JOURNAL OF SURGICAL RESEARCH 21, 415–424 (1976)

Evaluation of the Possible Role of Serum Factors in the Clearance of Endotoxin from Blood

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Submitted for publication February 20, 1976

Evidence that gram-negative organisms of enteric origin or the endotoxins derived from them play a significant role in the number of clinical conditions continues to accumulate. Thus, their possible contribution to experimental and clinical liver failure as suggested by Gans *et al.* [9, 11], Liehr *et al.* [18], Wardle [33], Cooperstock *et al.* [7], and Wilkinson and associates [35] and to some of the more unusual complications associated with blind intestinal loops, e.g., after intestinal bypass procedures for morbid obesity, as described by Simmonds *et al.* [27], by Hollenbeck and coworkers [14a], and by Wands and associates [32] constitutes a significant, recent finding.

We found that endotoxins, once they traverse the gutwall, escape equally readily into portal vein blood as into the intestinal lymphatics [10]. Where endotoxin leaving via the former route is readily eliminated and detoxified by a normal liver, that which enters the lymphatics also homes in on the liver; as Braude *et al.* [3] demonstrated, systemic endotoxins are predominantly eliminated by the Kupffer cells.

The clearance of endotoxin by macrophages, or its phagocytosis, far from being the only one present to reverse the adverse *in vivo* effects of endotoxin, has been the process that so far has received the most attention because it is the easiest one to study. Other mechanisms have also been implicated particularly by Tate *et al.* [29], May and co-workers [19], and Oroszlan and associates [24]; the significance of the mechanisms under these circumstances, however, remains to be assessed.

Animals can be rendered tolerant or refractory to endotoxin, a condition characterized by a diminished response to endotoxin, particularly to its lethality and pyrogenicity, its ability to induce the Shwartzmann phenomenon, and other effects, as a result of repeated injections with endotoxin. As Beeson [1] and Carey *et al.* [4] showed, those animals exhibit enhanced blood clearance rates. Several factors affect blood clearance; fibrin, as shown by Wilkins [34]; antibody or specific opsonins as described by Rowley [25]; certain complement components, as demonstrated by Muller-Eberhard [21]; and other presently ill-defined serum factors, described by Normann *et al.* [23] and Tullis and Surgenor [31], have been shown to facilitate the uptake of a number of substances by the macrophage. Because of the enhanced blood clearance for endotoxin observed in the tolerant animal, tolerance provides an interesting experimental model in which to evaluate the role that serum factors play in the process of phagocytosis of endotoxin.

Although endotoxin can induce intravascular clotting, we showed previously that fibrin probably plays little or no role in clearing endotoxin from blood since the clearance rates for endotoxin in the presence of heparin were not significantly different from those observed in the absence of this anticoagulant [8]. As a follow-up of that study we have at this juncture tried to determine what role complement and possibly other serum factors play in the clearance of endotoxin from blood. Results of our present studies indicate

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that neither agent plays an important role in this process, suggesting that rather than mediated by humoral factors, the uptake of endotoxin by the phagocyte is the result of a direct interaction of endotoxin with specific receptor sites on the macrophage membrane. Hence, the enhanced blood clearance rate for endotoxin observed during tolerance is probably not due to an immune mechanism. Instead it seems to be the result of an actual increase in the number of these specific membrane binding sites.

MATERIALS AND METHODS

Inbred, albino male Sprague–Dawley rats weighing 300–350 g were housed in individual cages during the described experiments, kept in a temperature and humidity controlled environment where they had access to liberal quantities of fresh tap water, and fed commercial rat chow. Every rat was weighed each time it was anesthetized and its weight was recorded.

Anticomplementary factor. Anticomplementary factor prepared from cobra venom factor (C.V.F.) was obtained from Cordis Laboratories, Miami, Florida in amounts of 500 units per vial.

Under ether anesthesia the rat was weighed, the abdomen shaved, and 100 units of the reconstituted C.V.F. was injected intraperitoneally for 3 consecutive days. Neither total body weight nor the liver weight changed as a result of these injections. Previously, Kournaunakis, Nelson, and Kupusta [17] demonstrated that in the rat a single intraperitoneal injection of a minute dose of purified C.V.F. produces complete depletion of C_3 for 3 days. Cochrane *et al.* [6], Schwartz and Naff [26], Nelson [22], Klein and Wellensieck [16], and Synder *et. al.* [28] have also shown the effectiveness of this material for *in vivo* decomplementation. In a parallel experiment, animals matching in weight were prepared with 1 ml of pyrogen-free saline, injected intraperitoneally for 3 consecutive days.

Endotoxin. Lipopolysaccharide B. *Escherichia coli* preparations, obtained from Difco Laboratories, Detroit, Michigan which consists of a macromolecular mixture of membrane components of the *E. coli* organism with an approximate molecular weight of 10^6, were used. Endotoxin was labeled with $Na_2{}^{51}CrO_4$, as described by Braude *et al.* [3], and modified by Chedid and co-workers [5] to a final concentration of 1 mg of endotoxin/ml. Previously Chedid *et al.* showed that this endotoxin preparation retains its label as well as its specific activity; we also found that the labeling technique does not affect the toxicity of this labeled preparation [10].

Heparin. Lipo-Heparin (Riker Laboratories), 1000 U/ml, was used to heparinize the rats. Before cross transfusion, and 10 min prior to performing the clearance studies, each animal was heparinized with 100 units of heparin iv. Sterile saline and sterile disposable tuberculin syringes were used to flush the catheters.

Endotoxin clearance studies in decomplemented rats. Animals prepared with C.V.F. or sterile saline for 3 consecutive days were then subjected to femoral vein cannulation under ether anesthesia. The clearance of a mixture of 0.5 mg of labeled and 0.5 mg of unlabeled endotoxin was studied during a 30-min period in fully awake, restrained animals. Blood samples (0.2 ml each) were obtained 1, 3, 6, 15, and 30 min after injection of endotoxin with a 2-syringe technique, using sterile, pyrogen-free, disposable tuberculin syringes and pyrogen-free saline for irrigation. Blood samples for counting were added to polyvinyl test tubes containing 2 ml 10% EDTA. Thirty minutes after injection the animals were sacrificed; liver, lung, and spleen were removed, weighed, and washed free of blood, and a 1-g aliquot of each was placed in polyvinyl test tubes.

Tolerant rats. Tolerance (or refractoriness), the diminished response to endotoxin as a result of repeated endotoxin injections, was induced by daily intraperitoneal injections of 0.5 mg of endotoxin for 3 consecutive days. As a result, most of the animals lost weight. Animals matching in weight and serving as controls received saline intraperitoneally in the same volume; they did not

TABLE 1
Weight of Animals and Their Liver and Spleen at Time of Sacrifice

	Weight ($g \pm SD$)		
	Tolerant animals ($n = 8$)	Saline controls ($n = 8$)	
Body	295 ± 34	320 ± 32	
Liver	11.9 ± 1.41	11.7 ± 1.58	
Spleen	1.5 ± 0.22	0.9 ± 0.05	$p < .01$

lose weight; in fact, some even gained a little weight (see Table 1). The femoral vein was intubated on the fourth day under ether anesthesia; then the animals were allowed to wake up and 0.5 hr later their ability to clear 0.5 mg of labeled endotoxin from the blood was studied.

Exchange transfusion. A second group of closely matched rats received saline intraperitoneally for 3 consecutive days. On the fourth day, after catheterization, these animals were exchange transfused against similarly treated saline control rats. A third group of saline control animals underwent exchange transfusion against matched tolerant rats. A fourth group of tolerant rats was exchange transfused against matched tolerant animals.

Exchange transfusions were performed in fully awake, restrained heparinized rats. The total amount of blood exchanged was 7 ml per rat. Berlin *et al.* [2] previously established that the blood volume in the rat is 4.59 ± 0.57 ml/100 g body weight. Hence, 7 ml, for the animals used in these experiments, represents approximately half of their blood volume. The exchange transfusion was performed as follows: 1 ml of blood was withdrawn into a tuberculin syringe over a 1-min period in each of two closely matched animals, and returned over the same period to the animals after switching the syringes. Between return of blood and subsequent withdrawal there was a 2- to 2.5-min waiting period; hence, the entire exchange operation lasted approximately 30 min. The animals, awake and restrained, were allowed to recover for 1.5 hr, at which time the clearance studies, as described above, were performed.

Determination of radioactivity. Radioactivity was determined in a well-type scintillator crystal and radioactivity was expressed in counts per minutes after substracting the background counts. Organ counts are expressed as counts per minute per gram of organ tissue.

Clearance curves. Clearance curves were prepared by plotting the actual counts corrected for background on semilog paper as a function of time, for each time the mean count was determined. To establish composite curves for purposes of comparison and determination of K values, $K = (\log C_1 - \log C_2)/(t_2 - t_1)$, the log of the actual count was plotted on regular graph paper as a function of time.

RESULT

Clearance curves in normal rats. The early disappearance of endotoxin after its injection into normal rats from the circulating blood occurs in an exponential fashion. The rate at which it occurs during the first 6 min is distinctly faster than that observed subsequently. Hence, we have come to regard this as the "early" disappearance of endotoxin. Thereafter the clearance rate declines; the slower rate observed at this time we have arbitrarily termed the "late" clearance. This biphasic response was observed with widely different doses of endotoxin (Fig. 1).

Effect of cobra venom factor injections for 3 consecutive days on the clearance of a sublethal dose of endotoxin. Previously complete or near complete decomplementation was obtained with small amounts of purified cobra venom in animals of different species, as shown by Cochrane *et al.* [6], Kournaunakis and co-workers [17], and Schwartz and Naff [26].

In the present experiment, quantities far in excess of those previously used to obtain complete decomplementation were administered.[2] This, however, had no effect on the early clearance of 0.5 mg of labeled and

[2]Serum samples of two rats pretreated with C.V.F. (100 U ip) for 3 consecutive days were found to have no demonstrable complement activity in the hemolytic assay.

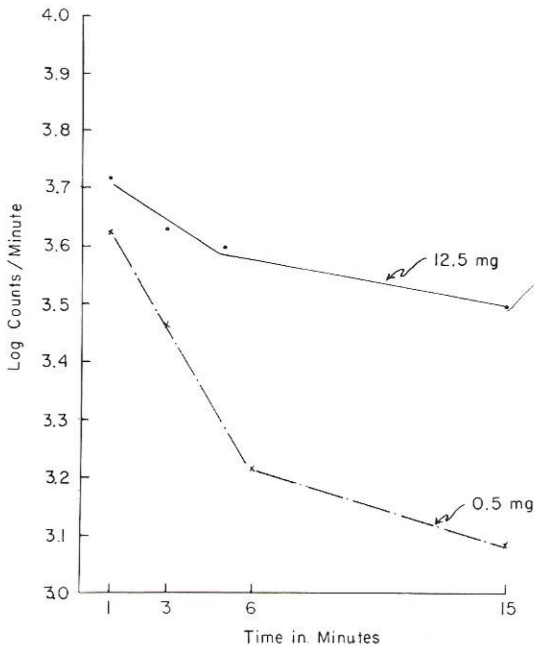


FIG. 1. Clearance curves for ^{51}Cr-labeled *E. coli* endotoxin in normal rats. The disappearance of endotoxin from blood during the first 5 min is rapid and occurs in exponential fashion. Subsequently clearance rates ("late" clearance) markedly decline. Examples of this biphasic response are shown here for two doses of endotoxin: 0.5 and 12.5 mg.

0.5 mg of nonlabeled *E. coli* endotoxin (Fig. 2a). In fact, the early disappearance rates of endotoxin in animals pretreated with saline is identical with that obtained in animals pretreated with C.V.F. (Fig. 2b).

We also found that the treatment with C.V.F. had no demonstrable effect on the distribution of the uptake of endotoxin by liver, lung, or spleen at the time of sacrifice of the animals 30 min after the injection of labeled endotoxin.

Effect of exchange transfusion on endotoxin clearance. Endotoxin clearance in tolerant rats after exchange with normal or tolerant rats was essentially the same, suggesting that normal serum had no effect on the early rate of endotoxin clearance observed in the tolerant animals (Fig. 3). The K value for 0.5 mg of labeled endotoxin in both conditions was 0.12 (Fig. 4).

In nontolerant control animals, the K value for early clearance of 0.5 mg of labeled endotoxin, after exchange against another nontolerant rat, was 0.055 (Figs. 3 and 4), while the clearance rate in nontolerant rats exchange transfused against tolerant animals was slightly but not significantly higher, 0.061 (Figs. 3 and 4). This is certainly not enough of an increase in the clearance rate to explain the observed difference between tolerant and nontolerant animals, suggesting that if humoral factors play a role in the early accelerated phagocytosis associated with tolerance, it is only a minor one.

Also, the clearance rate for endotoxin in tolerant rats was the same as that observed in tolerant rats after exchange transfusion indicating that exchange transfusion itself did not affect the clearance process significantly.

Organ uptake studies. Liver uptake in tolerant animals at time of sacrifice 30 min after endotoxin injection (mean, 23,954 cpm with a range of 21,882 to 26,128 cpm) was considerably larger than in nontolerant saline controls (mean count, 12,472 cpm, with a range of 10,137 to 17,608 cpm), the ratio being 1.92. Although after exchange transfusion organ uptake was slightly increased (mean liver count tolerant animals, 31,802 cpm, with a range of 20,780 to 44,520; and saline controls, 16,166 cpm, with a range of 12,070 to 21,711 cpm) the ratio was essentially the same as that observed in nonexchanged animals, namely, 1.96.

Lung uptake in tolerant animals (mean count, 3730 cpm; range, 2863 to 4962 cpm) was consistently less than in nontolerant saline control animals (mean count, 6458 cpm; range, 6022 to 7232 cpm).

DISCUSSION

Endotoxin, a mixture of macromolecules derived from the capsule of the *E. coli* bacillus with a molecular weight of approximately 1,000,000, is rapidly distributed after its injection over the circulating blood; hence, its first interaction with platelets and macrophages takes place within seconds following its injection.

Among its numerous activities, endotoxin activates the coagulation, the complement both via classical and alternate pathways as

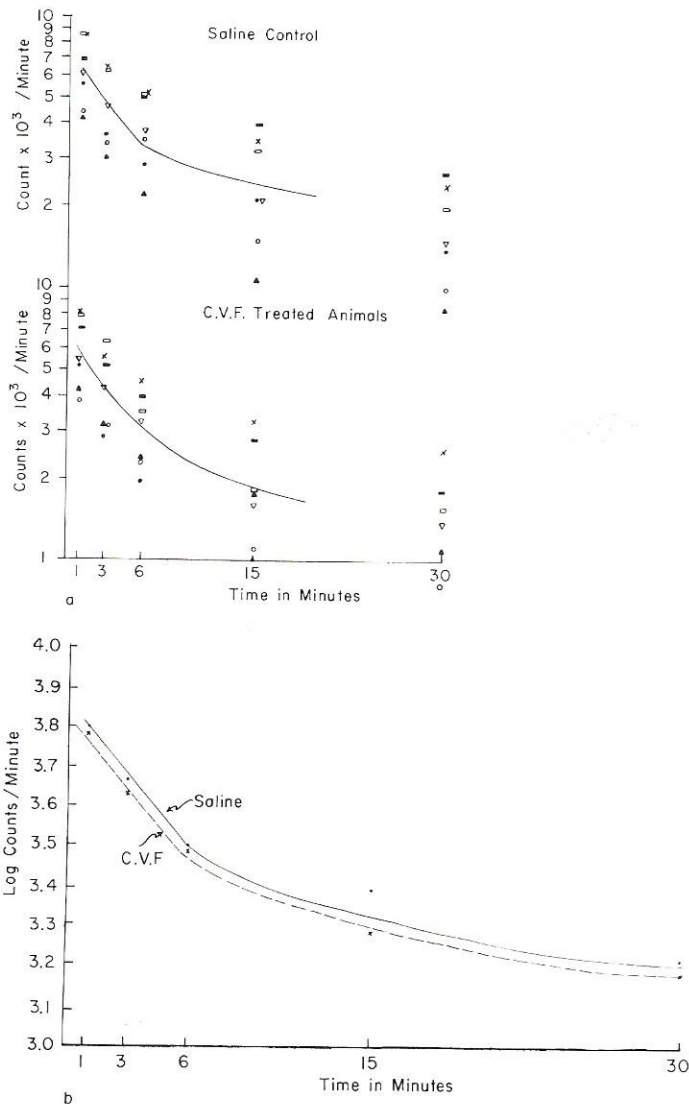
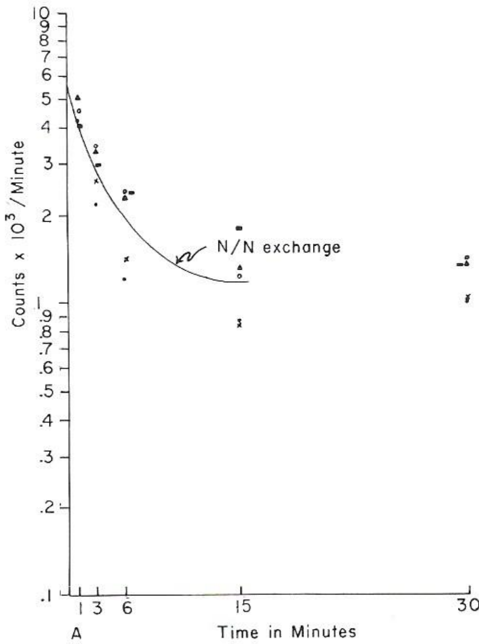


FIG. 2. Clearance curves for ^{51}Cr-labeled *E. coli* endotoxin in saline and cobra venom factor (C.V.F.) pretreated rats. The clearance rates observed in these two groups of animals are identical.

demonstrated by May and his associates [19] and the kinin system. All the described studies were performed in heparinized animals, thus we assume that fibrin played no role in any of the observed changes. In view of the marked affinity of endotoxin for complement and because of the ability of complement, particularly of C'_3 to coat various materials thereby faiclitating their adherence to and elimination by the phagocyte, one would expect that blood clearance rates for endotoxin would be greatly reduced in C.V.F. treated rats. The cobra venom anticomplementary factor activates the terminal complement components C_3–C_9 so that these are no longer available for a number of complement mediated reactions, including immune adherence, phagocytosis, neutrophil chemotaxis, anaphylotoxin generation, histamine release, and cytolysis. The latter process involves the enzymatic degradation of lipopolysaccharide membrane components much like those that constitute endotoxin; thus, it probably also plays a significant role



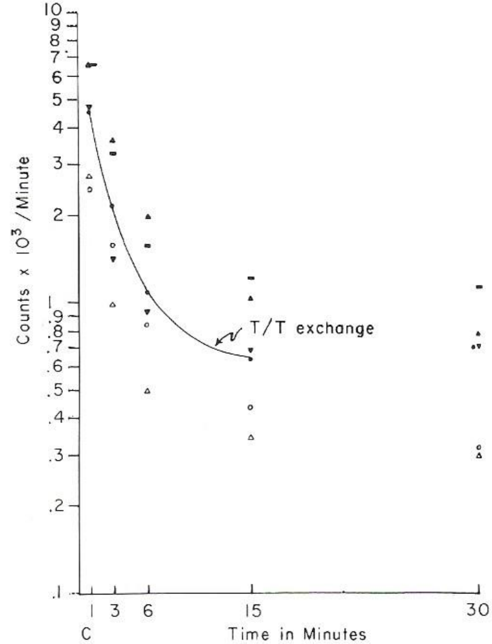


FIG. 3 (*continued*).

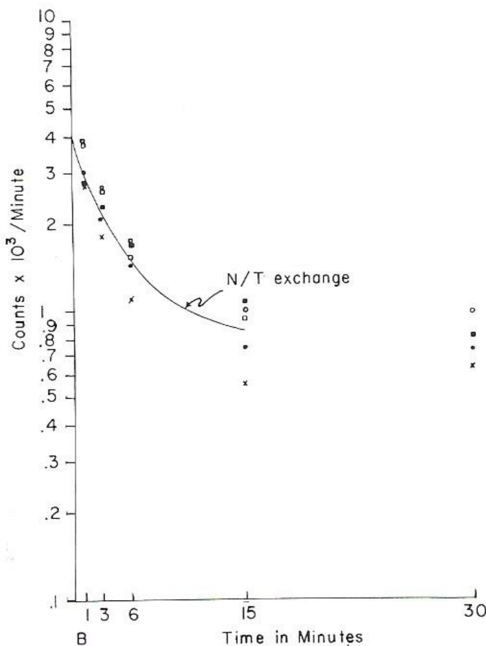


FIG. 3. Clearance curves for ^{51}Cr-labeled *E. coli* endotoxin in normal and tolerant rats: (A) after exchange transfusion between nontolerant rats; (B) after exchange transfusion between tolerant and nontolerant rats as determined in the nontolerant animals; (C) after exchange transfusion between tolerant rats.

in the *in vivo* disintegration of endotoxin as shown by May *et al.* [19].

Contrary to what we expected, we found that both early and late blood clearance rates for endotoxin in animals pretreated with C.V.F. are identical to those observed in matched saline controls. We have to assume, therefore, that complement mediated phagocytosis, either through immune adherence or by a phagocytosis promoting factor, plays no role in the elimination of endotoxin from blood.

Do other plasma or serum factors affect phagocytosis of endotoxin? The model used to try to resolve this question was the tolerant or refractory rat because the role of early phagocytosis in these animals in the present experiments is found to be twice as fast as that observed in the saline controls ($K_{\text{tol}} = 0.12$; $K_{\text{cont}} = 0.055$). Hence, if plasma or serum factors would play a significant role in the phagocytic process, their role would probably be magnified in this group of animals. The results of Good's studies however indicated already that tolerance is probably not an immunological reaction [12], thus

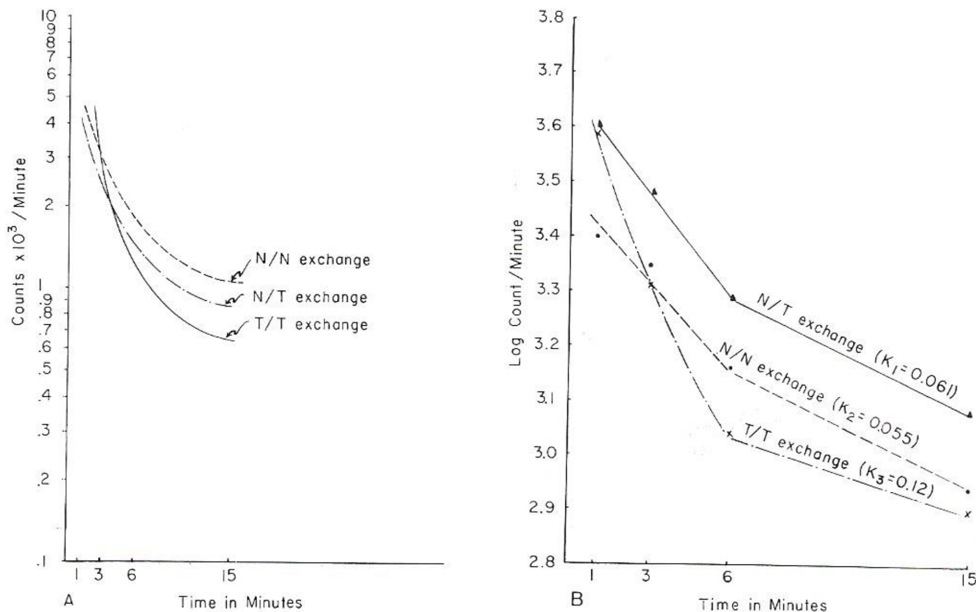


FIG. 4. Clearance curves for ^{51}Cr-labeled *E. coli* endotoxin in normal and tolerant rats after exchange transfusion: (A) composite of data in Fig. 3; (B) composite of the same data plotted to calculate K values.

confirming observations by Beeson [1] and Morgan [20] that the resistance to endotoxin that develops under these circumstances is independent of specific antibody formation, since tolerance for one endotoxin is effiective in protecting animals against the toxic action of immunologically distinct endotoxins of other gram-negative bacilli. In order to determine whether this was so, tolerant and nontolerant rats were subjected to exchange transfusions of approximately 50% of their blood volume. Clearance rates for endotoxin in normal rats, exchange transfused against tolerant animals, are slightly faster than those observed in nontolerant rats exchanged against matching nontolerant animals. (K_{ex} = 0.06.; K_{cont} = 0.055). However, this difference, though consistent, is minimal and not sufficient to explain the enhanced blood clearance observed in the tolerant animal. Thus, it would seem that under the described circumstances neither complement nor other plasma factors play a major role in endotoxin clearance. Previously, Beeson [1] observed that refractoriness to the pyrogenic response could not be passively transferred with serum to nontolerant recipients; however, subsequently Jenkins and Rowley [15] described opsonins, present in fresh serum, that seemed to be required for late endotoxin clearance. These and other differences in observation may possibly be explained by the recent finding by Greisman *et al.* [13] that after endotoxin injection the first antibodies start to appear 3 days later. We performed our exchange transfusion experiments 72 hr after the first endotoxin injection; hence, it is conceivable that the slightly enhanced endotoxin clearance observed during the present experiments after exchange transfusion may be due to the transfer of tiny amounts of antibody during the exchange of blood. Previously, Thomas *et al.* [30] demonstrated that antibody production is dependent upon antigen processing by the spleen. We found that spleen weights are significantly increased in tolerant animals (Table 1). Hence, to evaluate the role of antibody production in effecting the slightly increased clearance rate observed in control animals following exchange transfusion against tolerant animals, similar exchange transfusions between tolerant and nontolerant animals should be performed

also in splenectomized animals, since under those circumstances one may anticipate to observe less of an antibody response.

If, in the absence of antibody, plasma factors play no primary role in early endotoxin clearance we have to assume that endotoxin can interact directly with the phagocyte membrane, suggesting that the plasma membrane of the macrophage has specific receptor or binding sites for endotoxin. This is not a unique feature for the macrophage alone, however, since a similar interaction between endotoxin and sensitive specific membrane receptor sites has been demonstrated for erythrocytes by Young *et al.* [36] and for platelets by Hawiger and associates [14]. It should be recalled that after injection of endotoxin, platelet aggregation and sequestration occur nearly instanteously. Since endotoxin can interact directly with the platelet membrane, the possibility that part of the described change in the present study might be platelet mediated presents itself; if this could be considered a possible mechanism, the magnitude of its contribution to the observed phenomenon would require further assessment. However, so far no differences in platelet response to endotoxin between tolerant and nontolerant animals have been noted to make this a serious consideration.

The faster early clearance rates observed in tolerant or refractory animals may be due either to an increased number of receptor sites on the existing population of macrophages, to a larger phagocyte population, or to both mechanisms. Presently the evidence favors the latter explanation because the clearance rate for carbon or thorotrast is also faster in tolerant animals and because liver and spleen weight in these animals is consistently higher than in nontolerant rats, suggesting that the total number of cells is increased. In fact, the weight of the spleen is significantly higher (Table 1). We find that hepatic clearance in tolerant rats is nearly double that observed in nontolerant animals, hence we would expect liver weight to reflect this difference. Since the difference is not of this magnitude we assume that besides an increase in the number of phagocytes, the number of specific receptor sites per cell is probably also increased.

Our finding that lung uptake in the tolerant animal is less than that observed in nontolerant animals is in marked contrast to the observations of Carey *et al.* [4]. However, all our animals were heparinized; hence, we assume that the lung trapping they observed was probably a fibrin related phenomenon. Whatever the explanation, the practical significance of our reported findings is that early endotoxin clearance occurs independently of the different immune mechanisms. Instead a direct dependence upon the number of available receptor sites on the macrophage membrane can be implied from the observed findings. Since the liver is richly endowed with Kupffer cells, which contribute the bulk of the available macrophage population, the significance of their role in the early elimination of endotoxin from blood is quite obvious. Although phagocytosis is by no means the only process whereby the organism rids itself of endotoxins, it remains one of the more significant ones. Failure of this particular macrophage function, e.g., during hepatic failure, is anticipated to affect seriously this process.

SUMMARY

Circulating endotoxins are eliminated from the blood by phagocytes, predominantly the Kupffer cells. Neither complement nor other plasma factors appear to play a significant role in the initial stage of the phagocytosis of endotoxin. This suggests that the early phase of this process consists of a direct interaction between endotoxin and specific endotoxin receptor or binding sites on the macrophage cell membrane.

ACKNOWLEDGMENTS

We thank Dr. Margaret Polley for the complement determinations and John Kiev for his technical assistance.

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