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Interactions of the Complement System with Endotoxic Lipopolysaccharide: the Generation of an Anaphylatoxin

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Summary. The incubation of endotoxin derived from *Veillonella alcalescens* or *Serratia marcescens* with fresh guinea-pig serum leads to the production of a factor which contracts guinea-pig ileum. This factor has been identified as an anaphylatoxin since it causes tachyphylaxis and its activity is abolished by antihistamines. The activity is not generated if the serum has been heat inactivated or if the reaction is carried out at 0° or in the presence of EDTA. The complement system (C′) is implicated in the generation of this anaphylatoxin by the above data and by static and kinetic C′ consumption studies which show a correlation between the disappearance of the late acting C′ and the appearance of gut contracting activity. Endotoxoid preparations which do not utilize C′ are not able to generate anaphylatoxin. A further parallel between classical anaphylatoxin and the endotoxin-generated factor is found in the pattern of mammalian sera which support the reaction. In view of the similarities between the biological properties of anaphylatoxin and the syndrome of endotoxic shock, it is suggested that some of the manifestations of the latter might result from an interaction between endotoxin and the C′ system leading to the generation of an anaphylatoxin.

INTRODUCTION

Among the physiological changes which follow the administration of bacterial endotoxic lipopolysaccharide (LPS) to experimental animals and man is an alteration of smooth muscle tone and vascular permeability. At their extreme, these events are considered to contribute to the syndrome of 'endotoxin shock' (Spink, 1962). The biochemical basis for these changes is not yet clear. The small amounts of LPS capable of bringing them about has, however, led several investigators to surmise that LPS does not act directly upon tissue receptors, but rather acts indirectly via activation of serum enzymes (Thomas, 1954), such as members of the complement (C′) system (Spink, Davis, Potter and Chartrand, 1964).

Recent advances in the dissection of the C′ system and studies of the LPS–C′ interaction *in vitro* have provided an additional conceptual basis for this hypothesis. It is becoming increasingly clear that LPS and C′ exert dramatic effects upon one another when they interact in fresh mammalian serum. Lesions indicative of terminal C′ component activity appear on membranous LPS particles (Bladen, Gewurz and Mergenhagen, 1967) while, simultaneously, pronounced consumption of the six terminal C′ components ensues

(Gewurz, Shin, Mayer and Mergenhagen, 1968b). It is known that activation of these terminal C′ components is associated with the generation of several biologically-active factors. Prominent among these is 'anaphylatoxin', a serum product known to exert pronounced effects upon smooth muscle contractility and vascular permeability. This was first demonstrated by Osler, Randal, Hill and Ovary (1959) and has recently been confirmed with isolated components (C′3 and C′5) by several different groups. Hence, it seemed possible that the alterations of smooth muscle tone and vascular permeability initiated by LPS were mediated in part via the C′ system by generation of anaphylatoxin(s). The present experiments were designed to determine directly whether an anaphylatoxin is generated during the incubation of LPS with fresh mammalian serum as suggested by the work of Greisman (1960) and, if so, whether the C′ system participates in its formation.

MATERIALS

Endotoxic lipopolysaccharide (LPS)—the LPS used in most of the experiments was derived from *Veillonella alcalescens* by the phenol–water extraction procedure of Westphal and Lüderitz (1954). LPS also was prepared from *Serratia marcescens* by the trichloracetic acid extraction procedure as previously described (Nowotny, Thomas, Duron and Nowotny, 1963) (generously provided by Dr A. Nowotny). A detoxified derivative of this latter preparation ('endotoxoid') was prepared by deacylation with potassium methylate (Tripodi and Nowotny, 1966) (generously provided by Dr A. Nowotny).

Zymosan

Zymosan, a cell wall preparation of yeast, was obtained from General Biochemical Corporation, Chagrin Falls, Ohio (Lot No. 59844).

Immune complexes of bovine serum albumin (BSA) and rabbit antiserum (rabbit anti-BSA)

Crystalline BSA was obtained from Pentex Incorporated, Kankakee, Illinois. Rabbit anti-BSA was obtained from Hyland Laboratories, Los Angeles, California; precipitin analyses showed that it contained 240 μg antibody nitrogen/ml. BSA and rabbit anti-BSA were reacted at equivalence for 24 hours at 4° in the presence of 0·01 M EDTA. The precipitate was washed twice in EDTA–saline, twice in normal saline, and resuspended in normal saline. Final protein concentrations of the immunoprecipitate were determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

Aggregated human γ-globulin (HGG)

HGG was obtained as Cohn Fraction II from Hyland Laboratory, Los Angeles, California, and heated at 63° for 20 minutes.

Sources of various mammalian sera

Guinea-pig serum was obtained from Texas Biologicals, Incorporated, Fort Worth, Texas. Normal human serum was provided by young-adult male laboratory personnel. Rabbit serum was collected from adult New Zealand White rabbits. Sow serum was obtained from Minnesota miniature pigs. Mouse serum was collected from adult males of the BALB/c strain. Dog serum was pooled from mongrel animals. Rat serum was from Sprague–Dawley rats.

METHODS

Anaphylatoxin generation

Various concentrations of endotoxin, endotoxoid, antigen–antibody complexes, aggregated HGG, or zymosan were added in a volume of 0·1 ml to 0·9 ml of fresh or fresh frozen sera (−60°) of the species in question. The tubes were mixed, incubated for 60 minutes at 37° and then assayed by the addition of 0·2-ml aliquots to the Schultz–Dale apparatus as described below. In some experiments the specimens were frozen (−20°) before assay.

Assay for anaphylatoxin activity

The isometric Schultz–Dale apparatus described by Randall, Talbot, Neu and Osler (1961) was used with minor modifications in the recording system. An isotonic Krebs type buffer with 10^{-6} M atropine as described by Randall *et al.* (1961) was used in all experiments. The 10-ml muscle bath was maintained at 37° and a gas mixture of 95 per cent O_2 and 5 per cent CO_2 bubbled into it continuously. Gut sections, 3–4 cm in length, were obtained from 200- to 400-g male guinea-pigs. These were allowed to equilibrate in the bath for 10–15 minutes after which they were repeatedly challenged with 0·1–2 μg of histamine* (final concentration, 0·01–0·2 μg/ml) until a reproducible contractile force was obtained. This generally required 15–30 minutes. A maximal contraction was usually obtained with 0·5–1·0 μg of histamine added to the 10-ml bath. Samples to be tested were bracketed between several standard histamine challenges and were always tested directly after a level of histamine which induced a maximal response. Since the extent of tachyphylaxis was found to vary from gut to gut, only one challenge with anaphylatoxin containing samples was made with each gut section, unless the phenomenon of tachyphylaxis itself was under investigation. Once a maximal contractile force was achieved, the fluid in the bath was changed so that only the amplitude, and not the duration, of the contractile force was considered. Each observation was repeated at least twice.

Complement fixation

The ability of LPS, detoxified LPS, washed preformed BSA–anti-BSA complexes, zymosan and heat aggregated human γ-globulin to consume C′ was determined in reaction mixtures identical with those used in testing for their ability to generate the factor(s) with anaphylatoxin activity. In these experiments, 0·1 ml test reagent was reacted with 0·9 ml normal guinea-pig serum for 1 hour at 37°. Residual total C′ activity was measured by the method of Osler, Strauss and Mayer (1952), and residual C′1, C′4, C′2 and classical C′EDTA activities were measured with minor modifications (Gewurz, Page, Pickering and Good, 1967) of the methods of Mayer (1961) and Nelson, Jensen, Gigli and Tamura (1966).

The classical C′EDTA activity is now known to involve at least six separate proteins termed C′3, C′5, C′6, C′7, C′8 and C′9. The kinetics of anaphylatoxin generation and of the fixation of the complement components C′3 and C′5 was investigated by adding 1000 μg LPS to 5·0 ml guinea-pig serum at 37°. Aliquots were quick frozen at various times and residual C′3 and C′5 activities were quantitated by minor modifications (Shin and Mayer, 1968) of the methods of Nelson *et al.* (1966).

* Histamine standards were made fresh daily by dissolving histamine-dihydrochloride (Fisher H-295) in fresh Krebs buffer.

RESULTS

I. THE GENERATION OF ANAPHYLATOXIN BY ENDOTOXIN

When LPS is mixed with fresh undiluted guinea-pig serum and allowed to incubate at 37° for 60 minutes a factor is produced which causes the guinea-pig ileum to contract. No contraction is noted when endotoxin alone is added to the gut, and serum alone produces either no contraction or a minimal alteration of resting tension (Fig. 1).

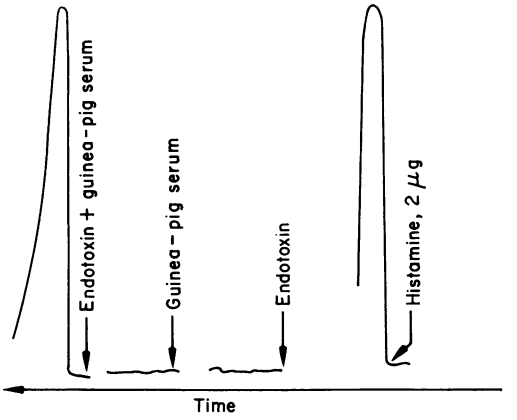

FIG. 1. The demonstration of a gut contracting factor following the addition of 0·2 ml from an incubation mixture of 200 μg of *V. alcalescens* endotoxin in 1·0 ml of guinea-pig serum. In this and subsequent figures the contraction induced by histamine is maximal for that gut and represents a contractile force of 5–8 *g*. The abscissa represents time; it is discontinuous between challenges. Paper speed was 1·5 cm/min.

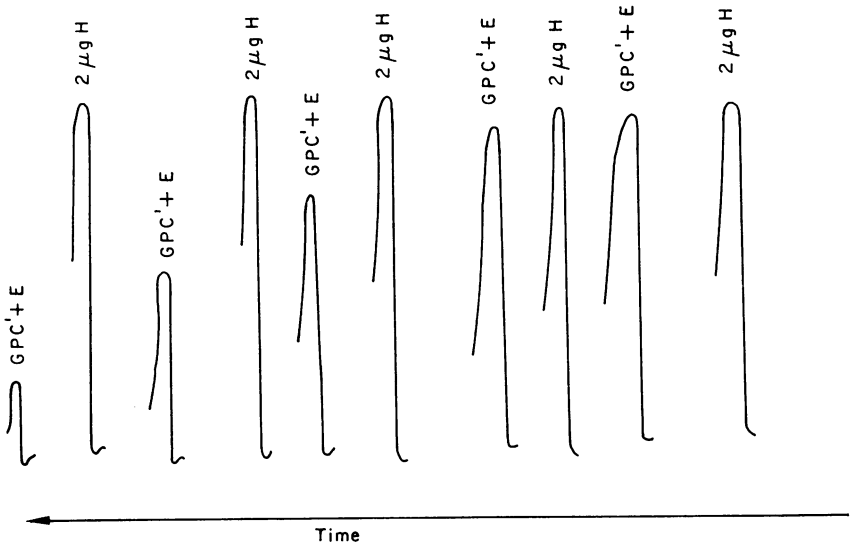

FIG. 2. Tachyphylaxis: the decreasing response of the ileal strip to repeated identical challenges with the serum–endotoxin incubation mixture. H, Histamine; GPC'+E, 0·2 ml from an incubation mixture of 200 μg of *V. alcalescens* endotoxin in 1·0 ml guinea-pig serum.

The product of the serum–LPS reaction appears to have the characteristics of anaphylatoxin. Tachyphylaxis, the progressively decreasing response to repeated identical challenges, is regularly observed (Fig. 2). The rapidity with which tachyphylaxis develops varies from gut to gut but usually a significantly decreased response is seen on the second or third addition of the LPS–serum mixture. In accord with the previously described properties of anaphylatoxin, the contraction caused by the LPS–serum product is completely inhibited by the presence of antihistamine (Fig. 3). Moreover, no activity is seen if the reaction is carried out in the presence of ethylenediamine-tetraacetate (EDTA), at 0°, or at 37° with serum which has been previously heated at 56° for 30 minutes (Fig. 4). Once the product has been formed, however, heating to 56° does not lead to any decrease in its activity. The LPS–serum product was also shown to mediate increased capillary permeability, as demonstrated by blueing of guinea-pig skin within a few minutes after intradermal injections in an animal given Evans blue dye intravenously. Thus the serum–LPS product appears to have the biological characteristics of an anaphylatoxin.

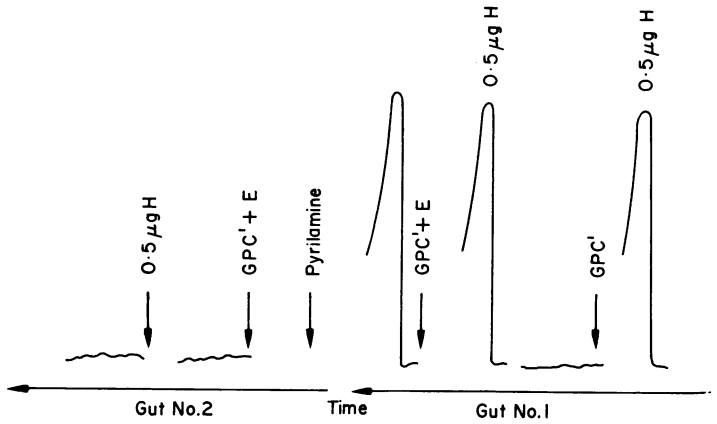

FIG. 3. Inhibition by the antihistamine pyrilamine maleate (10^{-6} M) of the contraction caused by the endotoxin–guinea-pig serum mixture. H, Histamine; GPC'+E, 0·2 ml from an incubation mixture of 200 μg of *V. alcalescens* endotoxin in 1·0 ml guinea-pig serum.

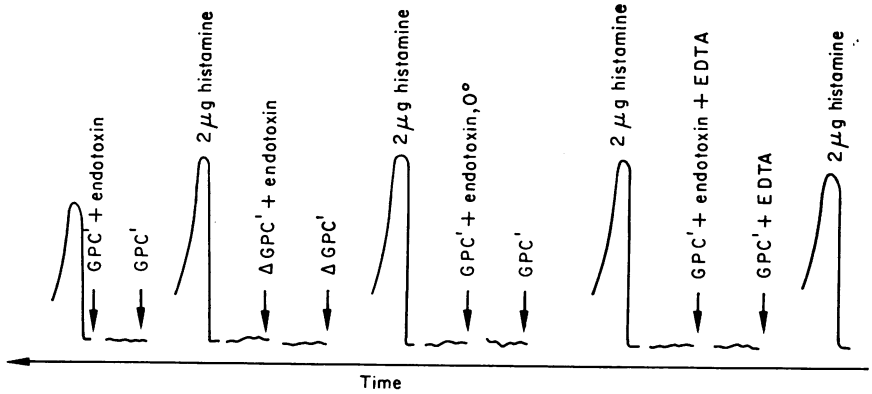

FIG. 4. The failure of endotoxin to generate anaphylatoxic activity when incubated with guinea-pig serum in the presence of EDTA, at 0°, or when the guinea-pig serum had been pre-heated at 56° for 30 minutes (Δ GPC'). GPC'–endotoxin mixtures as in Fig. 3.

Anaphylatoxin is known to be generated in guinea-pig serum by antigen–antibody interactions as well as by a wide variety of other substances including agar, zymosan and a cobra venom factor (Vogt, 1967). A 0·2-ml aliquot of a reaction mixture containing 10 μg of endotoxin per ml serum induces a definite response on the guinea-pig ileum and a maximal response is usually noted when the reaction mixture contains between 50 and 200 μg/ml serum. The ileal response is similar in all respects to that generated by BSA–anti-BSA precipitates whether preformed or formed in the presence of serum, and is of the same order of magnitude on a weight basis. Considerably larger quantities of zymosan and heat aggregated human γ-globulin are required. A maximal response was obtained with 1000 μg zymosan/ml serum but often the highest concentrations of aggregated HGG (1000 μg/ml serum) produced little or no contraction. The ileal response to the LPS–serum mixture was also similar to that generated with cobra venom factor (Jensen, 1967; Cochrane and Müller-Eberhard, 1968).

The older literature contains a number of reports dealing with the capability of the sera of various species to support the generation of anaphylatoxin (Vogt, 1967). With endotoxin as the inciting agent our results are generally in accord with these earlier reports. Pig and rat serum provide the best substrate for anaphylatoxin generations; guinea-pig serum is quite adequate and mouse serum only weakly active. Anaphylatoxin formation by endotoxin could not be clearly demonstrated in human, dog or cow serum.

II. THE RELATIONSHIP BETWEEN LPS GENERATED ANAPHYLATOXIN AND THE C′ SYSTEM

The role of C′ in endotoxin-generated anaphylatoxin is suggested by the experiments shown in Fig. 4. The admixture of LPS and guinea-pig serum in presence of EDTA, in the cold, or with heat inactivated serum (conditions which prevented activation of the terminal C′ components) completely inhibited the generation of anaphylatoxin. When anaphylatoxin was generated, however, C′ was consumed. The bar graph in Fig. 5 relates the fixation of the C′EDTA components with the generation of gut contracting activity by endotoxin. It is evident that the generation of anaphylatoxin with endotoxin parallels the consumption of the late acting C′ components. Consumption of the early C′ components could not be demonstrated. Fig. 6 strengthens this parallel with respect to the C′3

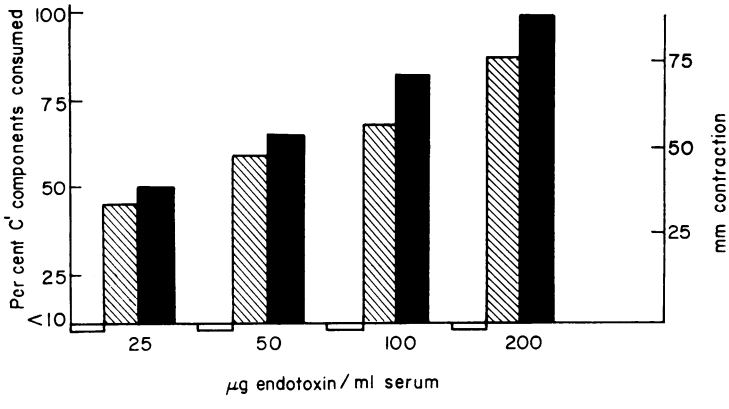

FIG. 5. Correlation of C′EDTA consumption and anaphylatoxin generation upon incubation of graded amounts of *V. alcalescens* endotoxin with guinea-pig serum at 37° for 1 hour. No consumption of C′1, C′4 or C′2 could be detected. Solid columns, Contraction; hatched columns, C′EDTA; open columns. C′1,4,2.

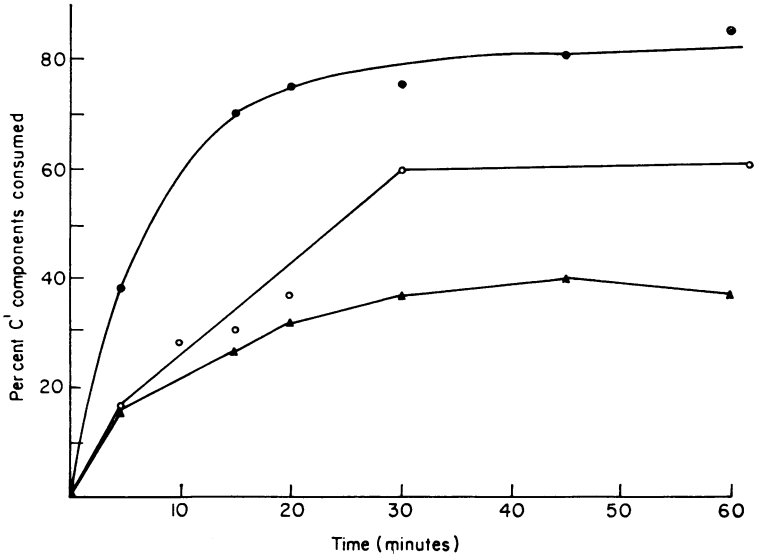

FIG. 6. Kinetic study showing a correlation between consumption of C'3 and C'5 components of complement and the generation of anaphylatoxin during the incubation of 1000 μg *S. marcescens* endotoxin with 5·0 ml guinea-pig serum at 37° for various periods of time up to 1 hour. ●, C'3; ▲, C'5; ○, mm contraction.

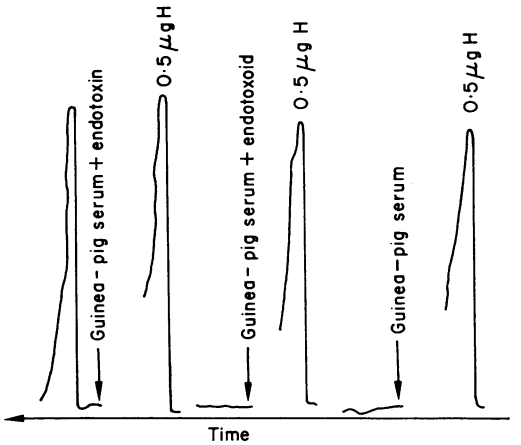

FIG. 7. The failure of endotoxoid to generate anaphylatoxic activity. Both the endotoxin and the endotoxoid were prepared from *S. marcescens* and reacted with guinea-pig serum at a concentration of 200 μg/ml serum. The endotoxin was toxoided by deacylation; following this treatment it no longer consumed complement. H, Histamine.

and C'5 components. In this experiment the reaction of endotoxin and serum was stopped by rapid freezing in an acetone ice bath. The rate of consumption of C'3 and C'5 was similar to the development of anaphylatoxin activity. In each case the reaction was essentially completed in about 30 minutes. This parallelism is compatible with a role for either C'3 or C'5 or both but it should be recognized that the kinetic assays of C'3 and C'5 consumption are more precise than that of gut contracting activity.

Another correlation between C′ fixation and anaphylatoxin generation comes from a study of chemically treated endotoxins. It has long been appreciated that the biological effects of endotoxin could be altered by several chemical manipulations which lead to the production of what have been called endotoxoids. A preparation 'toxoided' by deacylation loses its ability to fix detectable amounts of any of the C′ components (Gewurz, Mergenhagen, Nowotny and Phillips, 1968a). As shown in Fig. 7 such a preparation also loses the ability to generate anaphylatoxin on interaction with guinea-pig serum.

DISCUSSION

Endotoxic shock in man and animals has been extensively studied but the biochemical mechanism of the pathophysiology is not yet clear (Spink, 1965). While the syndrome has several similarities to anaphylactic shock the role of antibody in its induction has not been established. Recent work on the interaction of endotoxin and the C′ system has shown that the LPS is extremely effective in initiating the consumption of C′3–C′9 in normal serum (Bladen *et al.*, 1967; Gewurz *et al.*, 1968b). The pioneering work by Osler *et al.* (1959) several years ago established that the C′ system, especially what we now recognize as the latter components, was involved in the generation of anaphylatoxin from rat serum. Extending these observations the work of Dias da Silva and Lepow (1965, 1967), Dias da Silva, Eisele and Lepow (1967), Jensen (1967) and Cochrane and Müller-Eberhard (1968) has shown that factors with anaphylatoxin activity can be generated from isolated human C′3 and C′5 and from isolated guinea-pig C′5 by treatment with the earlier acting C′ components or with proteolytic enzymes. These developments made it reasonable to hypothesize that endotoxin might generate anaphylatoxin via the C′ system and that this product might be responsible for some of the biological effects of endotoxin. This report deals with the first part of that hypothesis.

The definition of anaphylatoxin is largely operational. At a minimum a product so designated should cause the guinea-pig ileum to contract, induce tachyphylaxis and be inhibited by antihistamines (Osler *et al.*, 1959). Its formation should require the presence of fresh serum or, based on recent work, isolated C′ components. Among other responses which have been reported to result from the action of anaphylatoxin are increased capillary permeability of the skin and histamine release from isolated rat mast cells and from chopped guinea-pig lung (Dias da Silva and Lepow, 1965; Silva, 1954). It now appears that there are at least two anaphylatoxins which have somewhat different spectra of biological activities (Dias da Silva *et al.*, 1967; Cochrane and Müller-Eberhard, 1968).

We have demonstrated that the interaction of fresh serum and endotoxic LPS produces a factor which contracts the guinea-pig ileum, induces tachyphylaxis, and is inhibited by antihistamines. The reaction requires fresh serum and is inhibited by cold or EDTA. No activity is generated if the serum has been heated before LPS addition but once made the product is heat stable. The time required for the gut response to the product is characteristic of anaphylatoxin, being slower than histamine but faster than slow-reacting substance (Brocklehurst, 1962). The product of the LPS reaction with serum is similar in these respects to that induced by an antigen–antibody reaction, by zymosan or by cobra venom factor. Finally, the variation in the ability of sera from different species to support the endotoxic generation of biological activity is similar to that described for anaphylatoxin. These observations would seem to establish that the product of LPS–serum interaction is an anaphylatoxin.

The role of complement in this reaction is suggested by the experiments with heated serum, EDTA treated serum and a 0° reaction temperature. There is a parallelism between the degree of consumption of the late acting C′ components (C′EDTA) by endotoxin and the generation of anaphylatoxin. Since work by several laboratories has shown that isolated guinea-pig C′5 and human C′3 or C′5 can be treated in various ways to yield products with anaphylatoxic activity (Dias da Silva and Lepow, 1965, 1967; Dias da Silva *et al.*, 1967; Jensen, 1967; Cochrane and Müller-Eberhard, 1968), we investigated the kinetics of C′3 and C′5 utilization under conditions which produce endotoxin-generated anaphylatoxin in whole guinea-pig serum. We found that the rate of appearance of anaphylatoxin parallels the rate of disappearance of C′3 and C′5. These observations are compatible with but do not prove the hypothesis that these are the components involved in anaphylatoxin formation in whole guinea-pig serum.

The similarities between the shock syndrome produced by endotoxin and anaphylatoxin, and the demonstration that the former can generate anaphylatoxin suggest that anaphylatoxin might mediate some of the biological effects of endotoxin. This must, however, be shown by direct experimentation. Especially with respect to man the role of anaphylatoxin remains to be established. We could not demonstrate anaphylatoxin production by LPS in human serum. Dias da Silva and co-workers, who have produced anaphylatoxin from isolated human C′ components, have likewise been unable to produce anaphylatoxin in whole human serum (Dias da Silva *et al.*, 1967; Dias da Silva and Lepow, 1967). The next steps in this investigation would, therefore, seem to be to characterize further the reaction as it occurs in whole human or animal sera and then proceed to investigate its effects *in vivo*.

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