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SECTION MEETINGS

IOWA	
University of Iowa	October 27, 1964
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MINNESOTA	
Mayo Foundation	October 2, 1964
SOUTHWESTERN	
Texas Medical Center, Houston	November 6-7, 1964

Anticomplementary Action of Endotoxin.* (29657)

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The anticomplementary action of endotoxins upon complement (C′) has been studied by numerous investigators resulting in general agreement that endotoxin exerts an anticomplementary effect *in vitro* when present in relatively high concentration in serum(1) and a depression of C′ levels as a result of injection into animals(2,3,4). In addition to other evidence based primarily upon certain similarities between reactions of hypersensitivity and endotoxin shock, the anticomplementary action of endotoxin is consistent with the theory that endotoxin produces injury, at least in part, by means of an antigen (Ag)-antibody (Ab) reaction. Thus, injection of endotoxin into an animal or addition of endotoxin to serum inevitably leads to formation of an Ag-Ab complex, since naturally occurring antibodies to the Gram-negative enteric bacteria and their corresponding endotoxins are present even in animals that have not been infected with the corresponding organism or deliberately immunized(5). The finding of Kostka and Sterzl(1), indicating relatively greater resistance of the serum of newborn piglets prior to their ingestion of maternal colostrum to the anticomplementary action of endotoxin compared to that of the serum of the adult pig, was in accord with this theory in view of the fact that adult pig serum contains normal antibody in larger amounts than the piglet serum. Despite these considerations, considerable doubt exists concerning the mechanisms responsible for the anticomplementary effects of endotoxin. Pearlman,

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Sauers and Talmadge(4) have indicated that, although their own findings also showed a decline of serum C′ after injection of adjuvant amounts of endotoxin, there was a lack of concordance with the *in vitro* findings of Kostka and Sterzl(1). The former group of investigators believed that the relatively small amount of endotoxin, as well as its rapid disappearance from the blood stream, suggested that the observed decline in C′ after endotoxin injection was probably not caused by the fixation of C′ to Ag (endotoxin)-Ab complexes. Similarly, Toussaint and Muschel(6) have shown that the inactivation of C′ by endotoxin *in vitro,* determined by phage neutralizing activity rather than hemolysis, may result from mechanisms other than C′ fixation by Ag (endotoxin)-Ab complexes. In view of these marked disagreements relating to the *in vitro* anticomplementary activity of endotoxin and its obvious bearing on the toxicity of endotoxin, the present work, involving a reexamination of the anticomplementary activity of endotoxin in serum, particularly its possible mediation by an Ag-Ab complex, was undertaken.

Materials and methods. The endotoxin preparations were obtained from Difco Laboratories. Titrations of hemolytic C′ of the sera of different species were performed by a standardized procedure(7). The anticomplementary activity of the endotoxin preparations was determined by addition of the endotoxin to serum and incubation of the serum with endotoxin for one hour at 37°C prior to the C′ assay. A serum control was incubated similarly without addition of endotoxin in all experiments. For absorption of sera by appropriate bacterial cells, the growth from a 16 hour culture on meat extract agar in 2 Petri plates (about 3×10^{10} cells/plate) were washed 3 times with saline, heat killed (56°C for 30 minutes), and suspended in 2.5 ml of serum. The serum was then removed after centrifugation at 4°C of the bacterial suspension.

Results. Comparison of the anticomplementary activity of endotoxins prepared by the Boivin (trichloracetic acid extraction of the organisms) and the Westphal (extraction with hot phenol water) methods. The Boivin endotoxin was considerably more anticomplementary than the Westphal preparation against both rabbit and guinea pig sera. With an amount of rabbit serum required for 95% hemolysis without endotoxin, 3 mg of the Boivin endotoxin of *Salmonella typhimurium* lowered the percentage of hemolysis to 60%, whereas that amount of the Westphal preparation scarcely produced a detectable decline. In a more precise experiment with guinea pig serum, about 1 mg of the Boivin endotoxin of *S. typhimurium* sufficed for a 50% loss of hemolytic activity whereas 2 mg of the Westphal preparation was required for a comparable loss.

TABLE I. Anticomplementary Action of *S. typhosa* 0901 Endotoxin (Boivin) upon Sera of Different Species.

Endotoxin, mg/ml	Serum: Guinea pig .01 ml	Serum: Rabbit .2 ml	Serum: Human 1 ml
None	85	95	85
.084	85	95	0
.17	70	90	0
.33	50	80	0
.66	0	65	0

Percent hemolysis after incubation at 37°C for 1 hr with different concentrations of endotoxin prior to addition of sensitized cells.

The low activity of human serum in this experiment (1 ml comparable to 0.2 ml of rabbit and 0.01 ml of guinea pig) is a result of the greater lability of human serum at 37°C. C′ titrations are performed generally without prior incubation of the test serum.

Quantitation of the anticomplementary action of endotoxins with the sera of different species. As Table I indicates, relatively high concentrations were required for a detectable anticomplementary effect with rabbit serum. Guinea pig serum was significantly more susceptible and human serum at least 8 times more susceptible than rabbit serum. Comparable results were obtained with endotoxins derived from *Salmonella abortus equi* and *Serratia marcescens* so that differences in Ab levels in the sera of the different species would seem not to be a likely explanation for the differences in the susceptibility of their C′ to inactivation by endotoxin.

Anticomplementary activity of endotoxin in sera from which normal Ab was removed

by absorption. To determine whether the anticomplementary activity of endotoxin was mediated by a reaction between endotoxin and normal antibodies to the endotoxins, the anticomplementary effect of *S. typhosa* 0901 endotoxin (Boivin) in guinea pig serum was compared with samples of the same serum absorbed with cells of *S. typhosa* 0901 and also with cells of serologically unrelated *Escherichia coli* 055:B5. The effect of different amounts of endotoxin on the hemolytic C′ activity of 0.01 ml of the untreated sample of guinea pig serum was as follows:

mg Endotoxin/ml	Hemolysis, %
No endotoxin	75
.083	70
.166	50
.333	25

The samples absorbed once, twice, or four times with each organism all gave identical results.

Anticomplementary activity of endotoxin in immune serum. Since Ag (endotoxin)-Ab complexes fix C′, it was expected that endotoxin would be more potent in destroying the C′ of an immune serum than that of normal serum. That proved to be the case. With a rabbit anti-*S typhosa* 0901 serum having an agglutination titer of 1:1280, reduction of its hemolytic C′ activity by 50% required an *S. typhosa* 0901 endotoxin (Boivin) concentration of 0.08 mg/ml whereas over 3 times that amount was needed for a comparable loss with normal serum.

Anticomplementary effect of endotoxin in serum of the piglet and of the adult pig. In contrast to previously reported results(1), the serum of 2 piglets obtained prior to intake of colostrum was exceedingly more sensitive to *S. typhosa* 0901 endotoxin (Boivin) than that of their sows. With 0.4 ml of the piglet's serum and 0.1 ml of the adult pig's serum each giving 90% hemolysis, as little as 0.02 mg/ml of endotoxin practically destroyed the hemolytic activity of the former while 0.66 mg/ml had only a scarcely detectable effect on the latter. Another experiment was performed in which the serum of the adult animal was diluted 1:4 with other adult pig serum decomplemented by an immune precipitate (ovalbumin-anti-rabbit ovalbumin) to equalize the serum concentrations in the C′ titrations of piglet and adult sera. This procedure did not alter the resistance of the adult pig's serum. Moreover, although less potent, Westphal endotoxin was similarly more destructive to the C′ of the piglet than to the C′ of the adult pig.

Attempted neutralization of the anticomplementary action of endotoxin by lysozyme and Ca ion. Since the acidic groups of the lipopolysaccharide endotoxins might contribute conceivably to their anticomplementary effects, lysozyme, a basic protein, was added to the test system. Egg white lysozyme (product of Worthington) in a concentration of 1.6 mg/ml was anticomplementary itself against guinea pig C′; smaller concentrations were ineffective in inhibiting the anticomplementary action of endotoxin. Ca ion, which plays a significant part in the stabilization of C′(8), was ineffective similarly.

Discussion. These results have confirmed the experimental observations noted in previous work relating to the *in vitro* anticomplementary action of relatively large amounts of endotoxin. Marked variation in susceptibility of the C′ in the sera of different species was noted. Human serum was over 8 times more sensitive than rabbit serum with guinea pig serum intermediate. The significance of this finding and its relation to the extreme sensitivity of the human subject in whom 0.5 μg of *S. typhosa* endotoxin injected intravenously produces a severe reaction(9) is doubtful, but may be worth bearing in mind.

As expected, the anticomplementary action of endotoxin upon a homologous immune serum was greater than its action against the C′ of a normal serum. Obviously, the immune complex formed by addition of endotoxin (Ag) to an Ab containing serum fixes C′. With normal serum containing relatively low concentrations of Ab, however, the anticomplementary action of endotoxin cannot be attributed exclusively to C′ fixation by an immune complex, but results primarily from other unknown mechanisms. The findings with the absorbed sera provide the strongest evidence in support of this view. When se-

rum was absorbed with cells of *S. typhosa* 0901 to remove its normal Ab against that organism, it was no less sensitive than the untreated, unabsorbed sample to the anticomplementary action of *S. typhosa* 0901 endotoxin. These findings determined by the assay of hemolytic C′ confirm those previously reported with the determination of C′ by phage neutralization(6). Other ancillary evidence that the anticomplementary action of endotoxin is not mediated *via* an Ag-Ab reaction includes the marked sensitivity of human sera to 3 serologically unrelated endotoxins and the relative insensitivity of rabbit or guinea pig sera to these endotoxins. It is unlikely that the greater sensitivity of human sera is a result of greater levels of Ab in human sera to 3 serologically unrelated endotoxins. Finally, the greater susceptibility of the serum of newborn piglets that had not received colostrum, compared to the serum of the adult pig, suggest also that endotoxin may exert an anticomplementary effect without the intervention of Ab, since piglet serum contains no detectable gamma-globulin(1) and lower levels of normal Ab than the adult animal. Though it is quite likely that the sera of different piglets may vary in the sensitivity of their C′ to endotoxin, which would reconcile our results with those previously reported(1), the piglet sera used in this study were also relatively lacking in gamma-globulin and antibody. Thus, the susceptibility of these sera to endotoxin must have resulted from the presence or absence of other serum constituents.

In brief, this study has confirmed former work relating to the anticomplementary action of endotoxin, but it remains to be determined how endotoxin exerts its anticomplementary effect on normal serum and, upon injection into the intact animal, gives rise to a depression of its C′ level. If an Ag-Ab reaction is involved in the toxicity of endotoxin, there would seem to be little basis at present for implicating the inactivation and participation of the C′ system although excellent experimental evidence has been obtained that a heat labile system in plasma may be involved in lethal endotoxin shock in dogs(10). The wide variety of the physiological effects produced by endotoxins(11) as well as the time lag in C′ depression after its injection into an experimental animal(4) suggests an indirect effect of endotoxin upon C′.

The failure of a basic protein such as lysozyme to neutralize the anticomplementary action of endotoxin indicates that the acidic groups of endotoxins are not involved probably in its anticomplementary action. The addition of Ca ion to the test system was based upon the premise that endotoxin may exert its anticomplementary effect by promoting the decay of C′ at 37°C, which can be prevented partially by Ca ion(8). However, this ion did not nullify the anticomplementary effect of endotoxin.

Summary. These results have indicated that the anticomplementary action of endotoxin upon normal serum is not mediated by an Ag (endotoxin)-Ab complex. Removal of Ab from normal serum had no detectable effect upon such anticomplementary action. In addition, the serum of the newborn piglet obtained prior to feeding and relatively lacking in Ab was more susceptible to endotoxin's anticomplementary action than the serum of its sow. Other ancillary evidence such as the marked difference in the susceptibility of the C′ of different species to endotoxin also supports a non-immunological mechanism for its anticomplementary action. Although an Ag (endotoxin)-Ab complex may possess potent C′ fixing action, other mechanisms, unknown at present, are responsible probably for the anticomplementary action of endotoxin in normal serum.

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Interaction of Autologous Complement with Red Cells in the Absence of Antibody.*† (29658)

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Significant advances have recently been achieved in relation to chemical characterization and elucidation of the mode of action of serum complement components and their biological significance. One of the important aspects in the study of serum complement is that of the molecular interaction between complement and cell membranes. The study of complement-membrane interaction is usually approached by using the classical hemolytic system consisting of sheep cells coated with non-agglutinating amounts of rabbit antibody, and guinea pig or human serum as a source of complement. In this system the analysis of complement-membrane interaction is complicated by the presence of antibody. This difficulty may be overcome by use of non-specific sensitizers which are capable of mediating the complement reaction without attaching themselves to the membrane.

Among the various substances that have been described as non-specific sensitizers, polyethylene glycol (PG) has been studied most thoroughly(1). McVickar(2) found that this agent is capable of enhancing the hemolytic effect of guinea pig serum on sensitized sheep erythrocytes. Subsequently, Cowan(1) demonstrated that PG can actually substitute for specific antibody in the sheep cell-guinea pig complement system, and that it can thus function as a non-specific sensitizer. He concluded that PG exerts this effect by combining loosely with the red cell membrane and by thus linking complement to its surface.

In the present paper, data are presented which favor an alternative mechanism to explain the effect of PG. To preclude the participation of antibody, all experiments were carried out using an autologous system consisting of human red cells and serum. Analysis of this model has furnished evidence for the occurrence of a direct link between the complement components β_{1E} and β_{1C}-globulin on the one hand and the red cell membrane on the other. In addition, evidence was obtained indicating that membrane-bound neuraminic acid counteracts the attachment of complement components in the absence of antibody. Removal of neuraminic acid from the red cell surface facilitates complement fixation in the PG system.

Materials and methods. Erythrocytes and serum were obtained from healthy white donors. Blood was collected in ACD solution, the plasma and buffy coat were removed and the red cells were washed 3 times with 20 volumes of Ca^{++} and Mg^{++} containing isotonic veronal buffer, pH 7.4. The packed red cells were suspended in the same buffer in a concentration of 2.5%. To obtain serum, a second aliquot of blood was withdrawn from each donor.

Enzymatic treatment of erythrocytes. Washed red cells were suspended in veronal buffer containing 1 mg/ml of either trypsin, chymotrypsin, β-glucuronidase or pancreatic lipase (Worthington Biochemical Corp., Free-

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