Influence of Endotoxin Tolerance on Detoxification of *Salmonella enteritidis* Endotoxin by Mouse Liver and Spleen[1] (36808)

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Endotoxin tolerance is the state of enhanced resistance of animals against the toxic effects of bacterial endotoxins. The tolerant state is usually induced by the prior administration of single (1) or multiple (2, 3) sublethal doses of endotoxin.

Although the mechanism of endotoxin tolerance has not been fully ascertained, evidence has suggested that tolerance involves the stimulation of the phagocytic function of the reticuloendothelial system (RES), primarily the macrophages of liver and spleen. Beeson (4, 5) using a method of passive transfer of serum, and later Braude, Carey and Zalesky (6) and Braude *et al.* (7) employing ^{51}Cr-labeled endotoxin, showed that endotoxin-tolerant rabbits clear intravenously injected endotoxins more rapidly than normal animals. Furthermore, the intravenous injection of colloidal substances which are removed from the blood stream by the RES and which produce a state of reticuloendothelial (RE) blockade completely abolish the state of tolerance (4, 5). Agents which alter RE function also render normal animals highly susceptible to endotoxin shock (4, 5). Based on these observations, the development of tolerance was attributed to an enhanced functional capacity of the RES, reflected by the increased ability of the RES to remove endotoxin from the blood stream. It was hypothesized that as a result of the increased removal of endotoxin from the vascular compartment, susceptible tissues were protected from endotoxin-induced injury (4, 5).

Inconsistent with the speculation that tolerance depends upon enhanced phagocytic capacity of the RES are the findings that a variety of RES stimulants such as BCG (8), zymosan (9), glucan (10), and triolein (11) sensitize rather than protect animals against endotoxin. The absence of a simple correlation between endotoxin shock and phagocytic state is also manifested with diethylstilbestrol (DES) which profoundly stimulates the phagocytic capacity of the RES, but does not modify the endotoxin-induced lethality (12). Additionally, contrary to enhanced endotoxin clearances in tolerant rabbits, rats or mice, the intravascular clearance of endotoxin was unaltered in endotoxin-tolerant dogs (13). Furthermore, the observation that tolerance to the pyrogenic effect of endotoxin in man has been shown to occur with normal phagocytic activity (14), has indicated that enhanced phagocytic function of the RES, *per se*, need not be a necessary step in the development of tolerance.

It has been demonstrated that liver and spleen, organs which possess extensive RE components, possess a potent endotoxin detoxifying ability (12, 15–18). The endotoxin detoxifying ability of the liver and spleen was shown to be a function of the RE or macrophage component (19). These findings, coupled with the lack of correlation between enhanced phagocytic activity of the RES and protection of animals against endotoxemia, suggest that the endotoxin detoxifying function of the RES may be a fundamental host defense mechanism against endotoxin (12, 16, 20). This concept is further supported by the observations that factors which altered endotoxin detoxification of liver and spleen also induced endotoxin hyperreactivity (15, 16, 21). Therefore, if endotoxin detoxifying activity of the RES is a factor in the prevention of the sequelae of endotoxemia, endotox-

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in-tolerant animals should possess a greater ability to detoxify endotoxin than control, nontolerant animals.

The present study was undertaken to determine whether endotoxin detoxification by liver and spleen, as assayed by a recently developed method (12, 15), is enhanced in endotoxin-tolerant mice. The findings demonstrate a significant enhancement of the liver and spleen endotoxin detoxification in association with the tolerant state.

Materials and Methods. Donor mice. CF No. 1 male mice (20–25 g) were purchased from Carworth Farms: "Tolerance" to the lethal effect of endotoxin was induced by the daily intraperitoneal administration of 12.5, 25, 50, 100 and 200 μg of *Salmonella enteritidis* endotoxin (Difco, LPS B control 599008). Control mice received isovolumetrically sterile, pyrogen-free, 0.9% saline solution. The development of endotoxin tolerance was manifested by the observation that the intraperitoneal administration of 1000 μg of endotoxin 72 hr after the last endotoxin injection, which induced a mortality of 66.7% in the saline-treated group within 24 hr, was nonlethal to endotoxin-injected mice.

Endotoxin inactivation assay. Seventy-two hr after the last endotoxin or saline injection, the animals were sacrificed and the liver and spleen were quickly removed and placed in cold buffered saline solution. Homogenates (1, 0.5, and 0.25%) of liver and spleen from endotoxin and saline-treated donors were prepared as previously described (12, 15). Four milliliters of liver and spleen homogenates were incubated with 1 μg (0.5 ml) of *S. enteritidis* endotoxin at 37° for 3 hr. Following incubation, 250 μg of actinomycin D (Meractinomycin, MSD) were added (0.5 ml) to each flask. Normal CF No. 1 white male mice were then injected intraperitoneally with 0.5 ml of the preparation. Control mice were injected either with homogenate preparations which were incubated with isotonic saline, or endotoxin incubated with buffer alone. Mortality was ascertained at 24 and 48 hr. The designated mortality was that occurring within 48 hr following the administration of the preparations. The employment of actinomycin D to assay endotoxin levels is based upon the observations of Pieroni *et al.* (22).

The presence of increased endotoxin detoxifying activity by liver and spleen in the tolerant group was determined by comparing the difference in mortality between the tolerant and the saline-treated group at the various homogenate concentrations. Mortality data were analyzed using the chi-square test corrected by Yates' factor and the level of significance was established at 95%.

Results. In agreement with our previous findings (12, 21), 1% liver and spleen homogenates prepared from saline-treated mice possessed significant ($p < .01$) endotoxin detoxifying activity. The endotoxin detoxifying activity was denoted by a reduction in the mortality rate of actinomycin D-treated mice which received endotoxin incubated in these homogenate preparations, compared to the mortality of mice injected with endotoxin incubated in buffer alone (Tables I and II). Similarly, liver and spleen homogenates at the 0.5% concentration also possessed a significant ($p < .05$) endotoxin detoxifying activity as determined by a significant reduction of the mortality. However, when endotoxin was incubated in normal liver or spleen homogenates at the 0.25% concentration, the degree of endotoxin inactivation was not significant ($p > .1$) from the buffer group (Tables I and II). These findings demonstrate an inability of liver and spleen homogenates at the 0.25% concentration to modify significantly the endotoxin and thus alter the mortality pattern.

The degree of endotoxin inactivation by liver and spleen homogenate preparations from endotoxin-tolerant mice was also determined. Note in Tables I and II that liver and spleen homogenates, prepared from endotoxin tolerant hosts at 1 and 0.5% concentrations, were associated with a 70 and 65% reduction in manifested mortality, respectively, compared to the mortality of actinomycin D-treated mice injected with endotoxin preparations incubated with liver and spleen homogenates prepared from saline-treated donors. However, as striking as the reduction in mortality was, the increase in the degree of endotoxin inactivation of endotoxin tolerant liver and spleen homogenates was not statistically ($p > .05$) different from the correspond-

TABLE I. Effect of Endotoxin Tolerance[a] on Detoxification of Endotoxin by Liver Homogenates.[b]

Group	Pre-treatment	Tissue homogenate (%)	Endotoxin (0.1 μg)	Deaths/total	Mortality (%)	p value[c]
1	—	Buffer	+	20/26	76.9	
2	Saline	Liver, 1	+	7/24	29.2	2 vs 1 <.01
3		Liver, 0.5	+	10/24	41.7	3 vs 1 <.05
4		Liver, 0.25	+	14/25	56.0	4 vs 1 >.1 (NS)
		Liver, 1	—	2/27	7.4	—
5	Endotoxin	Liver, 1	+	2/25	8.0	5 vs 2 >.05 (NS) 5 vs 1 <.001
6		Liver, 0.5	+	3/24	12.5	6 vs 3 >.05 (NS) 6 vs 1 <.001
7		Liver, 0.25	+	5/25	20.0	7 vs 4 <.02 7 vs 1 <.001
		Liver, 1	—	0/26	0	—

[a] Endotoxin tolerance was induced by the daily intraperitoneal administration of 12.5, 25, 50, 100, 200 μg of *S. enteritidis* endotoxin. Control mice received isovolumetrically sterile 0.9% saline solution.

[b] Liver homogenates (4.0 ml) (1, 0.5, 0.25%) were incubated with 1.0 μg (0.5 ml) of endotoxin in saline at 37° for 3 hr. After incubation 250 μg (0.5 ml) of actinomycin D were added and 0.5 ml of the mixture was administered ip to normal mice. Mortality was recorded 48 hr following the administration of the preparations.

[c] As determined by chi-square test.

ing saline-treated (control) group. In contrast, the degree of endotoxin inactivation by liver and spleen homogenates in the tolerant group at the 0.25% homogenate concentration was significantly greater ($p < .02$) than the corresponding 0.25% liver and spleen homogenates prepared from saline-treated mice. The enhanced endotoxin detoxification by liver and spleen in the tolerant group at the 0.25% level was further indicated by the observation that these same homogenates did significantly ($p < .001$) detoxify endotoxin compared to the group in which endotoxin was incubated in buffer. Indeed, liver and spleen homogenates from tolerant mice, when employed at the 0.25% concentration, detoxified endotoxin at a level which was equal to, or greater than that manifested by a 1% liver or spleen homogenate preparation of control mice (Tables I and II). In contrast to the effective detoxifying activity of liver and spleen from tolerant mice, homogenates of liver and spleen from saline-treated donors at 0.25% were not effective ($p > .1$) in reducing endotoxin-induced mortality (Tables I and II), denoting the absence of effective endotoxin detoxification at a low tissue concentration.

Discussion. The present findings demonstrate that during the state of endotoxin tolerance in mice, there is an increased ability of liver and spleen to detoxify endotoxin. The ability of liver and spleen of endotoxin-tolerant mice to detoxify endotoxin at the 0.25% homogenate concentration was not manifested by equivalent liver and spleen homogenates from saline-treated donors. A comparative evaluation of the data denotes an approximate fourfold enhancement in endotoxin detoxification in the tolerant state since reduction in mortality employing a 0.25% homogenate preparation was comparable to or greater than that obtained with a 1% homogenate preparation from normal mice. Our findings are in fundamental agreement with the observations of Rutenburg *et al.* (23) who showed an enhanced endotoxin detoxification by spleen homogenates from endotoxin-tolerant rabbits by means of the chick embryo test. The present findings support the hypothesis that tolerance is associated with an enhancement in endotoxin de-

TABLE II. Effect of Endotoxin Tolerance on Detoxification of Endotoxin by Spleen Homogenates.[a]

Group	Pre-treatment	Tissue homogenate (%)	Endotoxin (0.1 μg)	Deaths/total	Mortality (%)	p value[b]
1	—	Buffer	+	20/26	76.9	
2	Saline	Spleen, 1	+	6/24	25.0	2 vs 1 <.001
3		Spleen, 0.5	+	11/24	45.8	3 vs 1 <.05
4		Spleen, 0.25	+	17/28	60.7	4 vs 1 >.1 (NS)
		Spleen, 1	—	1/27	3.7	—
5	Endotoxin	Spleen, 1	+	2/24	8.3	5 vs 2 >.2 (NS)
6		Spleen, 0.5	+	4/24	16.7	6 vs 3 >.05 (NS)
						6 vs 1 <.001
7		Spleen, 0.25	+	5/24	20.8	7 vs 4 <.01
						7 vs 1 <.001
		Spleen, 1	—	0/26	0	—

[a] Spleen homogenates (4.0 ml) (1, 0.5, 0.25%) were incubated with 1.0 μg (0.5 ml) of endotoxin in saline at 37° for 3 hr. After incubation, 250 μg (0.5 ml) of actinomycin D were added and 0.5 ml of the mixture was administered ip to normal mice. Mortality was recorded 48 hr following the administration of the preparation.

[b] As determined by chi-square test.

toxifying ability of the RES (23).

The present findings, while delineating the role played by the RES in endotoxemia and the development of tolerance, do not define the molecular mechanism by which the administration of sublethal doses of endotoxin enhances endotoxin detoxification in RE cells. It is probable that the administration of endotoxin (substrate) induces a state of metabolic adaptation (23–25) of certain presently undefined lysosomal enzymes of macrophages which result in enhancement in endotoxin detoxification. Defining the molecular mechanism involved in the process of lysosomal enzyme inactivation of endotoxin (19) would contribute to an appreciation of the development of therapeutic measures to enhance endotoxin detoxification and reduce the lethal effects of endotoxemia.

Summary. The influence of tolerance, developed by the daily intraperitoneal administration of sublethal doses of *Salmonella enteritidis* endotoxin, on the endotoxin detoxifying capacity of the liver and spleen was evaluated. Liver and spleen homogenates from endotoxin-tolerant mice possessed greater endotoxin detoxifying ability than liver and spleen homogenates from saline-treated mice.

These findings further accent the importance of the liver and spleen macrophage cells in endotoxemia and indicate that endotoxin tolerance is associated with the enhanced capacity of the reticuloendothelial cells to detoxify endotoxin.

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