# Endotoxin Inactivating Activity of Rat Serum (42239)

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*Abstract.* The ability of rat serum to inactivate endotoxin (LPS) was assessed with the aid of the limulus amebocyte lysate assay. Following the addition of various amounts of endotoxin to normal serum the mixture was incubated for 1 hr at 37°C and the residual endotoxin activity determined. One milliliter of rat serum inactivated between 5 and 10 μg *Escherichia coli* LPS per hour. Heating serum for 45 min at 56°C resulted in loss of 80–90% of the LPS inhibitor (LPSI) activity. Serum from cobra venom factor (CVF)-treated rats inactivated between 0.5 and 2.5 μg LPS/ml serum. Serum from tolerant rats, even after heating for 45 min at 56°C, inactivates between 10 and 15 μg LPS/ml serum/hr; decomplemented tolerant rat serum neutralizes between 5 and 10 μg LPS/ml serum/hr. Clearly, the tolerant rat has large quantities of LPSI activity, which does not appear to be complement. The inhibitor found in tolerant rat serum is not species specific since it inactivates *Salmonella minnesota* and *Salmonella typhimurium* endotoxins to the same degree and in the same amount as *E. coli* endotoxin, the agent used to induce tolerance. Both heating serum (56°C) and lead acetate reduce LPSI activity. 

Minute amounts of endotoxin (lipopolysaccharide or LPS) escape from the gut (1, 2). Apparently these are sufficiently large enough to amplify immune responses elicited during acute hepatic injury (3–5) and to induce intravascular clotting (6–10) and possibly other changes also. These abnormalities associated with liver injury are thought to occur because the liver, which plays a major role in eliminating endotoxin from the circulation and in its subsequent detoxification (11, 12), fails to function properly. Hence endotoxins of enteric origin and other gut-derived antigens are expected to be found circulating in patients with advanced liver diseases (13). And, indeed, elevated antibody titers against gut-derived gram-negative organisms are found to occur in patients with liver cirrhosis (5, 14, 15), while circulating endotoxin has been demonstrated in their peripheral blood (16–22).

More recently, however, the presence of endotoxin in the blood of patients with liver cirrhosis has been difficult to substantiate (23–25) thus raising the issue of the reliability of either the observation or of the limulus amebocyte lysate test, the assay used to detect endotoxin. False negative test results, were they to occur, however, might also possibly result from the activity of an endotoxin inactivator in plasma or serum (LPSI or lipopolysaccharide inactivator). Such activity has indeed been shown to exist by several investigators (26, 28–42). Since these studies have provided conflicting results and because no data concerning any concrete quantitative aspects of this phenomenon have so far been presented, this subject was reevaluated.

**Materials and Methods.** *Animals.* Sprague–Dawley rats weighing 250–300 g were used for the reported studies. The animals were maintained on standard rat chow and water in a controlled environment.

Rats were made tolerant by intraperitoneal injection of 0.5 mg of *Escherichia coli* endotoxin on 3 consecutive days. Animals were rendered complement (C3–C9) deficient by intraperitoneal administration on 3 consecutive days of 100 units cobra venom factor (CVF; Cordis Laboratory, Miami, Fla.). Previously, this procedure was shown to result in complete loss of C3–C9 activity in the rat (26).

Reversed Eck fistulas were surgically constructed as previously described (27).

*Collection of blood samples.* Blood was collected by cardiac or aortic puncture from treated and untreated animals under sterile



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conditions, the former 24 hr after their last injection. The blood samples were placed under sterile pyrogen-free mineral oil (Beco Chemical Co., Compton, Calif.) and allowed to clot on ice for 1 hr. The serum, obtained by centrifugation, was stored under mineral oil at 4°C for subsequent LPSI assay. The mineral oil used was sterilized by Millipore filtration (Millipore, 0.45 μm, Bedford, Mass.). Unused serum was stored in pyrogen-free 16 × 25-mm plastic capped culture tubes (Falcon, Becton–Dickenson, Oxnard, Calif.) at −80°C for 2 weeks, to assess the stability of the inhibitor during storage.

Blood from reversed Eck fistula (REF) rats was collected from the abdominal aorta under sterile conditions 24 hr after the shunt was established.

*Endotoxin.* The commercially available 055:135 and 0111:B4 *E. coli* lipopolysaccharide (Difco Lab., Detroit, Mich.) was used for all described experiments. It was added to serum samples in 0.1 ml pyrogen-free saline in concentrations ranging from 0 to 10 μg. The actual concentrations used were 0–0.5, 5–10, 100, and 500 ng and 1, 2, 5, and 10 μg per 0.1 ml.

Sera from tolerant animals were also investigated for their ability to inactivate lipopolysaccharide W. *Salmonella minnesota* 9700 and lipopolysaccharide W. *Salmonella typhimurium.* All endotoxin preparations were obtained from Difco Laboratories, Detroit, Michigan.

*LPSI assay.* An entire LPSI assay was performed in a pyrogen-free 16 × 25-mm plastic capped culture tube (Falcon, Becton–Dickenson). To 0.2 ml serum was added 0.1 ml endotoxin in concentrations ranging from 0 to 5 μg. The mixture was placed on a rocker platform (Bellco Glass, Inc., Vineland, N.J.) in a 37°C incubator for 60 min. The test solution was then diluted with pyrogen-free water, 0.4 ml/tube, and boiled for 10 min (28). Of the test solution 0.2 ml was transferred with a pyrogen-free tuberculin syringe to an LAL vial mixed and incubated undisturbed at 37°C for 1 hr. The results were subsequently read after 1 hr.

*Lead acetate.* Pb · acetate · $3H_2O$ (MCB Reagents, Norwood, Ohio) was used in the endotoxin assay in concentrations of 0.02 mg/0.1 ml pyrogen-free water, added to the reaction mixture prior to incubation.

*Limulus lysate assay.* Limulus amebocyte lysate, obtained from Microbiological Associates, Walkersville, Maryland, was used to assay for endotoxin activity. All materials used in these experiments were tested for pyrogenicity. Sensitivity of the LAL test for 055:B5 *E. coli* LPS (Difco) for our lab was 0.125 ng/mg.

A firm gel that did not break on inverting the vial was considered a positive result. Control experiments with standard endotoxin solutions and with pyrogen-free water were performed with each experiment.

*Conversion factor.* Because 0.2 ml of serum was used in the different tests, the endotoxin concentration per milliliter of serum is obtained by multiplying the observed value by a factor of 5.

**Results.** Experiments performed following addition of endotoxin to rat serum and boiling the mixture without prior incubation provided positive results with even the smallest amount of endotoxin used in these experiments, 0.5 ng. Using sera from tolerant rats no endotoxin could be demonstrated.

Control rat sera inactivated between 1 and 2 μg LPS per test or between 5 and 10 μg/ml serum. Serum from CVF-treated rats neutralized between 100 and 500 ng LPS per test or 0.5–2.5 μg/ml serum (Table I). Control serum heated for 45 min at 56°C inactivated between 50 ng and 0.5 μg/ml. Tolerant, heated sera inactivated both *S. minnesota* or *S. typhimurium* endotoxin in amounts similar to those found for *E. coli* endotoxin, the material used to induce tolerance, namely, between 5 and 10 μg/ml serum.

Heated sera obtained from reversed Eck fistula rats inactivated the same amount of LPS as heated normal rat sera.

Lead acetate, which did not coagulate limulus lysate in the concentrations used in the absence of endotoxin, inhibited the inactivation of endotoxin by serum from normal as well as from tolerant rats (Table I). Sera, even those stored at minus 80°C, gradually lost their inhibitor activity. The rate at which this occurs remains to be determined.

**Discussion.** A number of investigators have demonstrated inhibitors for endotoxin in blood and serum. A reversible inhibitor was previously described by several laboratories (29–31). It is eliminated by diluting serum, by chloroform extraction (Levin *et al.* (29)), upon

TABLE I. AMOUNTS OF ENDOTOXIN INACTIVATED BY DIFFERENT SERA AS DETERMINED BY LAL TECHNIQUE

| Amount of LPS(*E. coli* 055:B5) added to serum | Normal rat serum | | | | Tolerant rat serum | | | | | | CVF Rx | | REF rat serum | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | W/O treat | 56°C 45 min | 56°C 60 min | Pb | W/O treat | 56°C 45 min | 56°C 60 min | Pb | 56°C 45′ + Pb | CVF treat | W/O treat | Pb | W/O treat | 56°C 45 min | 56°C 60 min | Pb |
| 10 μg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 5 μg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 μg | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 2 μg | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + | + |
| 1 μg | − | + | + | + | − | − | + | + | + | − | + | + | − | + | + | + |
| 500 ng | − | + | + | + | − | − | + | + | + | − | + | + | − | + | + | + |
| 100 ng | − | + | + | + | − | − | + | − | + | − | − | + | − | + | + | + |
| 10 ng | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 5 ng | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 0.5 ng | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 0 ng | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |

*Note.* Each column represents 10 animals. W/O: without treatment; 56°C 45 min: heated for 45 min at 56°C; 56°C 60 min: heated for 60 min at 56°C; CVF treat: CVF-treated tolerant rat serum; Pb: Pb acetate add to serum; 56°C 45′ + Pb: Pb acetate add to heated (56°C 45 min) tolerant rat serum; CVF Rx: CVF (cobra venom factor)-treated rat serum.

the addition of polyglucosesulfate (Oroszlan *et al.* (30)), or by boiling serum for 10 min (Cooperstock *et al.* (28)).

Irreversible inhibitors or humoral detoxifying mechanisms in blood have also been described (29–31) which reduce endotoxin lethality, tumor necrotizing properties, and the Shwarzman reaction. Furthermore, the results of several studies indicate that serum can alter the antigenic characteristics of endotoxins (32–34). A number of studies have characterized the active factor. Skarnes noted that the serum inactivator is heat labile, is suppressed by $Ca^{2+}$ ions, acts at a very slow rate, and is a lipoprotein with esterase activity (37, 38). Schultz and Becker failed to detect any detoxifying activity in normal serum but observed it in plasma of heparinized animals. From this they concluded that lipoprotein lipase exerts the endotoxin detoxifying activity (39). Since the development of the LAL assay, Johnson and Ward *et al.* isolated an LPS inactivator from normal human blood. The substance occurs in the α-globulin fraction and has a sedimentation velocity of approximately 4.5 S. It is neither a lipoprotein nor a serine esterase (40).

With the introduction of the limulus amebocyte lysate test it has become possible to demonstrate endotoxin in biological fluids in minute quantities. By determining whether endotoxin, added in known amounts to different sera, remains detectable or not after 1-hr incubation at 37°C, we established that most of the endotoxin detoxifying activity of normal serum is heat labile. Incubating serum for 45 min at 56°C prior to the assay resulted in loss of more than 90% of its activity. Also, C3–C9-deficient serum has only 10–20% of the inhibitor activity suggesting that the loss of endotoxin inactivator activity following heating may well be due to loss of complement. Thus Skarnes and Rosen's finding that complement is not involved in endotoxin inactivation by serum (37) could not be confirmed.

Since considerable activity remains in decomplemented blood, complement is apparently not the only factor responsible for the endotoxin neutralization by blood or serum. These findings are difficult to reconcile with the significant protection complement found to provide against the lethal effects of endotoxin *in vivo* (29–31). However, it confirms the observations by Johnson and Ward that the inactivation of endotoxin by serum is reduced in C6-deficient animals (42). The latter study fails to specify, however, the extent of complement's participation in endotoxin inactivation by serum or plasma. In our studies, normal rat serum heated for 45 min at 56°C, to remove the complement, resulted in 80 to 90% loss of LPSI activity.

Other features of the present study also deserve emphasis. The first concerns the rapid rate of the *in vitro* inactivation of endotoxin by serum. Major endotoxin inactivation oc-

curs within 1 hr. Second, the amount of endotoxin neutralized in that short time is large. Previously Skarnes and Rosen (37, 38) suggested as much. Where we noted that the capacity of the rat liver to eliminate and detoxify endotoxin *in vivo* approximates 1.5 μg/g liver/hr (27), results of the present studies suggest that the *in vitro* serum endotoxin inactivating activity far exceeds this capacity. Moreover, this is further enhanced in tolerant animals. The inactivator found in the tolerant animal after heating is greatly reduced far in excess to the loss of complement. Because it appears to be a labile substance, even upon storage at −80°C, which is inhibited by lead, it suggests that the inhibitor is an enzyme. Whether the LPSI that remains after heating tolerant serum is identical to the activity noted in complement-deficient normal rat serum but in greatly increased amounts or an altogether different material remains to be established. Because this inhibitor is present in very large quantities, we investigated whether it inactivates endotoxins other than the one used to induce tolerance. We found it did, suggesting that we are dealing here with a universal endotoxin inhibitor. The practical, therapeutic implications of this finding are considerable. As an autologous material appearing freely in the circulation of tolerant animals, LPSI is presumably nontoxic and nonimmunologic. This is in stark contrast to polymyxin B, the only other antiendotoxic agent currently available which is a highly toxic agent whose usefulness, therefore, is severely restricted.

Certain questions raised previously that we were unable to address adequately can now be studied. For instance, the issue whether reversed Eck fistula rats (REF rats) are tolerant or not (27) could be assessed by determining whether their plasma endotoxin inactivating activity conformed to that of a normal or of a tolerant rat. Sera obtained from reversed Eck fistula rats were studied to determine whether these animals develop tolerance. We found that heated sera of REF rats inactivate the same amount of LPS as heated normal sera; hence there is no evidence to suggest that tolerance develops in this animal model. Antibodies were previously found to play no role in the development of tolerance (37, 38). Hence the likelihood that antibodies are responsible for the rise in serum endotoxin detoxifying activity in tolerant animals is remote.

If this mechanism were to be operating *in vivo* to the same degree as *in vitro,* we have to assume that very large quantities of circulating endotoxin are rapidly and irreversibly detoxified in this manner. Tolerant rat's serum is particularly effective, thus providing a protective humoral mechanism against endotoxemia in addition to, and possibly more significant than, the previously described cellular (hepatic) mechanism (26, 28–42).

Also, the loss of endotoxin inactivation following heating of serum is greater than observed in complement-deficient plasma. Contrary to Rosen, Skarnes *et al.*'s observation that complement is not involved in endotoxin inactivation by serum, we find that it is, and that, besides complement, one of several other serum components is also involved in this process.

Since the mechanism of lead sensitization to endotoxin has never been fully explained and because the inactivation of endotoxin by serum appears to be such an important process, we studied the effect of lead salts on the inactivation process. The lead used in these experiments gave a negative LAL test. Yet we found that lead in this concentration *in vivo* inhibits this process, thus allowing endotoxin to remain in the circulation unaltered. This suggests another mechanism for the lead acetate induced endotoxin enhancing effect, one not previously described, an effect far greater than that exerted on the reticuloendothelial system. The source of the LPSI remains to be established. Its presence in large quantities in sera of tolerant rats, animals in which macrophage activation is expected to take place as a result of the repeated exposure to endotoxin, might suggest major inactivator observed in this model may possibly represent a macrophage activation or secretory product.

Finally, if the endotoxin inactivation operates *in vivo* to the same degree as *in vitro,* it must be assumed that if strict rules in blood collecting and the subsequent preparation of serum are not followed that part or all of the endotoxin in blood may become rapidly undetectable with technique. This could well account for the conflicting data reported for endotoxin determination performed on blood samples obtained from patients with acute or chronic hepatic failure (16–25). Hence the proper procedures to follow to effect minimal loss of endotoxin activity in serum or blood,

besides such fundamental questions as to its source, chemical nature, mechanisms of actions, presence in other biological fluids, and clinical therapeutic implications, remains to be assessed.

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