Alterations of Bacterial Clearance Induced by Endotoxin and Tumor Necrosis Factor

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The purpose of the study was to investigate the potential influence of endotoxin and tumor necrosis factor (TNF) on immune function in terms of systemic clearance and organ distribution of injected Escherichia coli in a rabbit model. To enable quantification of the clearance process, defined numbers of exogenous E. coli (1.3 × 10⁸ CFU) were injected intravenously 60 min after bolus application of TNF (4 × 10⁶ U, n = 6), after infusion of endotoxin (40 μg/kg of body weight) for 1 h (n = 6) or 4 h (n = 6), or after saline infusion (controls, n = 6). Parameters monitored were arterial pressure, oxygen uptake, and rates of bacterial elimination from the blood. At 180 min after E. coli injection, the animals were sacrificed, and tissue samples of liver, kidney, spleen, and lung were collected for bacterial counts. Endotoxin infusion produced a significant delay in blood clearance compared with saline and TNF pretreatment. The diminished systemic bacterial elimination was associated with significantly higher numbers of E. coli in the organs, thus reflecting reticuloendothelial system dysfunction. TNF had no major influence on the elimination kinetics of bacteria but affected the tissue distribution pattern with increased accumulation of E. coli in the lung (up to 100-fold of control values; P < 0.001).

Septic shock and multiple-organ failure continue to be major causes of death in severely injured patients (5). That a transient but profound depression of the reticuloendothelial system (RES) as well as intestinal barrier dysfunction has been observed after injury or surgical trauma suggests that it plays an essential role in the pathogenesis of septic events. Recent studies (11, 33) documenting an increased permeability of the gut under trauma and shock conditions, with spreading of endotoxin and bacteria into the circulating blood and translocation into other organs, emphasized the role of gut barrier failure. The reported pathomechanisms suggest that the incidence of septic complications may be partially caused, on the one hand, by a loss of barrier function, with invasion of great amounts of bacteria and endotoxin exceeding the normal capacity of bacterial clearance, and, on the other hand, by an impairment of clearance functions possibly due to reduced phagocytosis and lysis capacity of the RES, or by a combination of both.

On the basis of the concept that endotoxin and the release of cytokines, e.g., tumor necrosis factor (TNF) from macrophages which promote the inflammatory reaction, affect the systemic immune response (26), this study was designed to investigate the influence of endotoxemia on blood and organ clearance of injected Escherichia coli. Apart from the well-known effects on intestinal barrier function (10, 13), the central question addressed is whether endotoxin directly or indirectly influences phagocytosis and lysis capacity of the RES, thus enhancing bacterial growth in blood and tissues. Since TNF has been shown to be of critical importance in the development of septic shock in animals and humans challenged with endotoxin, further interest was focused on the relative role of TNF in mediating septic complications due to possible alterations of bacterial elimination. To simulate bacterial invasion from various compartments, such as the gut, the urogenital tract, wounds, or implanted catheters, into the circulating blood and to enable quantification of the clearance process, defined numbers of exogenous E. coli (1.3 × 10⁸ CFU) were injected intravenously after pretreatment with endotoxin (40 μg/kg/h) and TNF (4 × 10⁵ U). The elimination kinetics of exogenous E. coli from the blood and their tissue distribution in the liver, spleen, kidney, and lung were studied.

MATERIALS AND METHODS

Rabbit model. Standard-breed rabbits of either sex weighing between 2,900 and 3,300 g were anesthetized with pentobarbitral (60 to 80 mg/kg of body weight) and anticoagulated with heparin-sodium (1,000 U/kg of body weight) injected into an ear vein catheter. The animals were placed in a supine position on a tempered preparation table. After aseptic placement of a tracheostomy tube, the rabbits were mechanically ventilated with room air (tidal volume, 30 ml; frequency, 30 volumes per min) by means of a Starling pump (B. Braun, Melsungen, Germany) during the whole observation period. A polyvinyl chloride catheter (inside diameter, 1.4 mm) was inserted into the right carotid artery for arterial pressure measurements and blood sampling. Anesthesia was maintained by injection of small doses (30 mg) of pentobarbital under hemodynamic monitoring.

Monitoring. Arterial and airway pressures were monitored on-line via Statham strain gauge transducers connected to a Servomed recorder (Hellige, Freiburg, Germany). The method described by Neuhof (30) was used to determine...
oxygen uptake. The difference in oxygen volume between expiration air and inspiration air was continuously measured in an open system by means of an oxygen analyzer (Oxytest-S; Hartmann & Braun, Frankfurt, Germany). The oxygen uptake was calculated by the following equation and adjusted to standard temperature, pressure, and dryness conditions: \( O_2 \) uptake \( (\text{ml/min}) = (\text{air flow} \times \text{O}_2 \text{ difference} \times 100\text{ml/100}}) / 100 \). Blood samples were drawn intermittently for measurements of \( \text{pH}, \text{Pa}_2, \text{Pa}_2, \text{and } \text{HCO}_3 \), (ABL 330; Radiometer, Copenhagen, Denmark), hemoglobin and \( \text{O}_2 \) saturation (OSM2; Radiometer), hematocrit (Adams Autokrit centrifuge; Clay-Adams, Inc., New York, N.Y.), and lactate (test-combination lactate, fully enzymatic; Boehringer GmbH, Mannheim, Germany).

Experimental protocol. After a 30-min steady-state period of stable hemodynamics and oxygen uptake, the rabbits were randomly assigned to one of four experimental groups.

(i) Control group \(( n = 6 )\). After a 60-min period during which 0.9% \( \text{NaCl} \) (0.1 ml/min) was infused, \( E. \text{coli} \) \( (1.3 \times 10^8 \text{ CFU}) \) was injected via the ear vein catheter. Arterial blood was aseptically extracted for culture just before and after bacterial injection at 1, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min and thereafter at 30-min intervals. Blood gases, hematocrit, hemoglobin, and lactate concentrations were determined at hourly intervals. Three hours after bacterial injection, the animals were sacrificed with an overdose of pentobarbital, and, subsequently, tissue samples of liver, spleen, kidney, and lung were taken under aseptic conditions for quantitative bacterial determinations. The procedure from the time point of bacterial injection to 3 h later was identical in all experimental groups.

(ii) TNF group \(( n = 6 )\). After the steady-state period, human recombinant TNF \( (4 \times 10^5 \text{ U dissolved in } 2 \text{ ml of buffer}) \) was injected via the ear vein catheter. Sixty minutes after TNF application, \( 1.3 \times 10^8 \text{ E. coli} \) was administered intravenously. The same protocol as that described for the control group was followed. To exclude the possibility of bacterial translocation induced by TNF in the absence of injected bacteria, three experiments without bacterial application were performed.

(iii) Endotoxin 1 group \(( n = 6 )\). A nonlethal dose of \( E. \text{coli} \) endotoxin \( (40 \mu\text{g/kg/h}) \), as assessed in pilot studies, was infused for 1 h. Subsequently, \( E. \text{coli} \) \( (1.3 \times 10^8 \text{ CFU}) \) was injected intravenously. The same protocol was carried out without bacterial injection in three additional experiments.

(iv) Endotoxin 2 group \(( n = 6 )\). Prolonged endotoxemia was achieved by infusion of \( E. \text{coli} \) endotoxin \( (40 \mu\text{g/kg/h}) \) over a 4-h period prior to bacterial injection. Analogous to the other groups, bacteria \( (1.3 \times 10^8 \text{ E. coli} \text{ organisms}) \) were injected. To exclude the possibility of bacterial translocation being induced only by endotoxemia, three additional animals were subjected to the same protocol without subsequent bacterial injection.

Quantitative microbiology. Immediately after organ collection, cooled blood and tissue samples were prepared for bacterial culture. Blood samples were diluted by serial dilution into defined volumes of sterile saline. One hundred microliters of each dilution was plated in duplicate onto cysteine-lactose electrolyte-deficient agar plates as described by Sandys (36). Aseptically collected organs were weighed, and representative samples (0.8 to 2 g) of each organ were homogenized in a mortar with 5 ml of sterile saline. Serial dilutions of tissue suspension (50 \( \mu\text{l} \)) were plated onto cysteine-lactose electrolyte-deficient agar plates. The inoculated plates were incubated at 37°C for 24 h, and bacterial counts, appearing as CFU, were read. The final bacterial concentrations were calculated as the numbers of colonies per milliliter of blood and as colonies per gram of harvested tissue.

Materials. Recombinant human TNF was a generous gift from Knoll AG (Ludwigshafen, Germany). The protein concentration was 2.24 mg/ml, and the specific activity was 8.74 \( \times 10^4 \text{ U/mg of protein} \). Purity was >99%, and endotoxin content was <0.0027 ng/ml of protein (Limulus test). TNF was dissolved in sodium-phosphate buffer (50 mM) to achieve final concentrations of \( 2 \times 10^6 \text{ U/ml} \). The dose used of \( 4 \times 10^6 \text{ U of TNF} \) was selected to investigate the acute effects of TNF on clearance function independent of hemodynamic alterations. In pilot studies, this dose was shown to induce inflammatory reactions, such as significant histamine release and eicosanoid generation, a marked activation of granulocytes with elastase release, and a moderate increase in pulmonary vascular resistance and microvascular permeability but without significant changes in systemic arterial pressure or oxygen uptake.

Endotoxin from \( E. \text{coli} \) O111 was kindly donated by R. Urbanasch (Department of Immunology and Serology, Institute of Medical Microbiology, Faculty of Clinical Medicine Mannheim, University of Heidelberg). Lipopolysaccharide (LPS) was infused in a nonlethal dose, which did not induce severe organ damage (e.g., tissue necrosis in the gastrointestinal tract), as verified by light-microscope studies in previous experiments during the observation period.

Bacterial inoculum. \( E. \text{coli} \), an encapsulated, serum-resistant, nonhemolytic strain, freshly isolated from blood culture of a septicemic patient, was cultivated on blood agar plates. The grown colonies were scraped from the plates, carefully homogenized by vortexing in tryptic soy broth, serially diluted, and adjusted to a density of \( 1.3 \times 10^8 \text{ CFU/ml} \). They were stored in a freezer until use. The amount of exogenous \( E. \text{coli} \) used was based on pilot experiments, investigating the clearance and organ distribution of different doses of \( E. \text{coli} \) \( (10^{10}, 10^{9}, 10^8) \). For this study, a dose was chosen which showed an easily reproducible elimination kinetic but did not induce severe hemodynamic changes influencing clearance function by tissue hypoperfusion. The applied dose of \( 1.3 \times 10^8 \text{ E. coli} \) was completely eliminated during a time period of between 60 and 90 min in control animals and allowed registration of slight decreases as well as slight increases in elimination kinetics during the observation period. At higher doses, delayed clearance might be due to the measured depression of hemodynamics with increased vascular resistance, which was not the object of the present study. Shock-induced immune suppression has been reported in previous studies (1, 16). Experiments with lower doses, however, demonstrated such a rapid bacterial elimination (within 10 to 15 min) that possible minor increases in clearance function were not detectable.

Statistical analysis. Data are presented as the arithmetic mean ± standard error. The logarithm of the quantitative bacterial count was used for statistical comparison. Differences between groups were tested by one-way analysis of variance and subsequently verified by multiple-range test (Scheffé). Significance was accepted at \( P < 0.05 \).

This study was approved by the Animal Subject Protection Committee of the University of Giessen. The care and handling of animals were in accordance with the National Institutes of Health guidelines.
RESULTS

Hemodynamic and metabolic parameters. Mean measurements of arterial pressure and oxygen uptake as well as blood gases (PaO₂, PaCO₂, SaO₂, pH) did not differ significantly from baseline measurements throughout the study period in the control and TNF-treated animals. In the rabbits infused with endotoxin for 4 h, however, arterial pressure decreased progressively from 83 ± 4 mm Hg (baseline) to 58 ± 7 mm Hg (1 mm Hg = 133.322 Pa) at the end of observation (180 min after bacterial injection, P < 0.01 versus controls; by one-way analysis of variance), whereas no major changes were evident in those infused with endotoxin for 1 h. This pressure drop was paralleled by a decrease in pH from 7.48 ± 0.01 to 7.26 ± 0.05 compared with the other groups in which pH remained within the normal range. Likewise, the impaired hemodynamics in the endotoxin 2 group could be correlated with the reduction of oxygen uptake to 64% ± 7% of the baseline value. Minimal values of oxygen uptake were 87% ± 4%, achieved at the end of observation in the animals infused with endotoxin for 1 h. There was a continuous increase in lactate concentrations (normal values in plasma, 5.7 to 22 mg/dl) in all groups; this increase was most pronounced in the endotoxin 2 group, with a more-than-fivefold increase of the baseline value from 13.6 ± 3.6 to 81.3 ± 16.2 mg/dl. Minimal changes in hemoglobin and hematocrit, which were comparable in all groups, ensued from dilution effects due to blood sampling with isovolemic substitution with saline.

Microbiological cultures from blood samples taken before E. coli injection were sterile in all groups. The elimination kinetics of bacteria from the circulating blood (Fig. 1) differed significantly between endotoxin-infused rabbits on the one hand and TNF-treated animals and controls on the other hand. The rapid decrease of bacteria within the first 10 min, which was seen in all groups, was followed by a significantly delayed clearance with persisting numbers of E. coli until the end of observation in the endotoxemic animals. In contrast to this, blood cultures were sterile from 90 and 120 min in the control and TNF-treated groups, respectively.

In comparison with the results in the controls, the delayed clearance in the rabbits challenged with endotoxin was accompanied by significantly higher numbers of E. coli in the liver, spleen, lung, and kidney (Table 1) and a partially different tissue distribution pattern of viable bacteria (Fig. 2). Tissue cultures of the control group showed the highest bacterial numbers in the liver (57% ± 6.7% of total CFU, counted in organ cultures) and in the spleen (43% ± 6.6%), whereas very low counts (<70 CFU/g) could be detected in the lung (0.4% ± 0.1%) and kidney (0.2% ± 0.1%). In contrast to this, endotoxin (LPS) infusion for 4 h resulted in significantly higher amounts of bacteria (100- to 1,000-fold of control values) in all four organs (P < 0.001 versus all other groups) associated with significantly higher percentages of total bacteria detected in the lung (5% ± 1.5%); P < 0.001, endotoxin 2 group versus control) and kidney (6% ± 1.6%; P < 0.001, endotoxin 2 group versus control). Significant differences in the organ distribution pattern between both endotoxemic groups were evident, with predominant amounts of E. coli in the liver (72% ± 4.5%; P < 0.001 versus endotoxin 2 and TNF group) and minor numbers in the spleen (2.5% ± 0.8%; P < 0.001 versus endotoxin 2) group in the animals infused for 1 h, whereas higher counts in the kidney were detected in those infused for 4 h (P < 0.001 versus all other groups). TNF challenge produced distinct alterations in absolute counts and especially in the relative tissue distribution pattern of bacteria, with markedly increased numbers of bacteria in the lung (44% ± 2.6%; P <

![FIG. 1. Time course of bacterial elimination from blood after injection of E. coli (1.3 × 10⁸ CFU/ml) in the different groups. Mean counts of CFU were plotted semilogarithmically against time in minutes. The rapid decrease of bacteria within the first 10 min seen in all groups was followed by a significantly delayed clearance with persisting numbers of E. coli until the end of observation in the endotoxemic animals, whereas cultures were sterile from 90 and 120 min in the controls and TNF-challenged rabbits, respectively.](http://iai.asm.org/)

![FIG. 2. Comparative evaluation of organ distribution pattern of viable CFU of E. coli expressed as percentage of total detectable CFU in tissue cultures. Relative counts varied significantly from controls in endotoxin and TNF groups, with significantly higher values in the lung and kidney and lower values in the spleen. *, P < 0.05; **, P < 0.001 (by one-way analysis of variance).](http://iai.asm.org/)
immune suppression was emphasized and remia cating blood and other organs during the toxin infusion for either 1 (n = 3) or endotoxin infusion for either 1 (n = 3) or 4 (n = 3) h without subsequent E. coli injection. Consequently, the results represent the decreasing numbers of circulating E. coli resulting exclusively from clearance function and not falsified measurements of incalculable alterations due to translocated endogenous E. coli.

**DISCUSSION**

Severe injury predisposes the host to an increased susceptibility to infection, which often leads to acute respiratory distress syndrome or multiple-organ failure (5). In view of therapeutic strategies, several attempts have been made to delineate the underlying mechanisms of this complex relationship. A link between shock and development of bacte remia and endotoxemia was first suggested by Zweifach et al. (41) and Fine et al. (15). The role of shock-induced immune suppression was emphasized in studies reporting that animals subjected to hemorrhagic shock cannot sustain a normal inflammatory response (1) and have impaired peritoneal clearance of bacteria (16). Clinical evidence indicating that the gut is a reservoir for bacteria causing systemic infections in critically ill patients has accumulated. Loss of intestinal bacterial barrier functions, with invasion of bacte ria into the blood and translocation into other organs, appears to occur in certain clinical situations, resulting in systemic sepsis. Bacterial translocation in animal models (3, 12, 21, 25) and increased intestinal permeability to lactulose or mannitol in humans (8, 31) have been used as experimenta l markers of such gut barrier dysfunction occurring after hemorrhagic shock, endotoxin challenge, or thermal injury. Failure to eliminate translocated bacteria due to host immuno suppression may contribute to the persistence of enteric bacteria in mesenteric lymph nodes and other organs (22, 24, 32). It was previously reported that nonlethal doses of endotoxin appear to promote bacterial translocation from the gut primarily by injuring the gut mucosa in mice (9, 10). The potential causal association between endotoxin-induced intestinal injury and endotoxin-induced alterations in xen thine oxidase activities and bacterial translocation (14) is not fully clarified. In addition to questions on the effects of endotoxin on intestinal barrier function, another important question is whether endotoxin directly or indirectly influences phagocytosis and lysis capacity of the RES, thus enhancing bacterial growth. It appears that endotoxin produces its toxic effects in vivo by stimulating host cells, especially macrophages, to release various proinflammatory mediators that act as secondary messengers (20, 27). TNF is such a factor that has been found to play a pivotal role in the development of shock and tissue injury during septicemia (39, 40). TNF has been documented to induce shock, tissue injury, and necrosis of tumor cells as well as tissue necrosis in the gastrointestinal tract (37). Furthermore, it is known that TNF causes the release of other mediators such as interleukin 1, prostaglandins (2, 29), platelet-activating factor (6), and collagenases (7) and directly activates neutrophils and promotes adhesion between polymorphonuclear leukocytes and endothelial cells (17, 23).

Since endotoxemia is relatively common after severe injury and is associated with conditions leading to multiple organ failure, the aim of this study was to evaluate the potential influence of endotoxin on bacterial clearance and to determine the role of TNF in mediating septic complications. In contrast to others (3, 9–14), the present report was not intended to investigate possible enhanced translocation from the gut but whether systemic and organ clearance of systemically applied E. coli is impaired after infusion of endotoxin for different time periods and after TNF injection. The intravenous application of exogenous E. coli was chosen as a correlate of bacterial invasion from various compartments, e.g., the gut, the urogenital tract, wounds, or implanted catheters, into the circulating blood. To enable quantification of the clearance process, a defined number of E. coli was injected intravenously instead of inducing the invasion of uncontrollable numbers of bacteria from the gut into the circulation. To exclude possible miscalculation resulting from incalculable numbers of bacteria being translocated from the gut, additional experiments were performed to determine whether or not the LPS and TNF treatment itself leads to invasion of endogenous E. coli from the gut into the blood and to translocation into tissues during the observation period. These experiments revealed that bacterial translocation did not take place, as evidenced by sterile cultures in our model. Hence, enhanced translocation of intestinal E. coli, which might mask the effects on bacterial clearance function and tissue distribution, could be excluded. Thus, the differences in numbers of detected CFUs could be accounted for by alterations in clearance function due to the applied injury.

The present results demonstrate distinct alterations in bacterial elimination from the blood in the endotoxin-challenged groups and significantly different organ distribution patterns of bacteria in the endotoxin- and TNF-treated animals compared with those of saline-injected control rabbits. The elimination kinetics (Fig. 1) of injected E. coli suggest a biphasic time course, which is characterized by a rapid phase described by an exponential-like decrease of CFUs in the first 15 min, followed by a subsequent, slower phase of bacterial clearance. The second phase of bacterial clearance was significantly prolonged in both endotoxemic groups independent of the duration of the preceding endotoxin infusion, whereas the initial phase of rapid bacterial elimination was less altered in these groups. Whether a blockade of the RES by endotoxin, with consequentially reduced phagocytosis and lysis capacity, is one potential mechanism responsible for the failure to completely remove bacteria from the circulation remains to be clarified. The delayed bacterial clearance associated with significantly higher numbers of viable bacteria in organs was more pronounced after 4 h of endotoxin infusion than in the group infused for 1 h. In the latter group, it seems that the predominant amount of injected bacteria is filtered off and potentially phagocytized in the liver (72%), whereas after 4 h of infusion, a different distribution pattern with higher counts in the spleen, lung, and kidney was found. One can only speculate as to the underlying mechanisms leading to these findings. Apart from impaired phagocytic and lytic activity of various cell lines, such as neutrophils or circulating or tissue macrophages, a saturation of the phagocytic cells by ingested particles (e.g., endotoxin, coagulation products, debris), as well as a potential deficit of humoral phagocytosis-supporting factors (as discussed in previous
studies [24]), may contribute to the reported results. Similar findings, demonstrating an augmented extrahepatic, especially pulmonary, localization of systemically applied microparticles at times of hepatic clearance depression, were observed in other experimental studies in rats (34, 35). In attempting to analyze the mechanism leading to altered organ distribution patterns, regional blood flow was investigated. However, no correlation between decreased blood flow and organ distribution could be found (22). In a primate model, it was shown that the baboon responds to a nearly lethal dose of endotoxin, which severely depresses arterial blood pressure, without a decrease in mesenteric blood flow (38). Thus, if the RES was to be compromised by the hypertensive effects of endotoxemia, it does not appear to be caused by a decrease in organ blood flow. Hence, it is unlikely that altered organ distribution in the endotoxin 2 group is due to the decreased hemodynamics observed at the end of the study period. In our model, invasive measurements of regional blood flow were avoided because of potential infections and mediator release, which can presumably influence the microbiological results in an unpredictable manner.

To elucidate a possible modifying effect of the cytokine TNF on bacterial clearance independent of hemodynamic alterations, human recombinant TNF was injected 60 min prior to bacterial application in a dose which did not produce shock symptoms. Since a great variation in the levels of endogenous TNF production and responses to exogenous TNF administration among different species can be assumed (4, 18, 19), a bolus dose of 4 × 10³ U of TNF, like that of prior experiments with rabbits in our laboratory, was used. This dose produced moderate increases in pulmonary resistance and microvascular permeability, as well as in histamine liberation, and a marked activation of granulocytes but without hemodynamic effects (unpublished data). In contrast to the endotoxin infusion, the TNF treatment did not essentially influence the elimination kinetics, possibly because of TNF-induced neutrophil activation (23) and enhanced phagocytosis (28), but did distinctly alter the organ distribution of viable bacteria. Deviating from the pattern in the control and endotoxemic animals, with tissue counts constituting a relative organ uptake of 44% were discovered in the lung and low counts (2.5%) were found in the spleen. The high numbers of E. coli found in the lungs may be due to enhanced leukocyte sticking in the lung, reflecting a state of hyperphagocytic activity. Since leukocyte accumulation and adherence is a well-known cytokine-induced phenomenon, an increase of phagocytic cells in the lung, per se, may account for the elevated bacterial counts in the TNF-treated animals compared with those of the endotoxin-treated and control animals. No attempts were made in the present study to differentiate between intra- and extracellular viable bacteria, but such information might be of value as an indicator for local phagocytic activity. Future studies correlating bacterial growth with histological data and quantitative evaluations of phagocytotic cells are required to clarify this complex pathophysiological relationship.

Summarizing the reported results, endotoxin was shown to induce impaired bacterial clearance from the blood that was associated with increased bacterial counts in tissues, thus reflecting reduced phagocytosis and lysis capacity of the RES. These findings point towards a weaker resistance against bacterial colonization and translocation from inner and outer body surfaces and may account for the high incidence of sepsis in severely injured patients. The influence of TNF, which is known to modulate immune response, resulted in a distinctly different organ distribution pattern, with bacterial growth predominating in the lung, while elimination kinetics were unaltered. Since endogenous TNF may affect elimination kinetics not seen with bolus TNF administration, the question of whether TNF also exerts a mediating role on the bacterial blood clearance remains to be clarified in additional studies dealing with TNF antibodies. The current results warrant further investigations to elucidate the relative role of cytokines and the interrelationship with other mediators (platelet-activating factor, eicosanoids, free radicals) in the pathogenesis of endotoxin-induced RES impairment.

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