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ENDOTOXIN TOLERANCE. I. ITS INDUCTION BY EXPERIMENTAL PYELONEPHRITIS *

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The endotoxins of gram-negative bacteria are among the most pharmacologically active substances known and produce a variety of profound physiological alterations in both laboratory animals and man (1, 2). Many of the effects produced by endotoxins may be observed during infections caused by gram-negative bacilli. In fact, some investigators have suggested that the symptomatology and lethality of gram-negative bacteremia is due primarily to endotoxemia (3–5). These toxic effects become progressively less severe with repeated daily injections of endotoxin until they almost entirely disappear (6–8). This phenomenon of tolerance is neither species nor type specific and may be produced by unrelated strains of endotoxin-producing bacteria (6, 8). Although tolerance is readily produced by the injection of purified endotoxin or dead bacteria, it has not been clearly demonstrated during the course of clinical or experimental infections. Neva and Morgan presented evidence that patients convalescing from typhoid fever were endotoxin tolerant (9), but investigations by Heyman and Beeson in patients with a variety of infections including typhoid fever failed to demonstrate endotoxin tolerance in any of the bacterial infections studied (10). More recently, evidence has been presented that endotoxin tolerance does occur during experimental *Salmonella typhosa* infections in human volunteers (11).

The present investigation was prompted by clinical evidence that chronic renal infection exerted an ameliorating effect on the outcome of gram-negative bacteremia. A clinical study of gram-negative bacteremia has demonstrated that both shock (24%) and death (20%) were significantly less frequent ($p < 0.01$) in 78 patients with chronic pyelonephritis who developed bacteremia than in 95 patients (46%, 54%) with bacteremia who did not have chronic renal infection (12). Preliminary studies have suggested that experimental pyelonephritis does induce endotoxin tolerance (13). The present report is an extension of these studies and details the characteristics of endotoxin tolerance produced by experimental renal infection. A subsequent report documents the occurrence of endotoxin tolerance in patients with chronic pyelonephritis (14).

MATERIALS AND METHODS

Production of pyelonephritis. Pyelonephritis was produced in 2.0 to 3.0-kg male New Zealand rabbits by transient ureteral occlusion and the intravenous injection of *Escherichia coli* (O-111 : B-4). After the injection of 25 mg per kg of sodium pentobarbital, a right flank surgical incision was made and the right ureter was located and enclosed by a ligature. Both ends of the ligature were passed through the paraspinous muscles and tied loosely on the rabbit's back. Ureteral occlusion was produced the following day by tightening the ligature for 18 hours and 1×10^9 *E. coli* (O-111 : B-4) were injected into a marginal ear vein. Control rabbits underwent a similar operative procedure except that no ureteral occlusion was produced. An equivalent endotoxin challenge of 1×10^9 formalin-killed *E. coli* was administered to the controls. This group of controls will be identified as "operated controls" in the subsequent discussion. A second group of eight control rabbits that had not been sham operated on or injected with dead bacteria ("nonoperated controls") were studied to insure that these procedures did not alter the pyrogenic response in comparison to that observed in normal rabbits.

Seven of the rabbits with pyelonephritis subsequently were used for studies of the effect of reticuloendothelial blockade, and three received antimicrobial therapy to eradicate their renal infection as described below. Four of the 16 operated control rabbits were reoperated after endotoxin challenge and made pyelonephritic. Seven of the nonoperated controls were injected with 4×10^9 live

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E. coli immediately after initial pyrogen challenge and were rechallenged with endotoxin 10 to 14 days later to assess the effect of transient bacteremia on response to endotoxin.

E. coli pyelonephritis was produced in nine additional rabbits, and nine controls were sham operated on for use in studies of the dose-response relationship and a comparison of pyrogenic response in rabbits with pyelonephritis due to *E. coli* and *Streptococcus faecalis*. Enterococcal pyelonephritis was produced in three rabbits by a similar operation followed by the injection of 3×10^8 *S. faecalis*. At a later time, enterococcal pyelonephritis was produced in two additional animals. The latter were used as additional controls representing pyelonephritis caused by a nonendotoxin-producing species of bacteria.

Catheterized urine specimens were obtained at weekly intervals for quantitative bacterial cultures. The specimens were serially diluted in tenfold increments, and 1 ml of 10^{-2}, 10^{-4}, and 10^{-6} dilutions were thoroughly mixed with 15 ml of melted agar in sterile petri dishes. Colony counts were determined after 24 hours of incubation. Bacterial species were identified by colonial morphology, carbohydrate fermentation, indole production, citrate utilization, and acetylmethylcarbinol production.

Pyrogen challenge. Ten to 30 days after the induction of pyelonephritis or sham operation, the febrile response to purified *Salmonella enteriditis* lipopolysaccharide [1] was determined. All glassware was heated to 180° C for 3 hours prior to use, and pyrogen-free saline was used to dilute the lipopolysaccharide. During pyrogen challenge, the rabbits were maintained in wire cages without restraint in an air-conditioned room. Four rabbits were studied in each experiment, and at least one operated control and one pyelonephritic rabbit were included in each experiment as additional controls. All comparisons were made between animals who had been infected or had received formalin-killed *E. coli* at the same time throughout this study to insure that the duration of infection and the period of time after challenge with killed bacteria was the same. Temperatures were recorded on a Yellow Springs Telethermometer from either rectal thermocouples or thermocouples implanted deeply in the paravertebral muscles. Base-line temperatures were determined every 30 minutes during the 2 hours allowed for acclimitization. Rabbits with temperatures greater than 101° F or whose basal temperatures varied more than 1.0° during the control period were excluded from further study. Endotoxin was injected into a marginal ear vein, and temperatures were recorded every 30 minutes for an additional 6 hours. Temperature curves were plotted on 1.0-cm graph paper with each centimeter representing 0.5° F temperature on the vertical axis and 30 minutes time on the horizontal axis. An Ott Universal planimeter was used to measure the area above the base line, and the reading on the vernier was recorded as the fever index, each unit of which was termed a fever unit (F.U.).

[1] Difco Laboratories, Detroit, Mich.

Other studies. Reticuloendothelial blockade was accomplished by the injection of 9 ml of thorium dioxide (Thorotrast) [2] intravenously as described by Beeson (15). In four of the initial, operated control rabbits, the pyrogenic response to endotoxin was determined; then pyelonephritis was produced by the method outlined above, and the pyrogenic response to endotoxin was again determined.

Three rabbits in whom the pyrogenic response had been determined after the production of pyelonephritis were treated with colistin methanesulfonate,[3] 10 mg per kg daily for 3 weeks. The pyrogenic response to endotoxin was subsequently determined 4 weeks after the previous endotoxin challenge and the eradication of bacteriuria. Urine cultures obtained from these animals became sterile during the first week of treatment and remained sterile until their sacrifice. Post-mortem cultures of the renal cortex and medulla also were sterile. Similar treatment was administered to four other pyelonephritic rabbits, but since bacteriuria either persisted during treatment or recurred immediately after discontinuation of therapy, no further studies were performed on these animals. A similar number of rabbits with active pyelonephritis, as evidenced by persistent bacteriuria, that had been operated on and infected at the same time were challenged with endotoxin simultaneously to insure that no lapse in tolerance had occurred.

RESULTS

Renal infection. The injection of viable bacteria with and without transient ureteral occlusion was followed by lethargy and fever of 2 to 3 days duration. These symptoms also occurred in the controls who received formalin-killed *E. coli,* but were less severe and lasted only 18 to 24 hours. All rabbits were active, appeared healthy, and had been afebrile for at least 5 days before endotoxin challenge.

Catheterized urine specimens from the operated control rabbits were either sterile or contained less than 10^2 bacteria per ml. All rabbits who had viable *E. coli* injected after transient ureteral occlusion had bacteriuria in excess of 10^5 *E. coli* per ml of urine. *S. faecalis* bacteriuria of similar magnitude occurred in the five rabbits infected with this species. Bacteriuria ($> 10^5$ per ml) persisted throughout the entire study period in operated animals receiving viable bacteria except in those rabbits that received antimicrobial therapy.

All animals were sacrificed at the completion of the studies, and the kidneys were examined grossly and microscopically. Mild to moderate hydro-

[2] Testagar Co., Inc. Detroit, Mich.

[3] Colymycin, Warner Chilcott Co., Morris Plains, N. J.

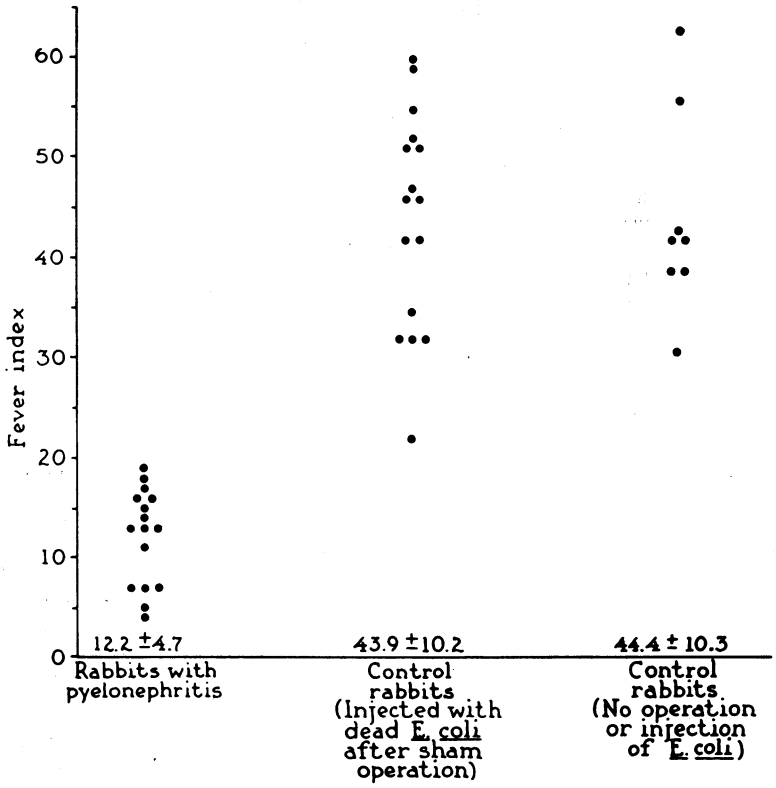

FIG. 1. FEBRILE RESPONSE OF 8 UNOPERATED CONTROL RABBITS, 16 OPERATED RABBITS, AND 16 RABBITS WITH *E. coli* PYELONEPHRITIS TO 0.5 μG OF HETEROLOGOUS ENDOTOXIN (*S. enteriditis*).

nephrosis, gross and microabscesses, scars, and polymorphonuclear and round cell infiltrate were observed in every kidney whose ureter had been transiently occluded before the injection of viable bacteria. No gross lesions were observed in the opposite kidneys of these animals, although in four rabbits there was evidence of mild pyelonephritis in the microscopic sections. No gross or histologic evidence of pyelonephritis was observed in either kidney of the control rabbits.

Response to endotoxin. The febrile responses to 0.5 μg of *S. enteriditis* of the initial group of 16 rabbits with *E. coli* pyelonephritis, 16 operated controls previously injected with dead *E. coli,* and 8 nonoperated controls who had not received *E. coli* are shown in Figure 1. No difference in the pyrogenic responses of the two groups of control rabbits was apparent. The fever indexes ranged from 22 to 60 F.U. with a mean of 43.9 ± 10.2 F.U. in the operated controls, while the fever indexes of the nonoperated controls ranged from 31 to 63 F.U. with a mean value of 44.4 ± 10.3 F.U. In contrast, all of the rabbits with *E. coli* pyelonephritis had fever indexes of less than 20 F.U. The mean fever index, 12.2 ± 4.7 F.U., of the rabbits with pyelonephritis was significantly less than that of the operated controls ($t_{30} = 10.6$; $p < 0.001$) or the nonoperated controls ($t_{22} = 10.6$; $p < 0.001$).

The febrile response of the 16 operated control rabbits and the 16 rabbits with pyelonephritis differed qualitatively as well as quantitatively. Composite fever curves were constructed by determining the mean temperature at each time interval and are shown in Figure 2. The mean fever curve for the 16 controls is illustrated by the heavy dashed line with the diagonally hatched area representing plus one and minus one standard deviation. The lower solid line is the mean fever curve for the pyelonephritic rabbits with ± 1 SD contained within the stippled area. The fever index of the composite fever curve was 42 F.U. for

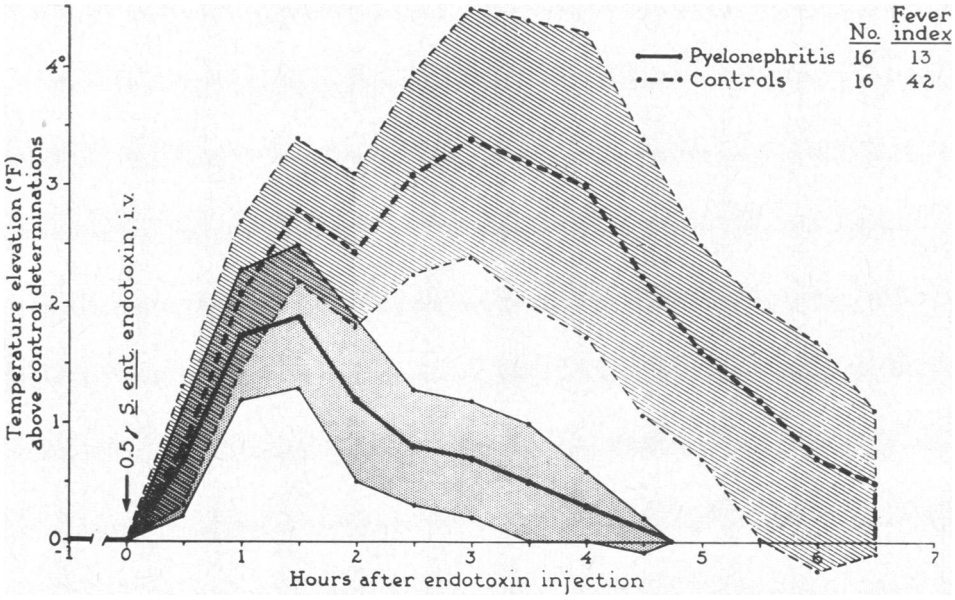

FIG. 2. COMPOSITE FEVER CURVES, ± 1 SD OF 16 RABBITS WITH PYELONEPHRITIS AND 16 OPERATED CONTROL RABBITS AFTER ENDOTOXIN CHALLENGE.

the controls and 13 F.U. for the rabbits with pyelonephritis, and corresponded closely with the mean of the fever indexes of both of these groups. The composite fever curve of the controls is typical of the febrile response to endotoxin in rabbits with an initial fever within 1 to 2 hours after the endotoxin injection, which is followed by a second and even greater temperature rise 3 to 4 hours after the initial challenge. In all but one of the 16 controls, the maximal fever occurred 2½ hours or longer after the injection of endotoxin.

In contrast, the composite fever curve of the rabbits with pyelonephritis is characteristic of the febrile response of endotoxin-tolerant animals. The initial febrile response is unchanged or slightly diminished, but rather than a second temperature rise, the temperatures progressively declined to base line levels. All of the pyelonephritic rabbits had their maximal temperature elevation within 2½ hours after the injection of endotoxin, and although six rabbits did exhibit a slight secondary rise, this did not exceed or equal the initial peak. All of the rabbits with pyelonephritis became afebrile within 5 hours, while 13 of the 16 controls remained febrile for 6 hours or more after endotoxin administration.

Response to endotoxin after bacteremia without localized infection. Eight of the unoperated con-

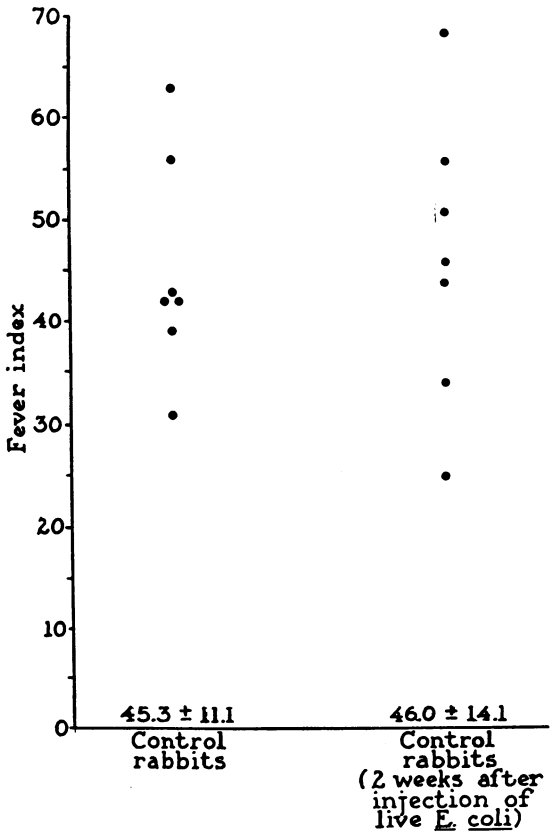

FIG. 3. FEBRILE RESPONSE OF SEVEN UNOPERATED CONTROL RABBITS BEFORE AND AFTER TRANSIENT BACTEREMIA FROM *E. coli*.

TABLE I

Febrile response to three doses of endotoxin in control and pyelonephritic rabbits

Endotoxin dose	Experimental group	Fever index
μg		F.U.*
0.5	Pyelonephritis	4
0.5	Pyelonephritis	7
0.5	Control	32
0.5	Control	34
1.0	Pyelonephritis	11
1.0	Pyelonephritis	18
1.0	Control	49
1.0	Control	56
2.0	Pyelonephritis	5
2.0	Pyelonephritis	7
2.0	Control	32
2.0	Control	32

* F.U. = fever units.

trols were injected with 4×10^9 live *E. coli* intravenously on the day after their initial challenge with 0.5 μg *S. enteriditis* endotoxin. One animal died 6 hours after the injection of live *E. coli,* while the remainder had a mild febrile illness of 2 to 3 days duration. Blood cultures obtained from all seven animals 96 hours after the injection of live bacteria were sterile. Ten to 14 days after the injection of live *E. coli,* the response to 0.5 μg of endotoxin was again determined. These results are illustrated in Figure 3. The febrile response was quite similar in the same unoperated controls before (45.3 ± 11.1 F.U.) and after (46.0 ± 14.1 F.U.) the injection of live *E. coli.* Although slight individual variations in the same animals did occur in the two experiments, all animals exhibited typical biphasic fever curves, and there was no evidence that endotoxin tolerance resulted from the intravenous injection of live *E. coli* when persistent infection did not occur.

Response to various doses of endotoxin. To insure that these differences were not localized to a single concentration of endotoxin, control and pyelonephritic rabbits were challenged with 0.5, 1.0, and 2.0 μg of endotoxin. The febrile response to this fourfold range of dose of endotoxin is shown in Table I. The control rabbits did not completely demonstrate the anticipated dose response in that the fever indexes of the two controls that received 2.0 μg of endotoxin were no greater than that of the two controls that were challenged with 0.5 μg. The rabbits with pyelonephritis, however, were tolerant at each of the three doses of endotoxin, and demonstrate that the tolerant state did extend over a fourfold range of endotoxin dosage.

Effect of reticuloendothelial blockade. Reticuloendothelial blockade was undertaken in rabbits with tolerance induced by pyelonephritis to demonstrate that these animals were capable of responding to endotoxin. Immediately after the determination of the pyrogenic response to 0.5 μg of endotoxin, 9 ml of colloidal thorium dioxide (Thorotrast) was injected intravenously into seven pyelonephritic rabbits. These animals were rechallenged with the same dose of endotoxin on the following day. The results of these studies are shown in Figure 4. The fever indexes in the seven pyelonephritic rabbits were 4, 7, 13, 13, 16, 16, and 19 F.U. with a mean of 12.6 ± 5.3 F.U. After the injection of Thorotrast, fever indexes in the same animals ranged from 35 to 47 F.U., with a mean value of 42 ± 3.8 F.U.

Effect of reversal of disease state on febrile response to endotoxin. The pyrogenic response to endotoxin was determined in four sham-operated

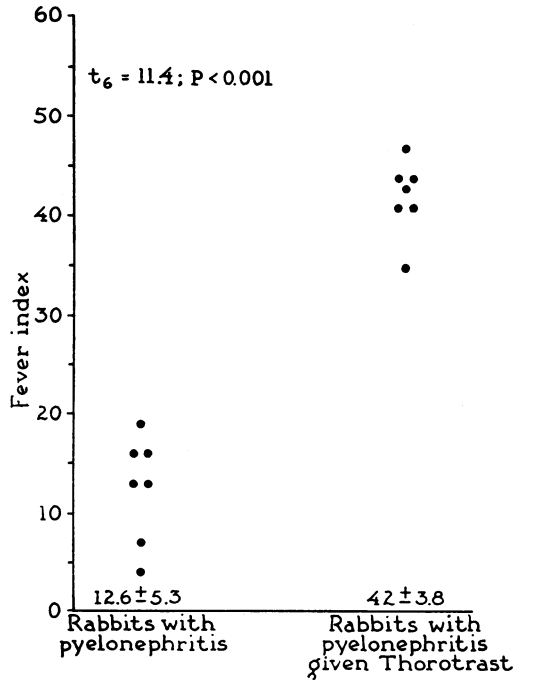

FIG. 4. PYROGENIC RESPONSE TO ENDOTOXIN OF SEVEN PYELONEPHRITIC RABBITS BEFORE AND AFTER RETICULOENDOTHELIAL BLOCKADE BY THORIUM DIOXIDE (THOROTRAST).

control rabbits, and pyelonephritis was produced the following day by the method previously described. An equal number of controls also were challenged with endotoxin at the same time, but were not made pyelonephritic. Both groups were subsequently rechallenged with endotoxin 3 weeks later. The fever indexes of both groups of controls (42.3 ± 16.0 F.U. and 47.8 ± 6.7 F.U.) were quite comparable initially, as Table II shows. Similarly the initial mean fever index of the controls in whom pyelonephritis had not been produced (47.8 ± 6.7 F.U.) differed only slightly from that observed 3 weeks later (43.8 ± 14.0 F.U.), and little individual variation occurred. The mean fever index of the controls made pyelonephritic was 42.3 ± 16.0 F.U. initially and was 12.5 ± 4.0 F.U. 3 weeks after the induction of pyelonephritis. A decrease in febrile response of 15 F.U. or more occurred in each animal after the development of pyelonephritis.

TABLE II

Febrile response to bacterial endotoxin in controls before and after induction of pyelonephritis and in pyelonephritic rabbits before and after treatment

Controls F.U.*	Controls made pyelonephritic (3 weeks after induction of pyelonephritis) F.U.
58	16
46	13
43	14
22	7
Mean 42.3 ± 16.0	12.5 ± 4.0
Controls F.U.	**Controls (Repeat challenge 3 weeks later) F.U.**
59	60
52	51
46	32
34	32
Mean 47.8 ± 6.7	43.8 ± 14.0
Pyelonephritic rabbits F.U.	**Treated pyelonephritic rabbits (4 weeks after beginning antimicrobial therapy) F.U.**
7	24
13	34
16	55
Mean 12.0 ± 4.6	37.7 ± 16.0
Pyelonephritic rabbits F.U.	**Pyelonephritic rabbits (4 weeks after initial challenge) F.U.**
4	7
7	11
16	18
Mean 9.0 ± 6.2	12.0 ± 5.6

* F.U. = fever units.

TABLE III

Febrile response to bacterial endotoxin in controls and rabits with pyelonephritis due to E. coli *and* S. faecalis

No pyelonephritis	Fever indexes in rabbits with *E. coli* pyelonephritis	*S. faecalis* pyelonephritis
*F.U.**	*F.U.*	*F.U.*
56	15	44
50	9	55
42	17	41
		39
		48
Mean 49.3 ± 7.0	14.0 ± 4.2	45.2 ± 6.4

* F.U. = fever units.

The lower part of Table II illustrates the febrile responses of rabbits with pyelonephritis before and after antibiotic treatment in comparison with the response of untreated pyelonephritic rabbits infected at the same time. The mean febrile response of three rabbits with pyelonephritis was 12.0 ± 4.6 F.U. initially and increased to 37.7 ± 16.0 F.U. after 4 weeks of antimicrobial therapy. An increase of 17 F.U. or greater was observed in each animal after treatment of pyelonephritis. The fever indexes in the untreated rabbits with pyelonephritis were quite similar at both periods, indicating that no lapse of tolerance had occurred in the untreated pyelonephritic rabbits.

Febrile response in enterococcal pyelonephritic rabbits. The pyrogenic response in three operated control rabbits, three rabbits with *E. coli* pyelonephritis, and three rabbits with pyelonephritis caused by nonendotoxin-producing species, *S. faecalis,* infected and challenged at the same time with 0.5 μg *S. enteriditis* endotoxin is compared in Table III. Two rabbits with enterococcal pyelonephritis produced at a later time are also listed. The three rabbits with *E. coli* pyelonephritis were tolerant to endotoxin and all had fever indexes of 17 F.U. or less, while rabbits with enterococcal pyelonephritis behaved like the uninfected controls, and both controls and rabbits with enterococcal pyelonephritis all had fever indexes of 39 F.U. or greater.

DISCUSSION

Clinical findings in gram-negative bacteremia suggested that endotoxin tolerance might result from chronic renal infection with gram-negative bacteria (12). These studies tend to confirm this

impression and demonstrate that persistent renal infection with endotoxin-producing bacteria is capable of inducing endotoxin tolerance. Rabbits with *E. coli* pyelonephritis not only had a significant diminution in the magnitude of the febrile response to endotoxin in comparison with control animals, but also demonstrated the characteristic single humped fever curve typical of that described in endotoxin-tolerant rabbits (6, 15). No differences were apparent between the febrile responses of nonoperated controls and sham-operated controls injected with an initial endotoxin challenge of formalin-killed bacteria equivalent to that given pyelonephritic rabbits, and both groups demonstrated typical biphasic fever curves. The failure of a single dose of endotoxin, unless considerably larger than the subsequent challenging dose, to alter pyrogenic response to endotoxin significantly is not surprising and has been noted previously (6, 7, 15, 16). The failure of either the injection of formalin-killed *E. coli* or the transient bacteremia following injection of live *E. coli* to produce tolerance suggests that the continued infection provides a greater and more persistent stimulus for the development of tolerance. The development of tolerance in animals previously demonstrated to respond normally to endotoxin after the induction of *E. coli* pyelonephritis and the failure of renal infection from nonendotoxin-producing bacteria (*S. faecalis*) to produce tolerance illustrated that the controls were capable of developing tolerance and that endotoxin-producing bacteria were responsible for the tolerant state. Finally, rabbits with pyelonephritis, previously demonstrated to be endotoxin tolerant, responded normally to endotoxin challenge after either reticuloendothelial blockade or the eradication of renal infection by antimicrobial therapy. Although it would have been desirable to have a larger number of the latter group, the difficulties in eradicating renal infection in fibrosed, partially obstructed kidneys precluded additional studies. Each of the three animals in whom renal infection was eradicated did exhibit a marked increase in endotoxin responsiveness, however, while no lapse of tolerance occurred in animals with active pyelonephritis of the same duration, indicating that these changes did not result from spontaneous lapse of tolerance. Other studies have demonstrated that relatively small numbers of animals may be used in comparing endotoxin effects (16) and suggest that these changes are probably meaningful.

Although the findings demonstrate that endotoxin tolerance does result from chronic renal infections, they do not necessarily imply that they are peculiar to pyelonephritis. Pyelonephritis was selected as a model infection only because of the previously mentioned clinical information, ease of production, and reproducibility, and because the presence of bacteriuria could be assessed readily. It seems likely that other types of chronic infection could induce endotoxin tolerance. Conflicting reports have been published on the occurrence of endotoxin tolerance in human infections (9, 10), but the occurrence of endotoxin tolerance in typhoid fever has recently been confirmed (11). Bennett was unable to demonstrate endotoxin tolerance in a careful study of the febrile response to endotoxins in rabbits with experimental pneumococcal infections or in *E. coli* peritonitis (17). The latter infection was quite similar to the bacteremia with *E. coli* in the present study in that it was either rapidly fatal or was terminated with apparently complete recovery in a few days. The present study was designed to circumvent this difficulty by utilizing an infection in which gram-negative bacteria persisted in the renal parenchyma and urine throughout the course of the experiment. Continuous proliferation and death of *E. coli* in the renal parenchyma ostensibly would provide both a quantitatively greater and more prolonged stimulus with endotoxin than occurred in *E. coli* peritonitis or in rabbits injected with either formalin-killed or live *E. coli*. Beeson's studies have demonstrated that both of these factors are important determinants of the development of tolerance (6, 15), but it was not possible to differentiate their relative importance in the present investigation.

SUMMARY

Tolerance to the pyrogenic effects of *Salmonella enteriditis* endotoxin was induced by the production of persistent renal infection with *Escherichia coli* (O-111 : B-4). Rabbits with pyelonephritis were tolerant over a fourfold range of endotoxin dosage. Tolerance was reversed by reticuloendothelial blockade with colloidal thorium dioxide and and by the eradication of renal infection with anti-

biotic treatment. The production of pyelonephritis led to the development of tolerance in rabbits previously demonstrated to respond normally to pyrogen challenge. *Streptococcus faecalis* pyelonephritis did not induce endotoxin tolerance.

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