INACTIVATION OF ENDOTOXIN BY A HUMORAL COMPONENT*

II. Interaction of Endotoxin with Serum and Plasma

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Immunization with endotoxin stimulates the production of specific antibody, however it does not provide the high level of specific protection conferred by the exotoxins. In addition to their antigenic activity, endotoxins elicit other characteristic reactions which are extensive and varied. Such effects may influence host resistance more significantly than does the stimulation of specific antibody. A non-specific increase in resistance to infection with various bacterial pathogens and a reduction or elimination of such reactions as fever, Shwartzman phenomena or lethality have been shown to follow injection of endotoxin. This altered host reactivity usually is of short duration and is independent of demonstrable antibody.

Although the mechanisms of such resistance have remained obscure for many years, recent studies on the humoral and cellular alterations in the host which follow the administration of endotoxins may provide meaningful information.

In this connection, the effect of endotoxin on serum properdin and on the activity of the reticuloendothelial system (RES) are noteworthy. Endotoxin has been shown (2) to increase the resistance of mice to experimental infections; there is a concomitant increase in serum properdin levels (3) and an enhanced ability of the RES to clear injected colloids or bacteria from the blood (4). However, these effects by themselves are not likely to account for the altered state of resistance (5).

In studies on the interactions, *in vitro* and *in vivo*, between endotoxins and properdin, attention in the past has been focused on the changes produced in properdin and the components of complement. More recent studies (6–9) have been concerned with the resultant change in endotoxin itself as a consequence of its interaction with serum. We have described a heat-labile component of serum, other than complement, properdin, or specific antibody, which altered endotoxic materials so that they no longer could elicit their characteristic host responses (1).

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* Reference 1 is the first paper of this series.

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This paper and the following one (10) record further information on this humoral component. In this communication, data are provided on its occurrence in the serum of various mammalian species and on the relative potency of serum and plasma from the same individual. The conditions for the interaction of this humoral component with endotoxin, and the bioassay technique necessary for the disclosure of the resultant reduction in activity of endotoxin, are described.

Materials and Methods

Endotoxins.—The tumor-necrotizing polysaccharide isolated from *Serratia marcescens* (Lot No. P-25) was employed in most of the experiments. In special situations other endotoxins were used which had been derived by various techniques, from colonially smooth or rough forms of *Salmonella* and *Escherichia*. The sources of these endotoxins are listed in Table V together with references to the publications which describe their properties.

Serum and Plasma.—Human blood specimens were obtained from healthy adult donors and allowed to clot at room temperature. After 1 hour the clots were rimmed and the serum decanted and clarified by centrifugation. Plasma was obtained by drawing blood into an anticoagulant solution (ACD,[1] unless otherwise stated); the formed elements were removed by centrifugation. Pools of serum or plasma were prepared from fifty or more donors, transferred to tubes and flame-sealed, and stored at −20°C.

Animals.—Etherized mice and rats were exsanguinated by bleeding directly from the heart; blood from groups of ten or more was pooled to provide sufficient volumes of serum or plasma. In the case of other species (rabbit, guinea pig, dog, horse, and sheep) blood from individual animals was used unless otherwise indicated. Plasma was obtained by collecting the blood in trisodium citrate (3.8 mg./ml.). These specimens were also stored at −20°C. in sealed pyrex tubes.

Interaction of Endotoxin with Serum or Plasma.—For measuring the capacity of serum or plasma to inactivate endotoxin, varying amounts were incubated with a standard quantity of the *Serratia marcescens* endotoxin at pH 7.4 to 8 for 1 hour at 37°C. The quantities of reactants employed were, in all instances, sufficient for subsequent assay in groups of ten mice each. In these assays, each mouse received as routine 10 μg. of this endotoxin (see following section on assay). A constant volume (0.10 ml.) of a stock solution of endotoxin containing 1,000 μg. per ml., enough for ten mice, was incubated with undiluted serum or plasma. After incubation, the volume was made up to 5.0 ml. with saline so that the amount administered intraperitoneally to each mouse was kept constant at 0.5 ml. The variable in these assays was the quantity of serum or plasma incubated with this fixed amount of endotoxin. The above procedure provided the material for injection into ten mice, but it is emphasized that the data are given in terms of the quantities administered per mouse. For example, when 0.1 ml. of plasma was incubated with 0.1 ml. of the stock solution of endotoxin and was then diluted to 5.0 ml., the 0.5 ml. aliquot given to each mouse represented the products of reaction of 10 μg. of endotoxin and 0.01 ml. of plasma.

Assay of Endotoxin Detoxifying Component (EDC).—The procedure employed for assessment of induced damage in tumor tissue was that employed in this Laboratory for many years (11). Sarcoma 37 tumor mash (0.05 ml.) was implanted in the thigh muscle of 2 or 3 month old CAF_1 mice. On the 6th day of tumor growth the mice were randomly distributed into groups of 10; usually 200 mice were implanted at the same time with aliquots of the same

[1] The formula is 22 gm. trisodium citrate, 8 gm. citric acid, and 25 gm. dextrose per liter of water; 15 ml. of this solution was added to each 100 ml. of whole blood.

tumor mash. Each such set of bioassays contained two control groups—an untreated one for determination of the presence or absence of spontaneous hemorrhage or necrosis, and a second one given 10 μg. of the endotoxin in saline for ascertaining the level of responsiveness of the tumors in each lot of 200 mice. This amount of the *Serr. marcescens* endotoxin was usually sufficient to induce marked hemorrhage and necrosis in the tumors of all mice in the group, and yet was small enough so that diminution of its potency could be detected. After incubation of graded volumes of serum or plasma with endotoxin as described above, 0.5 ml. aliquots of each test mixture were injected, intraperitoneally, into each of 10 mice. The animals were necropsied on the following day for examination of the tumors.

The tumors were inspected under low power magnification (×5) and induced tumor damage in the experimental groups was scored with reference to the two control groups. When the extent of tumor damage was undistinguishable from that in the endotoxin control group, no significant inactivation had occurred. When no tumor damage was evident in any of the tumors in a group, the endotoxin had been inactivated. Intermediate degrees of tumor damage, or evidence of damage in only some of the tumors, showed that partial inactivation had occurred.

General.—The findings reported in the present study are based on experiments repeated, in one form or another, many times. These involved an extensive series of assays in which more than 10,000 mice were used. The data obtained with this test system based on response of tumors were so voluminous that it is impractical to present them in full detail. Therefore the results of approximately 1,000 individual assays in tumor-bearing mice are given in summary form, except for certain illustrative protocols.

Until such time as its chemical nature is elucidated, we have, for convenience, assigned a functional designation to this humoral agent. For the time being, we shall refer to it as "endotoxin-detoxifying component" or EDC.

EXPERIMENTAL

Inactivation of Endotoxin by Serum.—We previously reported (1) that human sera, upon incubation with endotoxin, alter these polysaccharide complexes so that they no longer evoke the characteristic host responses. This effect of serum was shown to extend to a number of endotoxic materials irrespective of their derivation, *e.g.*, those obtained from tissues of higher plants and mammals (12), as well as from Gram-negative bacteria. The quantity of human serum required to inactivate *Serr. marcescens* endotoxin, as measured in the tumor damage assay, varied significantly among donors. For example, 0.1 ml. or less of some sera was sufficient to inactivate 10 μg. of endotoxin, while with other sera quantities ranging up to 0.5 ml. were required to bring about comparable inactivation. A few sera were encountered which did not inactivate endotoxin even at a level of 0.5 ml., the largest amount tested as routine. Sera from mice and rabbits were ineffective in altering endotoxin, while sera from the horse and guinea pig were comparable to human serum in their effects upon endotoxin.

It was demonstrated (1) that other host reactions caused by endotoxins were similarly affected after incubation *in vitro* of endotoxin and human serum. Endotoxin, so altered, no longer prepared for the local Shwartzman reaction, no longer elicited dermal hemorrhage and necrosis in epinephrine-treated rabbits,

and no longer produced fatal collapse in rabbits. Heat-inactivated serum, similarly incubated with endotoxin, failed to abolish these properties. It is therefore clear that serum contains a heat-labile system which is capable of producing striking alterations in endotoxins.

Distinction between EDC and Other Humoral Components.—Among the humoral components known to react with endotoxins are specific antibody, complement, and properdin. It was therefore necessary to determine what influence, if any, these substances had on the reported inactivation of endotoxins.

Accordingly, serum was rendered deficient in each of these components. Properdin was removed by treatment with zymosan at 17°C. for 1 hour (13), or bound by addition of an appropriate amount of rabbit antibody to human properdin (14). Elimination of the components of complement was effected by standard procedures (15, 16): serum deficient in C_1 (R_1) was prepared by dialysis for 48 hours against acetate buffer of pH 5.5, $\mu = 0.02$ at 0°C.; R_2 by heating serum for ½ hour at 52°C. (this procedure also inactivates the heat-sensitive factor (17)); R_4 by treatment with 9 mM hydrazine at 37°C. for 1 hour (this concentration of hydrazine also removes the hydrazine-sensitive factor (17)); complement-fixed (CF) serum by incubation with preformed antigen-antibody complex (18) (Pneumococcus Type III polysaccharide-rabbit anti-Type III serum); and streptokinase-treated (SK) serum (19) by incubation of serum with streptokinase (varidase, Lederle). Divalent cations were removed by treating equal volumes of fresh human serum with 6 mM EDTA for 45 minutes at 37°C. When required, reagents were checked for absence of hemolytic activity against sensitized sheep red cells and then tested for EDC activity with the assay procedure described.

To determine whether specific antibody was implicated in the effect of fresh serum on a given endotoxin (*i.e.*, from *Serratia marcescens* or *Salmonella typhosa*), the following tests were performed.

Fresh serum was examined for the presence of specific antibody in agglutination tests with the bacteria from which the endotoxin had been derived, and in hemagglutination tests with red cells which had been coated with homologous endotoxin. The sera employed were found to contain little or no antibody demonstrable by these methods. Moreover, the addition of specific anti-typhoid rabbit serum to incubated mixtures of human serum and endotoxin did not alter the subsequent inactivation of this endotoxin. Furthermore, the tumor-damaging potency of typhoid endotoxin itself was not diminished in the presence of specific antibody. The removal, by adsorption, of *Serr. marcescens* antibody from human serum did not alter its inactivating potency for the endotoxin from this organism. Aliquots of serum or plasma were rendered deficient in one component, *viz.* properdin or one of the components of complement, and were in every instance still fully active.

These findings are summarized in Table I and indicate that none of these known agents is responsible for such inactivation *in vitro* of endotoxin.

Comparison of EDC Activity in Serum and Plasma.—The possibility was considered that the component of blood responsible for the anti-endotoxic effect might originate from formed elements.

For this reason, blood was collected at 37°C. in siliconized glassware to avoid the use of anticoagulant. The plasma was carefully separated from the cellular elements by low speed centrifugation and the cell-free plasma collected in citrate to prevent clotting. The buffy coat was washed with saline to remove plasma; the leucocytes were then ruptured by alternate freezing and thawing.

TABLE I

Effect of Removal of Humoral Components on Inactivation of Endotoxin by Serum or Plasma

Component	Method of removal	Effect on inactivation of endotoxin
Properdin	Anti-properdin; zymosan	None
Complement C_1	Dialysis	"
Complement C_2	52°C. for ½ hr.	"
Complement C_3	Zymosan	"
Complement C_4	Hydrazine	"
Antibody	Specific adsorption	"

TABLE II

Comparison of EDC Activity in Paired Specimens of Serum and Plasma

Species	Volume required to inactivate* 10 μg. of *Serr. marcescens* endotoxin	
	Serum	Plasma
	ml.	*ml.*
Human	0.1–0.5	0.005–0.03
Chimpanzee‡	0.3	0.01
Horse	0.4	0.05
Sheep	>0.5	0.05
Dog	0.5	0.02
Rabbit	>0.5	0.01
Guinea pig‡	0.5–>0.5	0.20
Rat‡	0.02	0.01
Mouse‡	>0.5	0.01

* As measured in the tumor damage assay.

‡ Pooled.

This extract of buffy coat, in contrast to the cell-free plasma, was without effect on endotoxin, showing that EDC did not derive from blood cells. Upon testing the cell-free plasma at appropriate levels it became evident that this plasma exhibited far more EDC activity than serum. This marked contrast was convincingly demonstrated in the following manner.

Each blood sample from donors of various species was divided into two aliquots; one was allowed to clot, while the other was collected in citrate. These paired specimens were then assayed for EDC activity; the results are given in Table II. The data are expressed in terms of

the minimum volume of serum or plasma required for inactivation of the standard amount of endotoxin as measured in the tumor damage assay. Since the largest amount of serum tested as routine was 0.5 ml., a value of >0.5 ml. indicates that the test serum was without measurable EDC activity.

Human sera varied considerably in activity; an occasional specimen produced little inactivation at 0.5 ml. but most specimens were effective in amounts ranging from 0.1 to 0.4 ml. Sera of the chimpanzee, horse, and dog were generally comparable to human sera whereas those from sheep, rabbits, and mice were without measurable effect. However, except for the rat, the plasma aliquots from all species were highly effective in contrast to the paired serum aliquots which usually showed little or no effect. It is noteworthy that the plasma EDC activity was similar for eight of the nine species examined. In only one species, the rat, was high activity consistently exhibited by serum (0.01–0.1 ml.): this finding was affirmed in four different strains of this species.

Examination of a small number of citrated samples of human plasma had shown that the minimal amount required to inactivate 10 μg. *Serr. marcescens* endotoxin ranged from 0.005 to 0.03 ml. To obtain further information on levels of EDC in normal humans, citrated plasma from 35 donors was examined. The plasma specimens were tested at 0.02 ml., a level considered to be within an expected range of EDC activity. This quantity of each of the 35 specimens sufficed to inactivate the standard dose of endotoxin. Levels of EDC were followed in three selected subjects over periods of 1 to 7 months. EDC activity in the plasma of these individuals was found to be constant, within the experimental error of the assay. In contrast, serum specimens drawn from these same individuals over a period of many months were found to vary significantly in EDC potency.

Conditions for Interaction between EDC and Endotoxin.—The influence of experimental conditions on the inactivation of endotoxin by EDC was studied. In the determination of the requirements for inactivation, *in vitro*, it became evident that a number of factors importantly affected the outcome of the interaction. The influence during incubation of time, temperature, and pH was investigated. The quantity of *Serr. marcescens* endotoxin was kept constant at 10 μg. for the interaction with two different volumes of plasma; *viz.*, 0.01 ml., a small but not limiting amount and 0.1 ml., a substantial excess.

Influence of Incubation Time, Temperature, and pH.—The time required for inactivation of the standard dose of endotoxin was dependent upon the amount of EDC present. At a low level (0.02 ml. of plasma), 20 minutes incubation at 37°C. was needed to inactivate 10 μg. of the standard endotoxin, whereas at 0.1 ml., 10 minutes sufficed.

With the duration of incubation and the volume of plasma kept constant at 60 minutes and 0.01 ml., respectively, the temperature was varied from 4 to 45°C. At the lower temperatures (4, 20, and 30°C.) no inactivation was ob-

servable. At 37 and 45°C. inactivation was complete. In an excess volume of plasma (0.1 ml.), partial inactivation of the 10 μg. was obtained at 30°C.

The effect of pH on EDC activity was examined over the range between 5 and 9. Endotoxin was inactivated at pH values of 7.5 through 9, but at pH 6 or

TABLE III

Influence on EDC Activity of Incubation Time, Temperature, and pH

Volume of plasma incubated with 10 μg. endotoxin*	Conditions of interaction			Induced tumor damage‡	Inactivation of endotoxin
	Time	Temperature	pH		
ml.	*min.*	°C.			
0.02	0	37	7.6	9/9	None
	5			7/9	"
	10			6/10	Partial
	15			3/10	"
	20			0/10	Complete
0.01	60	20	7.6	9/9	None
		30		8/9	"
		37		0/10	Complete
		45		0/10	"
0.01	60	37	5.0	8/10	None
			6.0	4/9	Partial
			7.0	1/9	Complete
			7.5	0/9	"
			8.0	0/9	"
			9.0	0/9	"
None (endotoxin in saline)	60	37	7.0	8/10§	
None (no endotoxin)	—	—	—	0/10‖	

* *Serr. marcescens*, Lot P-25.

‡ No. of mice with damaged tumors/total No. of mice tested.

§ Control group for endotoxic effect of 10 μg. of endotoxin alone.

‖ Control group for tumors in untreated mice.

below inactivation was incomplete or absent. Even when a tenfold larger quantity (0.1 ml.) of plasma was employed, only partial inactivation of the endotoxin occurred at pH 6 and none was evident at pH 5. The results of these experiments are recorded in Table III.

Influence of Concentration of Reactants.—The EDC activity of plasma was found to be influenced appreciably by dilution prior to incubation with endotoxin. Thus, inactivation of the standard dose of endotoxin was brought about by 0.01 ml. of undiluted plasma, while three times this amount was required when the plasma was first diluted 1:16.

The inactivation of endotoxin by plasma to a degree below that detectable in the tumor response assay does not necessarily signify total inactivation. For example, 10 μg. of endotoxin reacted with 0.01 ml. of plasma exhibited no endotoxic activity. On the other hand, when two or three times this quantity was reacted with 0.02 or 0.03 ml. of plasma, respectively, endotoxic activity was still present. This indicated that in the first situation a small amount of endotoxin remained unaltered and that this unchanged material could be detected only when the absolute quantity of endotoxin was sufficiently great. This interpretation is based on results obtained in the titration of the tumor-damag-

TABLE IV

Effect of Concentration on Lability of EDC to Heat

Volume of plasma incubated with 10 μg. of endotoxin*	Pretreatment of plasma	Induced tumor damage‡	Inactivation of endotoxin
ml.			
0.3	56 C. for 1 hr.	0/9	Complete
0.2		2/9	Partial
0.05		4/9	"
0.01		7/9	None
0.01	None	0/9	Complete
None (endotoxin in saline)	—	8/10§	—
None (no endotoxin)	—	0/10‖	—

* *Serr. marcescens*, Lot P-25.

‡ No. of mice with damaged tumors/total No. of mice tested.

§ Control group for endotoxic effect of 10 μg. of endotoxin alone.

‖ Control group for tumors in untreated mice.

ing activity of *Serr. marcescens* endotoxin itself, in which less than 3 μg. of endotoxin ordinarily induced no tumor damage.

Stability of EDC.—Serum or plasma stored for several months at either −55 or −20°C. exhibited the same EDC potency as freshly drawn samples. Human plasma retained its full endotoxin-inactivating capacity after storage for more than two months at 4 to 8°C.; its activity began to decline somewhat after 4 months.

Human serum, after heating at 56°C. for 1 hour, could no longer inactivate 10 μg. of endotoxin at a volume of 0.3 to 0.5 ml. Human plasma (collected in ACD), heated in the same manner and tested at the same volumes, appeared to be heat-stable. However, when tested at an appropriately low level, EDC was seen to be heat-labile in plasma also. In general, 0.01 ml. of human plasma exhibited about the same potency in inactivating endotoxin as did 0.4 ml. of human serum. It is seen from Table IV that when a minimally effective volume

of plasma was taken for test, both serum and plasma were found to be equally sensitive to heat.

Interaction of EDC with Various Bacterial Endotoxins.—The initial observations in this work were made with *Serr. marcescens* endotoxin, and thereafter this product was used as reference material. Endotoxins obtained from different sources, and prepared by different techniques, were also investigated. We had found (1) that human serum could inactivate endotoxic substances not only from bacteria but also from tissues of mammals and higher plants.

In the present study the tumor-damaging potency of seven bacterial endotoxins was determined before and after incubation with plasma.

TABLE V

Quantity of Human Plasma Required to Inactivate Various Endotoxins

Endotoxin			Minimum dose inducing tumor damage	Volume of plasma required for inactivation
Preparation	Source			
	Organism	Colonial type		
			μg.	*ml.*
Westphal (20)	*S. schottmuelleri*	Rough	15	0.5*
" (20)	*S. enteriditis*	"	10	0.5*
Raynaud (21)	*S. typhosa* R-2	"	10	0.5
Staub (22)	*S. typhosa* 901	Smooth	3	0.05
Webster (23)	*S. typhosa* 901	"	3	0.02
Westphal (20)	*E. coli* 018	"	3	0.4
Shear (24)	*Serr. marcescens*	"	10	0.01

* Partial inactivation at this level.

First the potency of these products, *i.e.* the smallest quantity required for induction of hemorrhage and necrosis in tumors, was assayed and found to vary somewhat for the seven endotoxins tested. Then each of the endotoxins was incubated with different volumes of pooled human plasma, and assayed again in tumor-bearing mice. The quantity of plasma which sufficed to abolish the tumor-damaging effect of each of the endotoxins is given in Table V.

It is seen that endotoxins prepared from different sources and by different methods varied considerably in their alterability by EDC. Three of the four products derived from colonially smooth bacteria were inactivated with relatively small amounts of plasma. In contrast, all three products derived from colonially rough variants required large amounts of plasma to affect their tumor-damaging activity. Of these three endotoxins from rough variants, that from *S. typhosa* required the maximum volume of plasma employed; even this volume effected only partial inactivation of the other two products from rough *Salmonella*. The potency of these endotoxins for induction of tumor damage was not related to their sensitivity to EDC action. To what extent these and other

properties of endotoxins are a consequence not only of their source but also of the techniques of isolation is not known.

Attempts to Prepare EDC-Free Plasma.—Preliminary attempts were made to remove EDC from plasma by adsorption with Gram-negative bacteria. Human plasma was incubated at 4, 20, and 37°C. with washed cell suspensions of heat-killed *Salmonella typhosa* and of viable *Salmonella typhosa*, *Salmonella typhimurium*, *Salmonella newport*, and *Escherichia coli*. Adsorption for 18 hours at 4°C. resulted in little or no reduction in EDC activity of the plasma. Adsorption for 2 to 4 hours at 20 or 37°C. released endotoxin from the bacteria, as shown by the acquisition of potency to induce tumor damage by this adsorbed plasma itself. Although it is possible that EDC is adsorbed in this manner, it was not feasible to obtain evidence on this point inasmuch as the endotoxin released from the adsorbing bacteria interfered.

DISCUSSION

Endotoxins contribute significantly to the toxic state characteristic of infection with Gram-negative bacteria. Despite constant exposure to these noxious substances, mammals usually are free from their known harmful effects. Recently attention has been directed to the capacity of endotoxins to alter the natural state of resistance of experimental animals to infection. Interest has been focused on alterations in the level of properdin in serum and in the phagocytic activity of the reticulo-endothelial system (RES). This and the following paper (10) describe some of the characteristics of an additional humoral component which may also be involved in host resistance inasmuch as it is widely distributed among mammalian species and is capable, *in vitro*, of rendering endotoxins innocuous.

Although the endotoxin-modifying action of blood components has received much attention, its assessment has been restricted largely to changes in pyrogenicity. Moreover, the reports on such alterations in pyrogenic potency following exposure to serum, plasma, or formed elements have been contradictory.

Thus, several investigators have described augmentation of pyrogenicity while others have reported its neutralization or reduction.[2] In the latter category, Hegemann has published a series of papers describing the neutralization of endotoxin by human serum as shown by absence of pyrogenicity for man (6, 26, 27). Maximal neutralization required incubation in fresh serum for 8 to 12 hours; however, when these mixtures were tested in rabbits, the fever activity was found to be reduced rather than abolished. These findings were confirmed, in part, by Goodale and associates (28) who also found that the pyrogenicity for man of an endotoxin was significantly

[2] The voluminous literature on this subject is covered in detail in the excellent review on bacterial pyrogens by Bennett and Cluff (25).

reduced upon incubation for 3 hours with fresh serum or heparinized plasma. However, we have observed (29) that incubation for only 1 hour was sufficient to reduce the pyrogenicity of endotoxin for rabbits when citrated plasma was used.

Although the tumor response procedure for evaluating inactivation differs considerably from that of pyrogenicity, it is likely that the same humoral system is involved in both situations: the findings were similar with regard to heat lability, non-dialyzability, and variability among animal species. On the other hand, Hegemann's finding that prolonged incubation was essential to bring about reduction or loss of activity stands in contrast to an incubation period of less than 1 hour which we found to suffice.

Ho and Kass (8) found that serum or plasma from man, rat, or rabbit "attenuated" the lethality for rats of crude toxic extracts from *S. typhosa*; however, this effect was not observed with Boivin-type endotoxins. They showed further that neither specific antibody nor properdin was required for this attenuation but that incubation for 3 hours was necessary to obtain consistently a partial protection of rats. In our experience, purified Boivin-type endotoxins were readily inactivated after only brief incubation.

It is difficult to reconcile these apparent discrepancies inasmuch as the experimental conditions varied in so many important respects, *e.g.* the source and means of isolation of endotoxin, the species of animal from which the serum or plasma was drawn, the species employed for bioassay and the technique used to measure change in potency of altered endotoxin.

Rowley reported that an alkaline phosphatase in the serum of several mammalian species split phosphate from endotoxin (7). This serum activity differed from the one reported here in that it had a narrow pH optimum and was largely dependent upon the presence of divalent cation (Ca^{++} or Mg^{++}). He demonstrated this cation dependency by passing the serum through a cationic exchange resin, followed by treatment with EDTA. These procedures greatly decreased the serum activity which Rowley studied, whereas these were the same procedures employed by us (10) to greatly increase the EDC potency of serum. The relationship of such phosphate cleavage to the biologic inactivation of endotoxin remains to be demonstrated. In any event, it is evident that requirements for activity in these two situations are of opposite nature.

The alterations produced by blood in the properties of endotoxins have been examined by many investigators, of whom some employed serum or plasma while some used both. Until now, no difference between serum and plasma has been reported with respect to diminution of endotoxic activity. This difference which is described in the present work was not evident in previous investigations for two reasons: (*a*) pyrogenicity was usually taken as the principal indicator of endotoxin alteration, and (*b*) heparin was used as the anticoagulant in the collection of plasma. As is shown in the following paper, heparinized plasma

does not differ from serum in its ability to inactivate endotoxin, whereas citrated plasma does.

Although the nature of the anticoagulant is of prime importance this, by itself, is not sufficient to account for failure of this difference between serum and plasma to have become evident—the methods by which endotoxin activity is usually measured were not likely to reveal differences of this magnitude. Those reactions of laboratory animals to endotoxins which have been the object of such intensive study in recent years were found to be unsuitable as bioassay methods for this particular purpose. Though these reactions are capable of demonstrating the differences (*cf.* the following paper) once they were established by means of tumor response, they did not provide sufficiently consistent data to serve for quantitation. A major difficulty is the well recognized variability of response among individual animals to a given dose of the same material; this is particularly true of the rabbit.

These various reactions were examined by us for suitability as quantitative methods for measuring endotoxin. Lethality as an indicator was discarded when we found that it was not sensitive enough since it required a relatively large dose of endotoxin which, moreover, was too variable even in the controls to serve for the prompt recognition of inactivation. These considerations applied, in varying degrees to 10-day-old chick embryos, to mice and to rabbits. Local skin reactions in rabbits, both the customary Shwartzman phenomenon and the epinephrine potentiation of endotoxin, were examined but were found to be inconsistent because of variations among individual animals.

The pyrogenic response of rabbits, widely employed as an indicator of endotoxin activity was also examined. We found it to represent a special, if not an anomalous, situation. Under conditions in which endotoxin, after incubation with citrated plasma, was found to be inactive in the induction of tumor damage, in preparatory potency in the local Shwartzman reaction, and in lethal effects for thorotrast-prepared rabbits, it was still capable of eliciting fever of substantial magnitude (29). The reason for this seeming paradox is at present obscure, but at any rate these findings suggest that conclusions based on pyrogenicity in rabbits do not necessarily apply to other host reactions to endotoxin.

To meet the requirements of the present study, the induction of hemorrhagic necrosis in mouse Sarcoma 37 was employed. This reaction has been used in this Laboratory for many years as a measure of the potency of polysaccharides isolated at first from Gram-negative bacteria and, more recently, from mammalian tissues. In mice of known genetic constitution, tumor damage was readily and consistently induced with microgram quantities of purified endotoxic polysaccharides. Despite the limitations (11) of the reaction as a quantitative assay method for such endotoxic materials, it was found to provide results of sufficient sensitivity and uniformity to be useful for the study of inactivation of endotoxin.

The mammalian host possesses a number of humoral factors which react with endotoxins. These include specific antibody and a number of non-specific substances such as properdin, complement and the detoxifying component (EDC) described in this communication. Although each of these components can be shown to undergo different reactions with endotoxin, the possibility is now considered that these agents and perhaps still others do not function separately in the animal. It may be that they participate in a highly integrated series of reactions by which the host progressively modifies endotoxin to the point at which it is rendered inactive.

SUMMARY

A humoral substance which inactivates endotoxin *in vitro* has been shown to be clearly distinguishable from complement, properdin, and specific antibody. For the present, it is designated "endotoxin-detoxifying component" or EDC.

Animal species could be grouped in three categories with regard to the EDC activity of their sera; rat serum was highly potent; chimpanzee, dog, horse, and guinea pig sera were much less active; mouse, rabbit, and sheep sera exhibited no activity. The EDC potency of human sera varied widely, ranging from high to barely discernible activity. In contrast to the variations of EDC potency in serum, citrated plasma from all species manifested high potency of about the same magnitude.

The influence of time, temperature, pH, and concentration of reactants on the inactivation of endotoxin by EDC was examined. EDC activity in plasma and serum was found to be labile to heating at 56°C. for 1 hour. Bacterial endotoxins, derived by different isolation procedures from smooth and rough Gram-negative species, varied considerably in susceptibility to EDC action.

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