is rapidly cleared from the blood within 5 min and that over 95% of the inoculum is ingested by macrophages in the liver. It is also known (21) that, depending on bacterial virulence, liver macrophages are capable of inactivating over 50% of their bacterial load within the first 6 to 8 h of infection and that it is growth of the surviving organisms that gives rise to the infection which follows. The additional knowledge (21) that this initial bacterial destruction in the liver is unaffected by treatment with

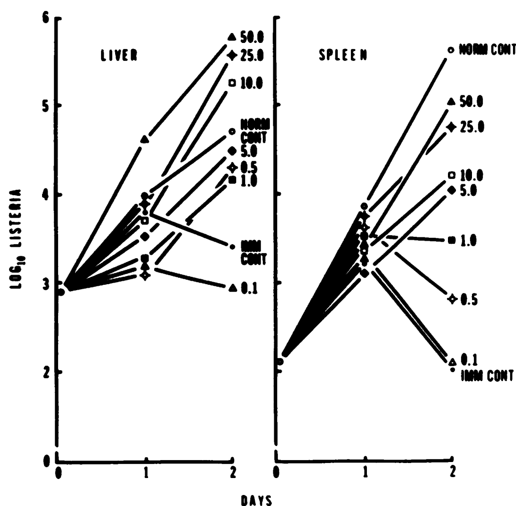

FIG. 3. *Dose dependence of suppressive effect of endotoxin on expression of adoptive immunity. Shown are the suppressive effects on the expression of adoptive immunity of doses ranging from 0.1 to 50 μg given to recipient mice 4 h before the infusion of immune spleen cells and a Listeria challenge of 10^5 cells. As little as 0.5 μg caused significant suppression of adoptive immunity, and the suppressive effect increased with increasing dosage. Means of five mice per time point.*

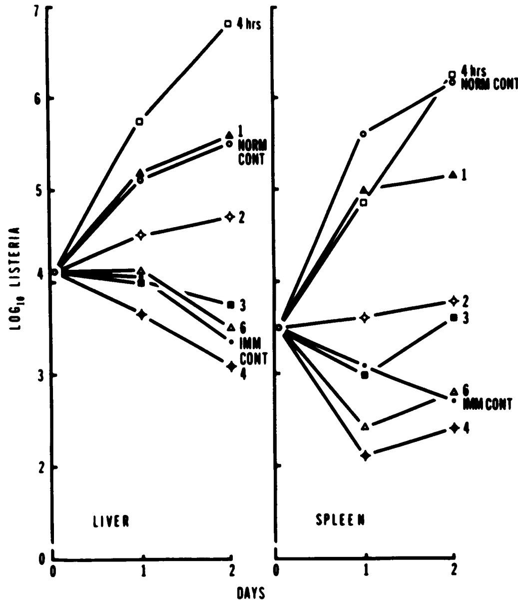

FIG. 4. *Suppressive effect of endotoxin relative to the time it is given before adoptive immunization and Listeria challenge. Shown are the suppressive effects resulting from endotoxin administration at 6, 4, 3, 2, or 1 day or 4 h before immune spleen cells were infused. The effect of endotoxin rapidly declined with time and was essentially gone by 3 days. Means of five mice per time point.*

corticosteroids or gamma irradiation points to its being mediated by the microbicidal activity of macrophages already resident in the liver at the time of infection. Therefore, it was important to determine whether endotoxin treatment suppressed the capacity of these resident liver macrophages to kill *Listeria* during the first 8 h of infection.

Figure 7 shows that intravenous injection of 25 μg of endotoxin 4 h before infection had no significant effect on the inactivation of *Listeria* by liver macrophages during the early stages of infection. Endotoxin-treated mice, like control mice, inactivated approximately 75% of the bacterial load in their livers by 6 h, and there was little difference between these two groups of mice at 24 h. It is evident from this finding, therefore, that endotoxin treatment suppresses antimicrobial mechanisms that begin to operate after 24 h of primary infection.

DISCUSSION

The data in this paper agree, in general, with those published by others (2, 4-7, 9, 10, 15, 16, 22-27), namely, that bacterial endotoxins possess potent infection-enhancing properties. The data show that intravenous administration of endotoxin to mice at any stage during infection with the gram-positive bacterial pathogen *L. monocytogenes* results in a rapid and striking increase in bacterial multiplication in infected tissues. They also show that pretreatment of normal recipient mice with endotoxin prevents them from being adoptively immunized against a *Listeria* challenge infection with spleen cells from immune donors. Endotoxin treatment of immune donors, on the other hand, had little effect on the capacity of their spleen cells to protect normal recipients against the same challenge. Thus, since it is known that adoptive T-cell-mediated, anti-*Listeria* immunity needs to be expressed by recipient macrophages, it seems reasonable to suspect that the infection-enhancing property of endotoxin resides in its capacity to interfere with macrophage function rather than T-lymphocyte function.

Indeed, a suppressive effect of endotoxin on in vivo macrophage function is well documented. It is known, for instance, that parenteral administration of endotoxin results in a suppressed capacity of the reticuloendothelial system for clearing intravenously infused colloids (1). This suppression of macrophage clearance capacity is short lived, in that it is followed after about 48 h by enhanced clearance capacity. Likewise, endotoxin-suppressed anti-*Listeria* resistance was shown here to be relatively short lived when given either before or during infection.

It is difficult to explain, however, the listeriosis-enhancing effect of endotoxin solely in terms of its ability to interfere with the function of fixed macrophages of the reticuloendothelial system, because these cells probably play only

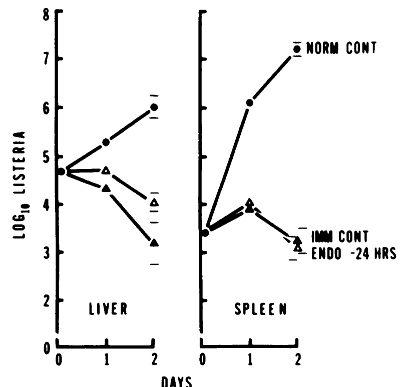

FIG. 5. *Evidence that endotoxin administration to immune donors had no significant effect on the capacity of their spleen cells harvested 24 h later to transfer immunity to untreated recipients (ENDO 24 HRS). Means of five mice per time point ± 2 standard errors.*

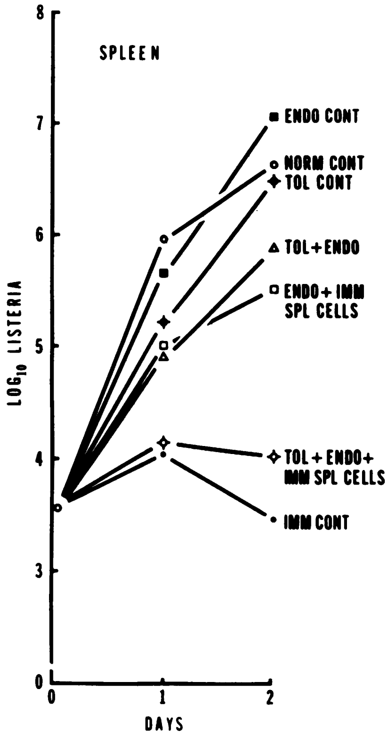

FIG. 6. *Evidence that recipient mice can be made tolerant to the infection-enhancing effect of endotoxin. Endotoxin had little suppressive effect on the expression of adoptive immunity in recipients that were made tolerant by repeated doses of endotoxin before being infused with immune spleen cells and challenged with Listeria 2 days later (TOL + ENDO + IMM SPL CELLS). These mice expressed almost as much adoptive immunity as immune controls (IMM CONT). Although being made tolerant alone (TOL CONT) caused bacterial growth comparable to that of normal controls (NORM CONT), it caused a reduction in the infection-enhancing effect of an endotoxin test dose in normal controls (TOL + ENDO). Means of five mice per time point.*

a minor role in the expression of anti-*Listeria* immunity (19). Indeed, the results shown in Fig. 7 provide evidence that endotoxin had no effect on the capacity of these cells to inactivate a large proportion of the ingested bacterial load in the liver during the first 8 h of a primary infection. It is more likely, therefore, that the infection-enhancing effect of endotoxin was caused by interference with the function of blood monocytes, since it is known (28) that these cells are the mobile antecedents of the macrophages that must rapidly populate infective foci where acquired immunity is expressed. The possibility that endotoxin administration results in a profound decrease in the number of circulating monocytes or causes a disturbance in their antimicrobial functions at infective foci is the subject of research currently in progress in this laboratory. This possibility is suggested by Fig. 1, which shows that endotoxin injection had a much more rapid and striking infection-enhancing effect when given on day 2 of infection and later than when given at the time of initiating infection. It is known that by day 2 infective foci begin to be heavily populated by monocytes from blood (19). It is also possible that a decrease in the number of circulating blood monocytes is part of the dramatic leukopenia that is known to follow endotoxin administration (18).

Whatever the target host cells with which endotoxin interferes, it seems fairly certain from this and other publications that this compound, under natural conditions, acts to promote infections caused by pathogenic, gram-negative organisms. It seems reasonable to suspect, moreover, that an acquired defense against infections

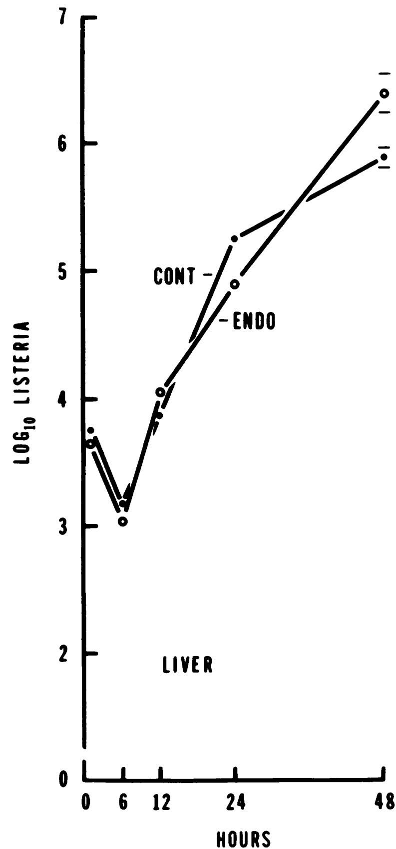

FIG. 7. *Endotoxin did not affect the ability of resident liver macrophages to inactivate a large proportion of the ingested bacterial load in this organ during the first 6 h of infection. Means of five mice per time point ± 2 standard errors.*

with these organisms probably includes a mechanism that counteracts the infection-enhancing effect of their endotoxins. This suggestion is supported by the results of this study which show that making mice tolerant to endotoxin prevents a later injection of this compound from promoting infection with a non-endotoxin-containing bacterium. Whether this tolerance is based on the generation of antibodies to endotoxin (8) or on an acquired refractoriness of host cells to endotoxin-induced physiological changes is currently under investigation. It is anticipated that the *Listeria* model will prove useful in investigating these possibilities.

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