HOST DEFENSE AGAINST BACTERIAL ENDOTOXEMIA: MECHANISM IN NORMAL ANIMALS*

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Studies on host mechanisms which protect against the harmful effects of endotoxins are relatively few and they have not provided a clear assessment of the importance of humoral or cellular defenses in detoxification. Although it is widely believed that cells of the reticuloendothelial system play a major role in detoxification, the evidence to date is not compelling. My own approach to the elucidation of those host mechanisms which are of primary concern in detoxification has been based on the belief that components in the circulating plasma are largely responsible for survival of the endotoxemic host.

Early studies on detoxification of endotoxins by serum or plasma did not encourage the view that humoral components were of much consequence in host defense. In vitro experiments indicated that the toxicity of endotoxins incubated for short periods in normal serum was often increased and that prolonged incubations were required to produce significant detoxification (1). Some clarification of the subject was obtained in 1958 when it was shown that the detoxifying capacity of serum or plasma from numerous species could be significantly augmented if calcium-binding agents were incorporated in the medium (25, 29). However, the need to reduce plasma ionized calcium in order to obtain rapid detoxification cast doubt on the functional importance of such a humoral system.

Nonetheless, the potential capacity of plasma, from all mammalian species tested, to detoxify endotoxins (29) and the additional observation that endotoxins circulate for many hours in the plasma after parenteral administration (5, 31) encouraged further efforts to define the role of the humoral system in defense against endotoxemia. These subsequent studies demonstrated that during detoxification in vitro, endotoxin complexed with two serum alpha globulins having esterase activity of the nonspecific, carboxylic type (32). The initial interaction of endotoxin with one of these, a heat-stable alpha lipoprotein (esterase), led to the degradation or disaggregation of the large molecules; detoxification of exposed toxic groups was presumed to be effected by the other, a heat-labile alpha globulin (esterase). Formation of the endotoxin–alpha lipoprotein (esterase) complex, a requisite for detoxification, was largely inhibited by serum Ca^{++} at physiologic concentration. The subsequent demonstration that endotoxin-lipoprotein (esterase) complexes form in the circulating plasma of mice, guinea

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pigs, and rabbits (33) implied that the ionized form of plasma calcium decreases and that activation of the humoral detoxifying system does occur in response to a sublethal dose of endotoxin. A substantial fall in plasma total calcium following a lethal dose of endotoxin has been reported (22).

After the recent development of the serum-Ca^{++} electrode, it became feasible to measure relative changes in ionized calcium in fresh serum obtained at various time intervals after injection of endotoxin. The data will show that the concentration of Ca^{++} gradually decreases in postendotoxin serum and that there occurs a concomitant increase in the heat-stable, carboxylic esterase level and a very marked increase in the rate of detoxification of endotoxin.

Materials and Methods

Endotoxins—Endotoxins were prepared from *Escherichia coli*, 0-111B_4 (LD_{80} = 1 mg/kg in rabbits), and from the Danysz strain of *Salmonella enteritidis* according to Boivin and Mesrobeanu (4). The final preparations were sedimented in an ultracentrifuge to obtain more homogeneous products (5, 31). Stock solutions of the pellet fractions were made up to 1 mg/ml in phosphate-buffered saline (pH 7.4). The *E. coli* endotoxin was administered to rabbits to obtain postendotoxin serum; the *S. enteritidis* preparation was used exclusively for assay of detoxification. Antiserum to *S. enteritidis* was raised in both horse and rabbit and combined in appropriate amounts for the immunodiffusion experiments (32).

Preparation of Pre- and Postendotoxin Sera—Young adult rabbits weighing 1.7–2.2 kg were immobilized and given a single intravenous dose of 100 μg/kg of *E. coli* endotoxin. Blood samples (1.5 ml) were taken by cardiac puncture from each rabbit according to the following schedule: (*a*) 5 min before endotoxin administration, (*b*) 15 or 30 min postendotoxin, (*c*) 1 or 2 hr postendotoxin, (*d*) 3 or 4 hr postendotoxin, (*e*) 5 or 6 hr postendotoxin. Each blood sample was put into a 10 × 50 mm tube and immediately covered with mineral oil. Control rabbits were bled according to the same time schedule after an injection of pyrogen-free saline. The blood samples were held at room temperature for 1 1/2 hr before clots were sedimented by light centrifugation. Serum was drawn off with a syringe and needle and transferred under oil to tubes in an ice bath. All procedures described in this report were carried out with serum freshly drawn on the day of the experiment.

Serum Calcium Ion Measurements—Individual serum samples were removed from beneath the oil layer with a 1 ml syringe and immediately put into a serum flow-through electrode (Model No. 99-20, Orion Research Inc., Cambridge, Mass.) to measure ionized calcium under essentially anaerobic conditions. Samples of these sera were also examined for esterase content and detoxifying capacity.

Immunodiffusion Assay for Detoxification—Detoxification was measured in vitro by an immunodiffusion method (32, 34) and also by a biologic assay employing actinomycin D–treated mice (23). The immunodiffusion assay is based on the transformation of the slow diffusing, toxic molecules (C antigen) into fast diffusing, nontoxic polysaccharides (A antigen). The disappearance of detectable C antigen precipitation (resulting in maximum A antigen precipitation) is taken as the point of complete detoxification. The validity of this method as an assay for detoxification has been judged against several different biologic assays (24, 27, 30–32).

In all tests for detoxification, care was taken to avoid contact of the serum with air so as to prevent critical changes in pH. The dose of *S. enteritidis* endotoxin (0.02 ml volume) was added to the bottom of small narrow tubes after which the test serum was added and the mixture immediately covered with mineral oil. No buffers or cation-binding agents were added.

The proportions of endotoxin to serum were 25 μg/0.4 ml in the immunodiffusion assay and 1 μg/0.1 ml in the biologic assay. In the former assay, samples of 0.1 ml were removed from incubating mixtures at 10, 30, and 60 min intervals and placed in an ice bath. An appropriate amount of each serum-endotoxin mixture (0.035 ml) was put into agar wells and diffused against the anti-*S. enteritidis* serum. The precipitation patterns were developed in a moist atmosphere at room temperature for a period of 36 hr. The agar patterns were then washed for 24 hr in a constantly stirred bath of buffered saline, rapidly dried, and stained (32, 34).

Biologic Assay For Detoxification—For the biologic assay of detoxification, two pools of serum were prepared from pre- and 5 hr postendotoxin bleedings of three rabbits. 6 μg of *S. enteritidis* endotoxin were incubated at 37°C (under oil) with 0.6 ml of each of the serum pools. Samples of 0.13 ml were removed from the incubating mixtures after 10, 30, 60, and 120 min and immediately diluted in 2.9 ml of cold saline. An equal volume (3 ml) of actinomycin D in saline was added to each sample before injection into common white mice weighing 27–30 g. Each animal received by intraperitoneal route 0.1 μg of endotoxin (5 LD_{75} doses) and 25 μg of actinomycin D in a volume of 0.5 ml. Deaths were recorded during a 36 hr period. Control groups of mice received 1 LD_{75} dose of endotoxin in saline plus actinomycin D or actinomycin D alone.

Serum Esterase Determination—Esterase levels were determined on pre- and postendotoxin serum samples which had been heated at 60°C for 25 min. Undiluted heated serum (0.12 ml) was added to standard cuvettes; this was followed by 2.5 ml of a saturated, filtered solution of betanephthyl acetate in 0.1 M phosphate buffer (pH 7.4). Diisopropylphosphofluoridate (DFP)[1] at a final concentration of 2×10^{-4} M was present in the substrate solution. The release of betanaphthol was measured spectrophotometrically at 328 mμ. Generally, zero order kinetics prevailed during 3–9 min of incubation at room temperature and the data given in Fig. 7 was compiled from the 3 to 6 min interval.

Acrylamide Disc Electrophoresis—Acrylamide gel electrophoresis (19) was carried out with 100 μl samples of pre- and 5 hr postendotoxin sera. Both fresh and heated (60°C for 25 min) samples were run at pH 8.4 during 105 min at 300 v, after which the gels were removed from the glass tubing. Bands with esterase activity were identified by incubating the gels for 40 min in saturated substrate with DFP, prepared as described above. Gels were rinsed free of substrate and immersed in a solution of diazo blue for 5 min to develop the blue-violet azo dye complex. After a final rinse, the gels were immersed in a solution of 3% acetic acid.

RESULTS

Calcium Ion Shift in Postendotoxin Serum—Previous results, showing that endotoxin-lipoprotein (esterase) complexes form in the circulating plasma of experimental animals implied that ionized calcium levels decrease following endotoxin administration (33).

To establish this point, experiments were designed to measure serum Ca^{++} at several time intervals after injection of endotoxin. These measurements showed that after the 1st hr postendotoxin a gradual decrease in $[Ca^{++}]$ occurred (Fig. 1). The maximum postendotoxin decrements in $[Ca^{++}]$ for individual animals ranged from -0.9 mv to -2.1 mv with an average maximum decrease of -1.3 mv which represents a 10 or 12% drop in ionized calcium

[1] Abbreviations used in this paper: DFP, diisopropylphosphofluoridate; RES, reticuloendothelial system.

(17). These decrements are expressed in millivolts since only relative changes were sought. However, by interpolation from a standard calcium ion curve, the average maximum decrease represents a downward shift from 1.30×10^{-3} to 0.95×10^{-3} M Ca^{++}. No measurements were made between 6 and 20 hr postendotoxin but in 9 of the 13 rabbits, $[Ca^{++}]$ was measured on the following day, i.e., 22–24 hr postendotoxin. By this time the ionized calcium had returned to preendotoxin levels.

Detoxifying Capacity of Postendotoxin Serum—According to earlier in vitro studies (25, 29), a decrease in serum Ca^{++} produces an increase in the rate of detoxification of endotoxin. Therefore, the observed in vivo calcium ion de-

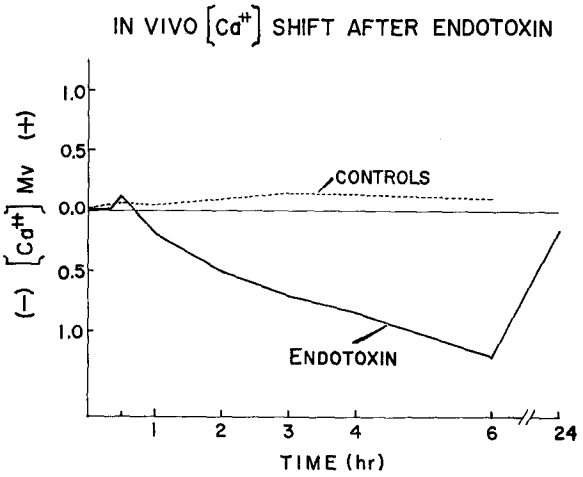

FIG. 1. Average decrease in ionized calcium levels in serum following intravenous administration of 100 μg/kg *E. coli* endotoxin to 13 rabbits. The control group of seven rabbits received sterile physiologic saline.

crease should signify an increase in the endotoxin-detoxifying rate of postendotoxin serum. A time study was carried out to demonstrate this point utilizing the immunodiffusion assay to measure detoxification. From Fig. 2 it is seen that detoxification was incomplete throughout a 60 min incubation period in both pre- and 2 hr postendotoxin sera. Weak precipitation of C antigen was apparent in 4 hr postendotoxin serum after a 10 min incubation (arrow) but detoxification was complete after 30 or 60 min, as judged by the absence of C antigen and by the maximum precipitation of A antigen. Detoxification was complete within 10 min in the 5 hr postendotoxin serum.

This result is in good agreement with the apparent detoxification rate observed in vivo in the circulating plasma of mice which have received ^{51}Cr-labeled endotoxin (Fig. 3). In this immunodiffusion pattern it is seen that little change

is evident in the amount of labeled, slow diffusing C antigen at 10 min to 1 hr postendotoxin, but that at 3 and 5 hr postendotoxin the amount of precipitable C antigen all but disappears. This apparent increase in the in vivo rate of detoxification between 1 and 3 hr postendotoxin was also observed in rabbits

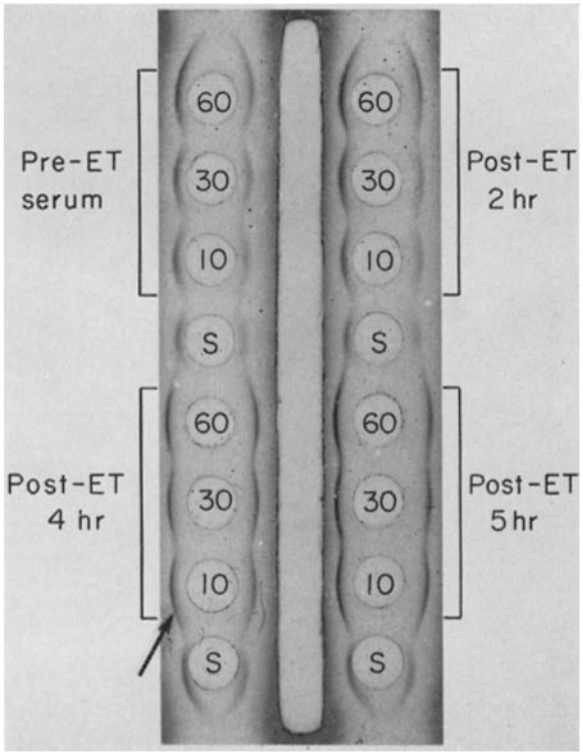

FIG. 2. A time study of the in vitro detoxification of endotoxin by pre- and postendotoxin serum. *S. enteritidis* endotoxin (25 μg) was incubated under oil for 10, 30, or 60 min with 0.4 ml of each serum sample. Each well in the immunodiffusion pattern received 0.035 ml of the indicated incubation mixture containing 2 μg of endotoxin; wells labeled *S* received 2 μg of endotoxin incubated in buffered saline (pH 7.4) for 10 or 60 min. Anti-*S. enteritidis* serum was put into the elongate troughs in this and all other immunodiffusion systems. The agar patterns were washed, dried, and stained with azocarmine (32).

but the proportion of endotoxin to total plasma volume in this species made it difficult to obtain well marked radioautographs.

Pre- and 5 hr postendotoxin serum pools were prepared from a group of three rabbits in order to compare the detoxifying capacity of each pool by both immunodiffusion and biologic assay. The immunodiffusion pattern in Fig. 4 showed that detoxification by postendotoxin serum was achieved within 10

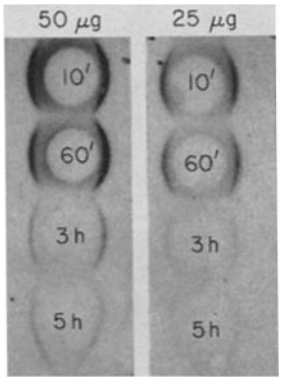

FIG. 3. Radioautographs of immunodiffusion patterns showing the rate of disappearance of ^{51}Cr-labeled C antigen precipitation from the circulating plasma of mice that received by intravenous injection either 25 or 50 μg of ^{51}Cr–*S. enteritidis* endotoxin. Details of the methods employed for labeling of endotoxin, collection of plasma, and preparation of radioautographs have been published elsewhere (5). Each of the two patterns represents a separate experiment in which plasma pools from groups of five mice were collected at the indicated time intervals postendotoxin. Each well received 0.2 ml aliquots of heparinized plasma; anti-endotoxin serum was added to troughs on both sides of the circular wells.

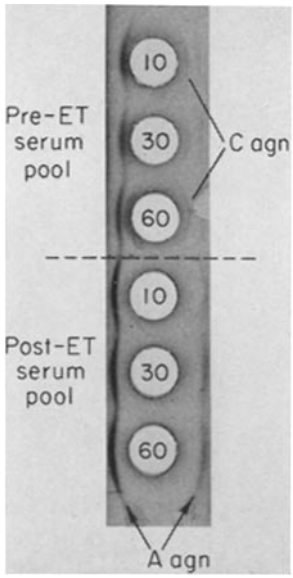

FIG. 4. Immunoprecipitation of *S. enteritidis* endotoxin following anaerobic incubation with pooled rabbit sera collected before and 5 hr after administration of *E. coli* endotoxin. Numbers in wells denote in vitro periods (in minutes) of incubation. The proportion of endotoxin to serum in this and all subsequent patterns were as given in Fig. 2, i.e., 2 μg endotoxin in each well.

min while the pre-endotoxin pool produced only a partial detoxification after 60 min of incubation. This difference in rate of detoxification was more strikingly demonstrated in the biologic assay (Table I). Whereas the immunodiffusion method revealed a greater than sixfold increase in the detoxifying rate

TABLE I

Detoxifying Capacity of Pre- and 5 hr Post-Endotoxin Serum*

Group	Incubation time	Incubation of *S. enteritidis* endotoxin‡ in		
		Preendotoxin serum	Post endotoxin serum	saline
	min			
1	10	8/10	0/10	—
2	30	7/10	0/10	—
3	60	7/10	0/10	—
4	120	3/10	0/10	—
5	120	—	—	10/13§

An additional control group of 10 mice received 25 μg of actinomycin D alone; no deaths occurred.

* No. of animals dead/total No. injected.

‡ Each mouse in groups 1–4 received 0.1 μg of endotoxin (equivalent to 5 LD_{75} doses) plus 25 μg of actinomycin D.

§ Endotoxin control group: each mouse received 0.02 μg of endotoxin (1 LD_{75} dose) plus 25 μg of actinomycin D.

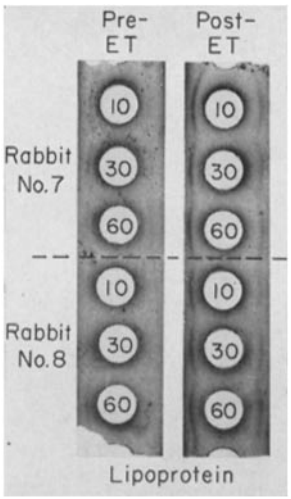

FIG. 5. Sudan black staining of immunoprecipitation pattern (33) to demonstrate the complexing of endotoxin with lipoprotein during in vitro incubation with rabbit serum collected before and 5 hr after injection of *E. coli* endotoxin. The bands of precipitation are stained deep blue.

of postendotoxin serum, the more sensitive biologic method showed this difference to be greater than twelvefold. The effect of a smaller initial dose of endotoxin (10 μg/kg) on the detoxifying capacity of postendotoxin serum was examined in a group of six normal rabbits. In five of the animals an important but somewhat less striking increase in detoxifying rate was obtained in serum collected 5 hr postendotoxin, detoxification being complete within 30 (3/5) or 60 min (2/5).

Endotoxin-Lipoprotein (Esterase) Complex in Postendotoxin Serum—The in vivo interaction of endotoxin with a plasma lipoprotein (esterase) has been

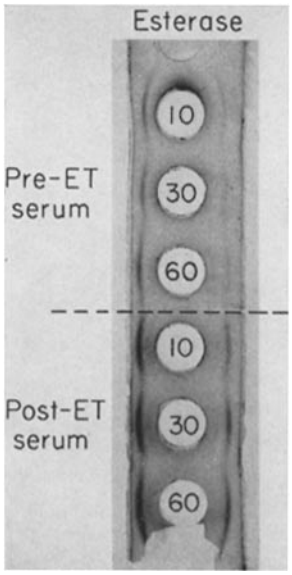

FIG. 6. Demonstration of esterase activity on precipitated endotoxin (33) following in vitro incubation with pre- and 5 hr postendotoxin sera. The precipitation bands are colored violet-blue by the formation of the azo dye complex.

shown to increase in intensity during a 6 hr period following endotoxin administration (33). The immunodiffusion patterns in Figs. 5 and 6 show that this interaction was also intensified upon incubation of endotoxin in vitro in 5 hr postendotoxin serum.

It was first thought that the increased rate of detoxification of endotoxin by postendotoxin serum was due solely to reduced [Ca^{++}]. To determine if this was so, the concentration of Ca^{++} in preendotoxin serum was reduced with sodium citrate to the same level as that attained in vivo in 5 hr postendotoxin serum, i.e., to give a decrement of -1.3 mv with the calcium ion electrode. Endotoxin was incubated with this serum as described in the Materials and Methods section and although the rate of detoxification was increased, it did

not approach the rate observed in 5 hr postendotoxin serum. The addition of greater amounts of citrate to preendotoxin serum further increased the detoxifying rate but not to the degree attained in postendotoxin serum. This implied that in addition to reduced [Ca^{++}], something more was required to account for the very rapid rate of detoxification observed in serum collected 5 hr postendotoxin.

Increase in Esterase Levels in Postendotoxin Serum—A series of experiments was done to determine changes in serum esterase levels after injection of

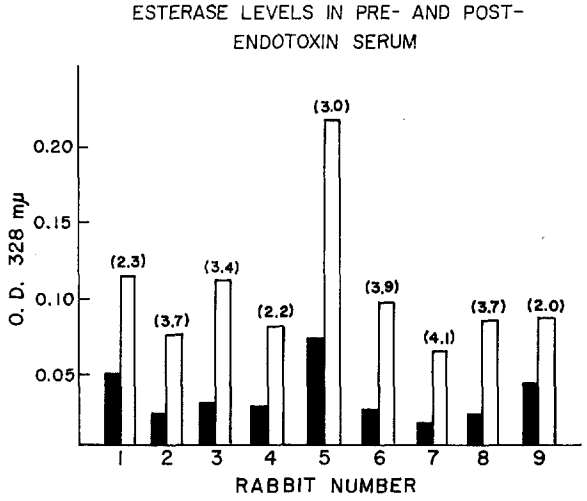

FIG. 7. Heat-stable, DFP-resistant esterase levels in pre- and postendotoxin serum. All serum samples were heated 25 min at 60°C prior to measurement of esterase activity in 0.12 ml of undiluted serum. The final concentration of DFP was 2×10^{4} M.

Black bars indicate esterase levels in preendotoxin serum. White bars indicate esterase levels in postendotoxin serum. Figures in parentheses denote relative increases in postendotoxin esterase levels in individual rabbits; the average postendotoxin increase was 3.1 times the preendotoxin level.

endotoxin. Fresh samples of pre- and 5 hr postendotoxin sera were diluted 1:20 with saline and 0.18 ml amounts were incubated with betanaphthyl acetate in the absence and presence of DFP (2×10^{-4} M). Not surprisingly, these tests showed no significant differences in esterase levels in pre- and postendotoxin serum samples. Sera were then heated at 60°C for 25 min and tested undiluted for esterase activity in the presence of DFP. The results of these tests are shown in Fig. 7 and it is seen that a threefold increase in the heat-stable, DFP-resistant esterase level takes place in postendotoxin sera. A few rabbits were bled 1 1/2 hr postendotoxin and their sera tested for heat-stable esterase activity but no increases were apparent at this time.

Acrylamide disc electrophoresis of heated samples of pre- and postendotoxin sera revealed that most of the increased esterase (DFP-resistant) was associated with the albumin–alpha$_1$ globulin region of postendotoxin serum (Fig. 8). However, attempts to demonstrate an increase in the alpha$_1$ lipoprotein (esterase) which migrates in the same region were not successful since the limited uptake of fat dyes in this gel medium did not allow for meaningful comparisons between pre- and postendotoxin sera. Acrylamide gel electrophoresis of fresh sera did

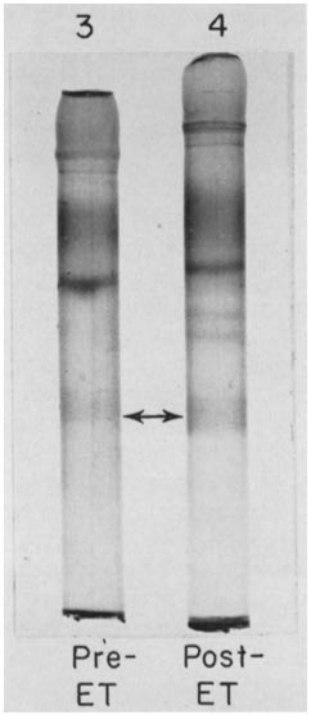

FIG. 8. Acrylamide disc electrophoresis of rabbit serum showing increase in heat-stable, DFP-resistant esterase in the albumin–alpha$_1$ globulin region (arrows) in 5 hr postendotoxin serum.

not indicate any significant increases in heat-labile, DFP-resistant esterase activity in the albumin–alpha globulin region of postendotoxin serum.

DISCUSSION

Most efforts to determine the in vivo localization of endotoxins and, by inference, tissue sites of detoxification are based on the distribution of radioactive labels following the parenteral injection of labeled endotoxins. These and other studies utilizing the fluorescent antibody technique have led to the

general view that detoxification takes place in cells of the reticuloendothelial system (RES). However, since none of these methods alone is capable of distinguishing between toxic and nontoxic moieties of endotoxic preparations, such results cannot be properly evaluated. The finding of nontoxic polysaccharide fragments of endotoxin in the urine of animals receiving sublethal doses (5), implies that the in vivo distribution of nontoxic moieties is ubiquitous and surely not indicative of actual sites of detoxification.

More definitive data on the in vivo distribution of sublethal doses of endotoxin have been obtained with a ^{51}chromate-labeled endotoxin studied in conjunction with immunologic and biologic tests (5, 6, 31). These results showed that whereas the presence of label and immunoprecipitability of endotoxin were affirmed in several sites, toxicity could be demonstrated only in the circulating plasma. It was also established that modest doses of endotoxin are not cleared rapidly from the circulation but that they continue to circulate for at least 6 hr in toxic form and for at least 24 hr as nontoxic moieties. This observation, in itself, provided a strong argument against an important role for the RES in the uptake and detoxification of circulating endotoxins. Rather, the observed persistence of endotoxin in plasma suggested that detoxification takes place in the vascular compartment. Immunodiffusion patterns of plasma samples taken from animals subsequent to injection of endotoxin (5) have shown that there occurs in the circulating plasma a gradual disappearance of the large, toxic molecules (C antigen) and an appearance of fast diffusing, nontoxic fragments (A antigen). This same transformation also had been observed during incubation of endotoxin with serum or plasma in vitro but only under conditions which resulted in detoxification (30, 31). These observations gave impetus to the view that the humoral system represented an important host mechanism of defense against endotoxemia and prompted efforts to identify the enzyme(s) concerned.

The fractionation of human serum on DEAE cellulose resulted in the separation of an alpha globulin fraction which could degrade but not detoxify the large endotoxin molecules (31). The degradation or disaggregation gave rise to a preparation which was more toxic than the original undegraded endotoxin. The alpha globulin fraction was stable to heating and its degrading action was inhibited by calcium; the observation of which led to the definition of detoxification as a two-stage reaction, initial degradation followed by detoxification of exposed toxic groups.

Further chromatographic work led to the separation of a potent detoxifying fraction (32) containing two alpha$_1$ globulins shown to interact with endotoxin: a lipoprotein which was responsible for initial degradation and a second heat-labile globulin presumed to be responsible for eventual detoxification. Both proteins exhibited esterase activity of the nonspecific or organophosphate-resistant type. It was found that the Ca^{++} inhibition of detoxification was due to a blocking of the initial interaction between endotoxin and the alpha$_1$ lipoprotein (esterase), thus preventing degradation. The subsequent demonstration that endotoxin-lipoprotein (esterase) complexes formed in the circulating plasma of experimental animals (33) furnished indirect evidence that ionized calcium decreases in the endotoxemic host and that the humoral system can be activated in vivo.

The present data provide direct evidence of a downward shift in [Ca^{++}] which develops gradually during the first few hours following endotoxin administration. The host mechanism responsible for the observed decrease in [Ca^{++}] in postendotoxin serum is not known. However, it is likely that Ca^{++} binding results from increases in plasma lactate and phosphate as well as from increases in lipoproteins; all have been reported to increase within a few hours after exposure to endotoxic products (10, 12, 13, 15, 16).

The detoxification studies on serum samples taken from rabbits before and subsequent to endotoxin administration clearly demonstrated the development of a marked increase in the detoxifying capacity of the circulating plasma. The time study (Fig. 2) showed that little change took place in the rate of detoxification during the first 1–2 hr postendotoxin but that the rate increased markedly thereafter and paralleled the downward shift in ionized calcium. The timing of this change in rate of detoxification was also observed in vivo (Fig. 3 and reference 5) as judged by the marked reduction in C antigen precipitation which occurred in circulating plasma after the 1st hr postendotoxin. The increase in the rate of detoxification witnessed by immunodiffusion assay was observed more strikingly in the biologic assay which indicated a greater than twelvefold increase in the detoxifying rate of 5 hr postendotoxin serum. The data also demonstrated that the interaction of endotoxin with the lipoprotein (esterase) was much more intense in 5 hr postendotoxin serum than in preendotoxin samples. This would be expected in circumstances where increased detoxification takes place (32) and is in accord with in vivo observations showing a gradual intensification in the interaction of endotoxin with lipoprotein (esterase) during a 6 hr postendotoxin period (33).

The enhanced detoxifying capacity of postendotoxin serum was not due to reduced [Ca^{++}] alone. A significant increase in the heat-stable, organophosphate-resistant esterase level most probably contributed to this enhanced capability. Attempts to demonstrate that increased esterase activity was associated with an increase in the $alpha_1$ lipoprotein (esterase) were only partly successful. In acrylamide gel electrophoresis, the esterase zone in the albumin–$alpha_1$ globulin region was stronger in postendotoxin serum but lipoprotein staining in the gel medium was too weak to permit a conclusive statement. However, such an increase could be anticipated in view of the above mentioned reports of lipidemia and lipoproteinemia following exposure to endotoxins. The finding of no significant increases in heat-labile, DFP-resistant esterase zones in postendotoxin serum made it appear that the detoxifying esterase level did not change. These points require further investigation, however, before it can be established that the enhanced detoxifying capacity of postendotoxin serum is due only to decreased [Ca^{++}] and increased levels of endotoxin-degrading lipoprotein (esterase).

The in vitro detoxification studies were designed to approach as closely as

possible in vivo conditions. Blood samples were kept from contact with air to prevent changes in pH and only fresh, undiluted serum was employed. Except for the small volume of endotoxin, no other agents or buffers were added to the serum samples before or during incubation. Only the fact that serum rather than whole plasma was used in the detoxification experiments made the in vitro conditions significantly different from those in an in vivo milieu. This difference is probably of little consequence in these experiments since we had shown previously that the clotting mechanism, per se, has no effect on the detoxifying capacity of plasma (25).

The present results, together with previous observations (5, 31–33), provide firm evidence of the functional importance of the humoral detoxifying system in host defense against endotoxemia. The increase in detoxifying capacity of postendotoxin serum coincides in timing, both in vitro and in vivo, with the gradual (*a*) drop in [Ca^{++}], (*b*) increase in heat-stable, organophosphate-resistant esterase level, (*c*) intensification of the endotoxin-lipoprotein (esterase) complex, (*d*) loss of C antigen precipitability and toxicity as determined by immunodiffusion and biologic assays. The activation of this humoral mechanism in rabbits receiving modest, sublethal doses of endotoxin, requires 4–5 hr, at which point the circulating plasma becomes a very potent medium for the detoxification of endotoxins. The obvious implications of the present findings to the endotoxin-tolerant state will be considered in a subsequent report.

Plasma enzymes other than the organophosphate-resistant, carboxylic esterases have not been implicated in the detoxification of endotoxin. Evidence to date has shown that neither hemolytic complement (29) nor serum lipase (28) contribute to the detoxification reaction. My own experience with a large number of purified and semi-purified preparations of esterolytic enzymes has shown none to be capable of reducing the toxicity of endotoxins. These included several different preparations of lipases, phosphatases, and lysozymes (esterase-contaminated) as well as cholinesterase, phospholipase, phosphodiesterase, trypsin, chymotrypsin, papain, and pronase.

Reports have appeared purporting to show that the plasma-inactivation of the lethal action of endotoxin is reversible and nonenzymatic in nature (20, 26). The evidence for this was derived from experiments in which the detoxifying capacity of plasma was exceeded by the large doses of endotoxin employed. Therefore, it is probable that a significant portion of the endotoxin is not detoxified but only bound reversibly to plasma proteins in a nonenzymatic interaction. It is unlikely that reversible inactivation or, more appropriately, reversible neutralization will be observed if the dose of endotoxin per milliliter of plasma does not exceed 50 μg, the maximum detoxifying capacity of mammalian plasma in vitro under the best of circumstances.[2]

Although the reticuloendothelial system has been widely implicated as the system of major importance in the uptake and detoxification of circulating endotoxins, direct evidence in support of this view is lacking. It is reasonable to assume that in normal

[2] Skarnes, R. C. Unpublished data.

circumstances the RES is quite able to remove and to detoxify the very small quantities of free endotoxins which may reach the general circulation from the gut or via bacterial cells during transient low-level bacteremias. In special circumstances, however, it has been shown that cells of the RES are capable of both rapid uptake and detoxification of substantial amounts of endotoxins. The studies of Fine and his coworkers (21, 27, 36) established that denervation of RES organs, induced by surgery or anesthesia, will allow for a greater participation of the liver and spleen in detoxification and will protect animals from the lethal action of endotoxins. This is most probably accomplished by prevention of vasoconstriction and the resultant maintenance of normal blood passage through RES tissues. This interpretation receives support from the observation that endotoxin disappears more quickly from the circulation of animals with denervated RES tissues (24). In nonoperated animals, however, endotoxins given parenterally are excluded in large part from the RES and remain in the circulating plasma for many hours (5, 24, 31, 33).

The rapid clearance from the blood of endotoxin–antibody complexes formed in vitro (7) may well be due to a reduced vasoconstrictive action of the complexed toxin. Likewise, the reversibly neutralized endotoxin–serum protein complexes (20, 26) may be less vasoactive than free endotoxins and largely removed by the RES. This then would have the effect of broadening the defensive capability of the host by making cells of the RES available for detoxification. It is emphasized that these are special instances of RES participation which probably have little relevance to the usual type of experimental endotoxemia or to endotoxemias which may develop during Gram-negative infections. Intracellular detoxification of such complexed endotoxins as well as of endotoxins bound to bacterial cells may well be accomplished by organophosphate-resistant esterases like the one identified in spleen tissue (34).

In the present study, as in the vast majority of other studies, endotoxemia was induced by the injection of endotoxin freed from the bacterial cell. The equivalent to this degree of endotoxemia in natural infections would most probably be encountered in animals with a developing bacteremia, in which case the failure of the RES-clearing mechanism can be expected to lead to an increasing endotoxemia. Pronounced interference with the uptake of colloids by the liver following injection of endotoxins has been reported by several investigators (1, 3, 14, 18) and as little as 10 μg of endotoxin in rabbits can effectively interfere with RES function for many hours (2). Consequently, a persisting bacteremia may well lead to an endotoxemia which is of sufficient magnitude to exclude the RES from participation in detoxification. The main burden of defense would then be shifted to the humoral phase, resulting in activation of the plasma-detoxifying system in the manner described above. In the present report, activation of the plasma-detoxifying enzymes was induced in rabbits by the injection of 100 μg of endotoxin per kg but even as little as 10 μg per kg evoked a substantial increase in the detoxifying capacity of 5 hr postendotoxin serum.

The treatment of animals with chemical or biological agents which increase

sensitivity to endotoxins and interfere with granulopectic activity of the RES (1) or which alter the detoxifying potential of extracts of liver (9, 11) has been done with the aim of implicating the RES in host defense against endotoxins. Interpretations of results based on such experiments deserve reconsideration in terms of the effect of these agents on the plasma detoxifying system. For example, a plasma concentration of 0.075% thorium dioxide (Thorotrast, Fellows Medical Manufacturing Co., Inc, Detroit, Mich.) which is only one-tenth of the dose required to blockade the RES will completely inhibit the plasma esterases which detoxify endotoxin.[2] It is likely that some of the reported discrepancies between enhanced RES activity and increased susceptibility to endotoxins (1, 8, 35) will be explained by an induced deficiency in plasma-detoxifying capacity brought about by the particular agent employed to stimulate RES function.

SUMMARY

The present study defines the early response of normal rabbits to the intravenous injection of a single, sublethal dose of endotoxin. Within the first few hours following endotoxin there occurs in the circulating plasma of recipients a decrease in ionized calcium, a threefold increase in the heat-stable, organophosphate-resistant esterase level, and a striking increase in the endotoxin-detoxifying capacity. These results are fully consistent with the thesis that circulating plasma represents a principal site of detoxification and that plasma esterases of the nonspecific, carboxylic type are of major concern in defense against circulating endotoxins.

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