POTENTIATION OF THE LETHAL EFFECT OF ENDOTOXIN BY HETEROLOGOUS PLASMA*

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Although the presence of endotoxin has been suspected in the circulating blood of patients suffering from Gram-negative infections, it was not possible until recently to produce evidence to support this contention.

The unusual sensitivity of the rabbit skin to endotoxin in the presence of epinephrine (accelerated Shwartzman reaction) led us to use this reaction as a test for circulating endotoxin (1). As described by Thomas (2), a striking hemorrhagic necrosis develops at the intracutaneous site of epinephrine injection, if the animal has received an intravenous injection of endotoxin during the preceding 4 hours. When plasma from infected patients is utilized for the intravenous injection, positive results are reliably obtained in patients with protracted hypotension or shock. When attempts were made to estimate the quantity of endotoxin present by injecting known amounts of endotoxin in a vehicle of normal human plasma, and in saline, it was found that human plasma mixed with endotoxin not only enhances the local reactivity of the rabbit to intracutaneous injections of epinephrine, but also potentiates the lethal effect of endotoxin administered intravenously by approximately 10,000-fold.

Plasma has been reported both to potentiate, and to inhibit the effects of endotoxin. Farr (3) found a tenfold enhancement of pyrogenic and leucocytic effects of *Salmonella typhosa* vaccine in the rabbit, when mixed with rabbit plasma. LeQuire (4) confirmed this potentiation and showed that it was not abolished by prior treatment with heparin. Grant (5) found that the potentiation of pyrogenic effect by plasma could not be demonstrated in animals rendered tolerant to endotoxin. Farr *et al.* (6) confirmed that the pyrogenic effect of endotoxin in rabbits was augmented by both human and rabbit plasma, and showed that the serum of tolerant animals failed to produce this augmentation. They believed this to be due to humoral inhibitors. All of these investigators were concerned with pyrogenic effects, and the amounts of human plasma and endotoxin utilized by Farr *et al.* (6) are too small to produce effects of the kind to be reported.

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Inhibition of the pyrogenic effect of endotoxin by incubation with homologous human plasma was reported by Hegemann (7, 8) and confirmed by Goodale *et al.* (9). Inhibition of pyrogenic effects was also noted on incubation with homologous plasma of rabbits (10–13), rat (18), and the horse (15). Hegemann (16) attributed the inactivation to the presence of properdin. Landy *et al.* (14) were able to inhibit the effects of endotoxin by incubation with heterologous plasma (human) and testing the mixture in the rabbit. These workers were unable to demonstrate plasma inhibition in an homologous system (rabbit), but did find inhibitors present in guinea pig and mouse plasma, tested in the rabbit. The same authors also observed an inhibition of tumor-damaging potency of lipopolysaccharides, as well as other biologic effects of endotoxins, on incubation with human serum. Ho and Kass (18) found the inhibitor to be in Cohn fractions III and IV in human plasma tested in rats (17). Rowley *et al.* found that alkaline phosphatase in serum has a high degree of specificity for the phosphate ester bonds of lipopolysaccharides (19). Westphal, who suggested that the active portion of lipopolysaccharides is lipoid fraction "A," noted that this was inactivated by incubation with horse serum (20). Rosen *et al.* (21) were of the opinion that inactivation may be due to the iron content of serum or plasma. Most authors have found that the inhibitory action is destroyed by temperatures above 60°C for several hours but not at 56°C for 30 minutes (13, 14, 17, 22, 23).

Materials and Methods

Test Animals.—Albino rabbits of either sex, weighing between 1.0 and 1.5 kg were used. No animal subjected to previous experimentation was used, and tests were carried out during summer, fall, and winter months.

Plasma Samples.—At first, fresh serum or heparinized plasma (1 mg heparin/1 ml of blood) from blood samples obtained from human volunteers was used. It was then found that plasma obtained from citrated bank blood was equally effective, and this was the source of plasma for the experiments reported here. Plasma obtained by centrifugation in a refrigerated centrifuge (15 minutes at 4000 RPM) was kept frozen until used. Serum was prepared under standard conditions; the blood was allowed to clot for 2 hours at 37°C. It was then centrifuged and the supernatant removed.

Endotoxin.—Purified lipopolysaccharide *Salmonella enteritides No. 127402* (Difco Laboratories, Inc., Detroit) was used. The material was stored in dry form in a refrigerator and dissolved in 0.9 per cent saline prior to use. In all instances, pyrogen-free plastic syringes and tubes were used.

Drugs.—Hydrocortisone sodium succinate (solucortef), The Upjohn Company, Kalamazoo, Michigan; Heparin sodium U.S.P., The Vitarine Company, New York; Normal serum albumin (human) (albumisol) 25 per cent, Merck Sharp & Dohme, West Point, Pennsylvania; Poliomyelitis immune globulin, Cutter Laboratories, Berkeley; Epinephrine U.S.P. (1:1000), Vitarine.

Standard Injection Procedure.—Endotoxin, reconstituted daily in 0.9 per cent saline, was diluted to proper dose and mixed immediately prior to injection with plasma or saline as the vehicle. 7 ml of plasma was used in all experiments, unless otherwise stated. Injections were made in the marginal vein of the rabbit ear. Animals were observed hourly for 4 hours, and again at 24 hours.

Skin Tests.—Epinephrine was diluted so as to contain either 100 μg or 50 μg in 0.2 ml of 0.9 per cent saline. Duplicate skin sites were injected intradermally on the shaved abdomen of the rabbit. The skin tests were read at 4 hours and at 24 hours. A test was considered positive when both sites at a given dosage showed hemorrhagic necrosis.

Tabulation of Results.—The end-point in these studies was death of the test animal. This frequently occurred within 1 hour, and usually within 4 hours. Animals alive at 24 hours after injection were considered to have survived. The results are expressed as a fraction, in which the numerator is the number of animals which died, and the denominator the number of animals tested. Thus a result of $^{3}/_{10}$ would indicate a 20 per cent mortality.

RESULTS

1. Potentiation of Lethal Effect of Endotoxin by Human Plasma or Serum.—Table I shows that the majority of the rabbits survived the injection of endotoxin in saline, in single doses up to 1000 μg. When human plasma (and in a few instances, serum) was used as the test vehicle, amounts of endotoxin as small as 0.1 μg produced a high mortality rate. Comparable batches of animals given endotoxin in saline, and endotoxin in plasma, were tested at the same time.

TABLE I

Potentiation of Lethal Effect of Endotoxin by Human Plasma

Dose of endotoxin	No. of rabbits dying /no. of rabbits injected	
	0.9 per cent saline	Human plasma
μg		
1000	0/5	5/5
500	0/5	5/5
200	0/4	5/5
100	1/5	5/5
50	2/8	9/9
20	2/10	9/10
10	0/8	6/8
5	0/6	5/5
1	0/8	4/7
0.5	0/5	4/9
0.25	0/5	3/9
0.1	0/5	3/4
0.05	0/5	0/4
0		0/12

The death of these animals was quite characteristic. The rabbits became lethargic, dyspnoic, and stuporous, and frequently expired within an hour. On lifting the dead animal, bloody urine was usually found. At autopsy, there was the lack of significant findings associated with death of animals given large injections of endotoxin in saline. Gross and microscopic hemorrhage was noted in the urinary tract, including the tubules, but there was no evidence of fibrin deposition, and the remaining organs were normal, both on gross and microscopic examination.

2. Enhancement of Skin Reactivity to Epinephrine in the Presence of Endotoxin, by Human Plasma or Serum.—Results of skin tests for reactivity to epinephrine in the presence of circulating endotoxin are shown in Table II. Although it was originally planned to carry out skin tests on all animals subjected to tests for lethal effect, it was found that the early death of animals given endotoxin and human plasma precluded determination of the skin reaction in many instances. For this reason the data in Table II are not a representative sampling of experiments carried out simultaneously at different dose levels for endotoxin in plasma or saline, but instead the results represent the accumulated observations on survivors. Although these results convey the impression that potentiation of skin reactivity to epinephrine in the presence of endotoxin is produced by plasma, experiments were not carried out extensively in the dose range below 0.1 μg to substantiate this point.

TABLE II

Skin Reactivity to Epinephrine in the Presence of Endotoxin

Dose of endotoxin	No. positive/No. injected	
	0.9 per cent saline	Human plasma*
μg		
20	3/4	
10	2/2	1/1
5	4/5	
1	3/5	2/2
0.5	4/5	6/6
0.25	3/5	4/6
0.1	3/5	1/1
0.05	1/5	3/4

* In surviving animals

TABLE III

Comparative Effects of Heterologous and Homologous Plasma in Potentiation of Lethal Effect of Endotoxin

Vehicle	Endotoxin dose	
	10 μg	20 μg
0.9 per cent saline	2/10	0/8
Human plasma	9/10	6/8
Guinea pig plasma	4/4	3/3
Rat plasma		2/3
Rabbit plasma (homologous)	1/5	0/4

The hemorrhagic reaction developed at the site of epinephrine injection in

all of 15 animals given endotoxin (1 to 200 μg) human plasma mixtures which had first been incubated at 56°C for 30 minutes, in order to abolish the lethal effect of the mixture.

3. The Effect of Heterologous and Homologous Plasma in Potentiation.—

In a separate series of experiments, rabbit plasma was compared with heterologous plasma in its capacity to potentiate the lethal effect of endotoxin. Blood was obtained from rabbits, guinea pigs, and rats, of no particular strain, by cardiac puncture, and plasma removed after centrifugation of heparinized samples. Endotoxin dosages of 10 and 20 μg were utilized, since at this level 75 to 90 per cent of animals given human plasma die, while 80 to 100 per cent survive if saline is used.

As Table III shows, homologous plasma does not cause a significant rise in mortality, whereas mixtures containing human, guinea pig, and rat plasma strikingly increase the lethal outcome. In individual experiments, the impression was gained that the lethal effect of mixtures with guinea pig and rat plasma was not as great, since such animals died between 12 and 24 hours, in contrast to the early mortality when human plasma was used.

4. Effect of Heat on Potentiating Activity.—

Three groups of rabbits were used. Group 1 received 100 or 200 μg of endotoxin in human plasma, the mixture having been heated at 56°C for 30 minutes prior to injection. Group 2 received human plasma heated in the same way, with a similar dose of fresh endotoxin added just prior to injection. Group 3 received 100 or 200 μg of endotoxin, heated at 56°C for 30 minutes, and added to fresh human plasma just prior to injection.

TABLE IV

Heat Inactivation of Plasma Potentiation of Lethal Effect of Endotoxin

Vehicle	Endotoxin dose	
	200 μg	100 μg
Endotoxin in saline	0/4	1/5
Endotoxin in plasma	5/5	5/5
Endotoxin in plasma, heated (56°C for 30 min.)	0/5	0/5
Endotoxin plus heated plasma	1/5	
Heated endotoxin plus plasma	5/5	

Incubation of the endotoxin-plasma mixture at this temperature completely nullified the potentiation of lethal effect. Table IV shows clearly that this is not due to inactivation of the endotoxin by heat, but to inactivation of the plasma factor.

5. Plasma Fraction Responsible for Potentiation.—

Groups of rabbits were given 10 μg of endotoxin to which was added, respectively, 1.3 ml of human serum albumin, 0.42 ml of human gamma globulin, and 1.3 ml of albumin plus 0.42 ml of gamma globulin. These represent the approximate quantities of these protein fractions in 7 ml of human plasma. A fourth group was given 2.6 ml of human serum albumin, the approximate total protein content of 7 ml of normal human plasma.

TABLE V

Plasma Protein Fractions in Relation to Potentiation of Lethal Effect of Endotoxin

Vehicle	Endotoxin, 10 μg
In saline	0/8
In plasma	6/8
In 1.3 ml human serum albumin	0/5
In 0.42 ml human gamma globulin	0/5
Albumin plus globulin	0/5
In 2.6 ml human serum albumin	0/5

TABLE VI

Relation between Dose of Endotoxin and Quantity of Plasma Needed for Potentiation

Plasma	Endotoxin			
	200 μg	100 μg	20 μg	10 μg
ml				
7	5/5	5/5	9/10	6/8
6	5/5	5/5	6/7	
5		5/5	2/5	0/2
4			1/5	0/2
3		2/5	0/5	
2	5/5	2/5		
1	1/5			

None of these (Table V) separately, in combination, and in amounts comparable to total protein content, potentiate the lethal effect of endotoxin. Moreover, since serum is as effective as plasma, fibrinogen is apparently not involved. It is probable that the potentiating factor, if it is a protein, will be found in the alpha or beta globulin fraction.

6. Relationship between Endotoxin Dose and Amount of Plasma Required for Potentiation.—When the dosages of endotoxin and plasma are varied (Table VI) it is evident that potentiation of lethal effect is a function of the dosage of both endotoxin and plasma. When 10 to 20 μg of endotoxin are used, a lethal effect is not consistently seen unless 6 ml or more of plasma are used. Although a dose-weight study was not done, it was felt that the smaller rabbits (in the

range of 1 to 1.5 kg used) were somewhat more susceptible to lethal effect of endotoxin. Approximately 5 ml/kg of human plasma are required to consistently potentiate the lethal effects of small doses of endotoxin in the rabbit.

7. Effect of Induced Tolerance in the Test Animal on Potentiation.—Four rabbits were rendered tolerant by administration of increasing doses of endotoxin, until they were refractory to 200 μg over a 2 week period. On the 15th day, each animal was given an intravenous injection of 20 μg of endotoxin in 7 ml of human plasma, and intracutaneous injections of epinephrine were made. As previously shown, the mortality at this level is 90 per cent (Table I), and skin tests are uniformly positive. All of the tolerant animals survived, and all skin tests were negative. Thus, tolerance to endotoxin abolishes the lethal potentiality of an endotoxin-human plasma mixture.

8. Effect of Timing and Sequence of Dosage.—To investigate the possibility

TABLE VII

Effect of Timing and Sequence on Plasma Potentiation of Endotoxin

Time interval	5 min.	30 min.	3 hrs.	7 hrs.
7 ml human plasma FOLLOWED by 20 μg endotoxin	3/4	6/9	3/4	1/5
7 ml human plasma PRECEDED by 20 μg endotoxin	4/4	8/9	4/6	2/5

that endotoxin must first combine, or form a complex with, the potentiating factor in human plasma, in order to produce an increased lethal effect, both the timing and sequence of plasma and endotoxin injections were varied (Table VII).

In one group of rabbits, the intravenous injection of 20 μg of endotoxin in saline was followed by the injection of 7 ml of plasma at intervals of either 5 minutes, 30 minutes, 3 hours, or 7 hours. A second group of animals received 7 ml of plasma as the initial injection, followed by the same dose of endotoxin in saline at either 5 minutes, 30 minutes, 3 hours, or 7 hours.

The results indicate that the order in which the injection of endotoxin and plasma is made does not influence the development of an enhanced lethal effect, and suggest that potentiation may occur even when the injections are separated by hours.

9. Effect of Heparin and Pretreatment with Cortisone on the Lethal Effect.—Pretreatment with heparin, which prevents the generalized Shwartzman reaction, and cortisone, which does not, was investigated in relation to the enhanced lethal effect.

Fifteen rabbits were given 50 mg of heparin, and 5 rabbits were given 100 mg of heparin, 1 minute prior to the intravenous injection of 20 μg of endotoxin in 7 ml of human plasma. Control experiments showed that this dosage of heparin effectively blocks blood coagulation

in the rabbit. Five rabbits were given 25 mg of hydrocortisone intramuscularly twice a day for 3 days. On the 4th day, a similar dose was given and 20 μg of endotoxin and 7 ml of plasma injected intravenously.

The results of these experiments are shown in Table VIII. The mortality figures are essentially the same as these seen with endotoxin and plasma alone, at these dosages.

10. Inhibition of the Lethal Effect by Incubation of Endotoxin and Plasma.— Inasmuch as data in the literature indicated the presence of inhibitory factor in plasma, experiments were done to detect inhibition of the lethal effect.

TABLE VIII

Effect of Pretreatment with Heparin or Cortisone on Potentiation of Lethal Effect of Endotoxin by Plasma

Pretreatment with	20 μg endotoxin, plus 7 ml human plasma
Heparin, 50 mg	10/15
Heparin, 100 mg	5/5
Cortisone, 25 mg *b.i.d.* for 3 days	5/5

TABLE IX

Inhibition of Lethal Effect of Endotoxin by Incubation with Heterologous Plasma

Endotoxin	Incubation time			
	0	2 hrs.	4 hrs.	24 hrs.
μg				
500	5/5		3/5	
200	5/5	5/6	2/3	1/4
100	5/5	3/6		
20	9/10	3/6	0/5	

Endotoxin, in amounts ranging from 20 to 1000 μg, was incubated at 37°C with 7 ml of plasma, for periods of 2, 4, and 24 hours. These mixtures were injected into test animals, and compared with suitable endotoxin saline controls. Incubation of endotoxin alone, or of human plasma alone, did not affect their potency when mixed together just prior to injection. In some experiments, fresh endotoxin was added to the incubated mixture, with restoration of the lethal effect.

In order to demonstrate plasma inhibition of the lethal effect of endotoxin, it would be desirable to show that the lethal effect of the incubated mixture is less than that of a comparable mixture of endotoxin in saline. At the dosages used, consistent mortality did not occur with endotoxin in saline, so that this

comparison could not be made. However, a comparison with the mortality rates in the other experiments in this study suggests that inhibition does occur (Table IX). This is supported by the observation that addition of fresh endotoxin to the incubated mixture restores the lethal effect; and this suggests further than the plasma factor responsible for potentiation is distinct from that concerned with inhibition.

DISCUSSION

The above experiments demonstrate the presence, in human plasma, of a substance which produces a remarkable potentiation of the lethal effect of endotoxin administered intravenously to a rabbit. In the low dose range studied, there is an inverse plasma-endotoxin dose relationship, such that smaller doses of endotoxin require larger quantities of plasma to produce an equivalent effect. A similar enhancement of the lethal action of endotoxin was found with the use of plasma from guinea pigs and rats, though not with rabbit plasma. It is not clear whether this represents a specific deficiency of the potentiating factor in the rabbit, or indicates an immune mechanism based on species differences.

It is of considerable interest that prior incubation of endotoxin with plasma is unnecessary, and that during a period of 3 hours, neither the sequence nor the timing of endotoxin and plasma injections appears to interfere with evocation of an increased lethal effect. In previous work on the augmentation of pyrogenic effect of endotoxin (24, 25) it was speculated that an endotoxin-plasma factor complex forms *in vitro*, and exhibits greatly increased activity *in vivo*.

In view of the clearance curves of Cr^{51}-labeled endotoxin in the rabbit (26) which indicate more than 90 per cent disappearance from the circulation in 8 minutes, it is improbable that endotoxin, in the amounts used (20 μg), persist in the circulation long enough to permit the *in vivo* formation of a "complex" 3 hours later. Similarly, altered function of the reticuloendothelial system as an explanation of the increased lethal effect seems unlikely, in view of the small quantities of endotoxin used. This is supported by preliminary studies which fail to demonstrate depression of RES phagocytic function by injections of plasma in the amounts used in this study.

An alternative mechanism would be that endotoxin and heterologous plasma both act directly, and interchangeably, upon the reactive system of the rabbit in such a manner as to produce death, when both components are present.

That endotoxin is a vital component in this enhanced lethal reaction is indicated not only by the minute doses which are effective, but also by the fact that tolerance to endotoxin in the test animal abolishes the potentiation of lethal effect produced by human plasma.

Pretreatment with heparin does not protect the recipient animal against the lethal effect produced by combination of endotoxin and plasma. This is in

accord with the failure to find, at autopsy, fibrin deposition in pulmonary and other capillary beds pointing against rapid polymerization of fibrinogen as a cause of death. Moreover, in contrast to the protection ordinarily conferred by pretreatment with cortisone against lethal effect (though not against the generalized Shwartzman reaction) (27), in our experiments cortisone failed to protect against the potentiation of lethal effect by plasma.

Since the demonstration of a plasma inhibitor distinct from the potentiating factor would require a reduction in lethal effect below that found with injection of endotoxin in saline, these studies do not permit a positive statement on this point. However, the lethality of incubated mixtures of endotoxin and plasma, as compared to that produced by fresh endotoxin and plasma injected immediately, strongly suggests that inhibition (or decay of endotoxin activity) occurred during incubation. The restoration of lethal effect upon addition of fresh endotoxin, still in doses too small to be effective if given in saline, indicates that the loss of activity produced by incubation of endotoxin and plasma at 37°C is not achieved at the expense of the potentiating factor.

SUMMARY

The intravenous injection of endotoxin in human plasma into rabbits produces a marked potentiation of lethal effect, when compared to the mortality associated with comparable doses of endotoxin in saline alone. A similar enhancement was noted with other heterologous plasma (guinea pig, rat) but not with homologous plasma. The potentiating factor is not in the albumin or gamma globulin fractions, is not concerned with fibrinogen, and is heat-labile. Tolerance of the recipient animal to endotoxin destroys the lethal effect. Within a period of 3 hours, endotoxin and human plasma may be administered separately, without regard to timing or sequence, without loss of the lethal effect. The enhancement of lethal effect is not avoided by pretreatment with heparin or cortisone. Preliminary experiments indicate that the loss of lethal effect found after incubation of endotoxin-plasma mixtures may be due to a separate inhibitor, but is not due to loss of the potentiating factor.

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