Anaphylatoxin in Its Relation to the Complement System

Abstract. *The fifth component of complement that reacts hemolytically in the guinea pig or rat complement system, C′3b, is intimately associated with the generation of anaphylatoxin. Anaphylatoxin was formed (i) if this purified component reacted with a washed, intermediate complex consisting of antigen, antibody, and the first four components of complement; (ii) if the component was treated with trypsin; or (iii) if the component was incubated with a material isolated from cobra venom together with a fraction from serum other than complement. The significance of this fifth component as anaphylatoxinogen is discussed, and a common mechanism is proposed for the generation of anaphylatoxin in whole serum.*

Anaphylatoxin can be generated in whole serum or plasma of several animal species by treatment with seemingly unrelated materials, such as immune precipitates, agar, various dextrans, bacterial endotoxins, or cobra venom.

Stegemann *et al.* (*1*) have isolated, purified, and characterized a basic glycoprotein (molecular weight 30,000) from rat and pig plasma which had been incubated either with Sephadex or with a purified enzyme obtained from cobra venom (*2*). This material caused the typically abrupt, slightly delayed contraction of the guinea pig ileum and subsequent tachyphylaxis, the characteristic response to anaphylatoxin in a Schultz-Dale experiment. Stegemann *et al.* also demonstrated that anaphylatoxins obtained from rat and pig plasma showed crossed tachyphylaxis and had an almost identical amino acid composition, similar molecular weights, and heat stabilities.

Although now chemically defined, the origin of anaphylatoxin has remained obscure, especially in view of the many conditions under which it can be generated. The complement system may be involved in the generation of anaphylatoxin; however, supporting experimental evidence is mainly indirect. This toxin could not be generated in serum or plasma deprived of its C′-activity (*3*) by physical or chemical treatment or if conditions did not allow the "activation" of C′; on the other hand, in many experiments its generation followed closely the fixation and depletion of C′ and certain C′ components (*4*); furthermore, human C′1 esterase generated anaphylatoxin in guinea pig serum presumably by interaction with the complement system (*5*).

Guinea pig C′ consists of nine distinct components which react with antigen-antibody complexes in the following sequence: C′1, C′4, C′2, C′3c, C′3b, C′3e, C′3f, C′3a, C′3d (*6*). Evidence is presented which strongly indicates that anaphylatoxin is liberated during the C′3b reaction step and that the C′3b molecule is the anaphylatoxinogen from which anaphylatoxin can be released.

Anaphylatoxin was generated in four different ways as follows: (i) by treatment of whole serum or serum fractions with a semipurified, nontoxic material obtained from lyophilized cobra venom (*Naja haje*) by chromatographic separation on DEAE- and CM-cellulose columns; this material, here referred to as "venom factor," inactivates C′3c in vivo and in vitro, is relatively heat-stable, has a sedimentation constant of approximately 6.1*S*, is antigenic, and is inactivated by trypsin (*7*); (ii) by treatment of serum or serum fractions with an immune precipitate; (iii) by incubation of purified C′3b with crystalline trypsin; and (iv) by reaction of EAbC′14 sequentially with the next three components of guinea pig C′.

Anaphylatoxin was assayed by its effect upon the isolated guinea-pig ileum contained in an 8.0-ml bath of aerated Tyrode solution. The contractions were registered isotonically by a kymograph.

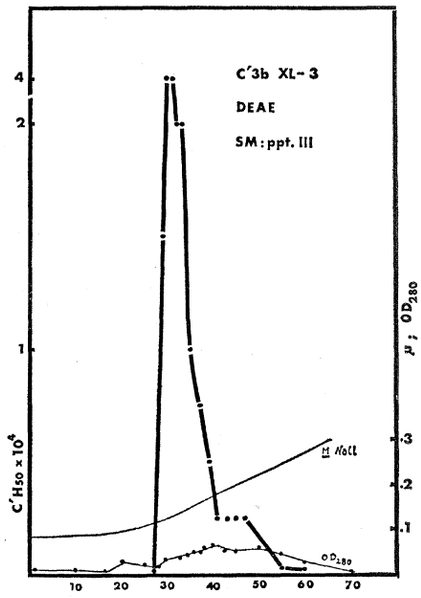

Fig. 1. Purification and concentration of C′3b by column chromatography with DEAE-cellulose at *p*H 7.5.

The supernatant obtained from treated guinea pig serum (venom factor) after two successive precipitations at *p*H 7.5 and 5.0 (the ionic strength was 0.04) served as control for anaphylatoxin. Near maximum or maximum contractions of the gut were obtained with 0.1 ml in a 1:10 dilution in terms of the original serum. By several criteria this material behaved like purified anaphylatoxin (*1*). It was heat-stable at low *p*H, was not retained by DEAE-cellulose at low ionic strength, was eluted from Sephadex G-100 after the albumin, and exhibited crossed tachyphylaxis with rat or guinea pig serum treated with immune precipitates, zymosan, Sephadex, or cobra venom. Tyrode solution was used in the bath and served as diluent for all reaction mixtures.

Purified preparations of C′3b were obtained from C′1-depleted serum by precipitation at *p*H 5.0 (ionic strength 0.04*M* NaCl). The precipitates were dissolved, sometimes precipitated again, and then chromatographed on DEAE-cellulose (*6*).

The low protein content of a functionally pure and highly reactive C′3b preparation obtained from a DEAE-cellulose column by salt-gradient elution is apparent from Fig. 1. No separation of a distinct anaphylatoxinogen from the C′3b component of complement could be observed on gel filtration (Sephadex G-200) of such purified preparations.

1) Anaphylatoxin was formed if nine parts of guinea pig or rat serum, diluted 1:3.5, were incubated with one part of venom factor (that is, 20 unit/ml) for 60 minutes at 30°C. (One unit of venom factor depletes 95 percent of C′3c in whole guinea pig serum.) Anaphylatoxin was not formed if the serums were first depleted of C′3b by precipitation (*6*), which results in a 1:3.5 dilution of the serum. Such depleted serums could be reconstituted by addition of purified homologous C′3b (500 to 1000 $C'H_{50}$ unit/ml), although anaphylatoxin was not formed if the component alone, even at ten times higher concentrations, was treated with the venom factor. Fractionation of C′3b-depleted serum revealed that an unidentified cofactor, but no other component of complement, was required for the generation of anaphylatoxin from C′3b by the venom factor.

2) Anaphylatoxin was formed if 500 to 1000 $C'H_{50}$ units of purified C′3b were incubated with 5 μg of

trypsin (crystallized three times) for 10 minutes at 25°C in a total volume of 1 ml. Similar treatment of any one of the other eight purified components at concentrations as high as 5000 $C'H_{50}$ units did not result in the formation of anaphylatoxin. Equal amounts of crystalline trypsin inhibitor obtained from soybean prevented the formation of anaphylatoxin. Purified C'3b, rendered hemolytically inactive by heat (80°C) or by repeated freezing and thawing, still formed anaphylatoxin if treated with trypsin, whereas its hemolytic reactivity was necessary for anaphylatoxin formation in the immune system.

3) Incubation of guinea pig serum with an immune precipitate (66 μg of antibody nitrogen per milliliter of guinea pig serum) consisting of bovine serum albumin (BSA) and the corresponding rabbit 7*S* antibody resulted in the formation of anaphylatoxin only if C'1 was not previously removed from the serum by precipitation. A serum depleted of C'1, however, became toxic if incubated with a well-washed intermediate complex consisting of the same immune precipitate and purified C'1. This complex was obtained by incubating 2 ml of the suspended precipitate (666 μg of antibody nitrogen per milliliter) with 8 ml of purified C'1 (7500 $C'H_{50}$ unit/ml) for 2 hours at 0°C in glucose-gelatin-barbital buffer (*6*). Again, removal of C'3b from the guinea pig serum before its reaction with the complex abolished its faculty for anaphylatoxin formation which could be restored by addition of purified C'3b.

4) Sheep erythrocytes in the state EAbC'1423c were prepared by incubating 50 ml of EAbC'14 (1×10^8 cell/ml) with 50 ml of a mixture of highly purified C'2 and C'3c containing about 80 and 50 $C'H_{50}$ unit/ml, respectively, for 30 minutes at 30°C. The buffer was glucose-gelatin-barbital. The cells were then centrifuged, washed three times in ice-cold buffer, suspended in Tyrode solution to 2.5×10^8 cell/ml, and dispensed in 1-ml quantities. If such cells were incubated immediately with an equal volume of purified C'3b containing approximately 2000 $C'H_{50}$ unit/ml for 30 minutes at 30°C and then centrifuged, the supernatant was strongly positive for anaphylatoxin in amounts of 0.5 to 1.0 ml, but only if the C'3b preparation was hemolytically reactive. The cells formed in this reaction were washed and analyzed; with standard titration procedures (*6*), 92 percent of the cells underwent lysis with a reagent containing purified C'3e, C'3f, C'3a, and C'3d, while 4.5 percent underwent lysis with only C'3f, C'3a, and C'3d. Therefore, the cells were in the state EAbC'1423c3b. In view of the short half-life of the C'2 site (*8*), EAbC'1423c decays rapidly to an intermediate cell, EAbC'143c, that does not react hemolytically with C'3b unless fresh C'2 is supplied. The same C'2 dependence was observed for the formation of anaphylatoxin.

The foregoing results demonstrate that C'3b, the fifth component in the hemolytic sequence, is intimately associated with the generation of anaphylatoxin. The experimental data are interpreted to mean that C'3b is the anaphylatoxinogen from which anaphylatoxin is released as a relatively small part of the molecule. The release could be accomplished either directly in the fluid phase by trypsin and possibly other enzymes, or through interaction with active C'3c sites of an immune complex. The second mechanism would constitute an integral part of the immune reaction and would require a reactive combining site on the C'3b molecule (presumably the heat-labile site); the interaction would bring the molecule in close proximity to the C'3c site, which may exert peptidase activity (*9*), and would result in the release of anaphylatoxin and possibly in the preparation of the complex for the next component, C'3e. If this interpretation should be correct, the current concept of C'-participation in immune-pathological conditions might be altered. Anaphylatoxin could be generated directly by the interaction of an activated enzyme with C'3b. As a result, histamine would be released, and possibly other tissue reactions would be initiated (*10*). The formation of anaphylatoxin mediated by the venom factor could serve as a model for this hypothetical situation, especially since it also occurs in vivo, as J. Maillard observed in this laboratory: the labile serum cofactor would be the postulated zymogen which, activated by the venom factor, would react with C'3b in a manner analogous to trypsin. The role of C'3b as an anaphylatoxinogen must also be considered in attempts to suppress cytotoxic immune reactions via specific inactivation of the complement system (*7*).

A common mechanism for the generation of anaphylatoxin is suggested. The wide distribution in nature of antigenic substances consisting of or containing varied polysaccharides is reflected by the common occurrence of cross-reactive natural antibodies against such antigens (*11*). Incubation of whole serum with these antigenic materials (dextrans, zymosan, agar, bacterial lipopolysaccharides, and others) under conditions which allow fixation or activation of the complement system has long been known to result in formation in vitro of anaphylatoxin. Under these circumstances, generation of anaphylatoxin seems not merely coincidental but seems rather the result of an immune reaction between natural antibody, antigen, and C', during which anaphylatoxin is released as a consequence of the participation of C'3b. The release mediated by trypsin or the venom factor could be viewed as a short-cut mechanism in contrast to the multistep immune reaction.

JOERG JENSEN*
Laboratories of Microbiology, Howard Hughes Medical Institute, Miami, Florida 33136

References and Notes

1. H. Stegemann, W. Vogt, K. D. Friedberg, *Z. Physiol. Chem.* **337**, 269 (1964); H. Stegemann, G. Bernhard, J. A. O'Neil, *ibid.* **339**, 9 (1964); H. Stegemann, R. Hillebrecht, W. Rien, *ibid.* **340**, 11 (1965).
2. W. Vogt and G. Schmidt, *Experientia* **20**, 207 (1964).
3. The following abbreviations are used throughout this paper: BSA, bovine serum albumin; DEAE, diethylaminoethyl; C', the complement system; C'1, C'4, and so forth, first, fourth components of the complement system; CM, carboxymethyl; $C'H_{50}$ unit, the amount of C' or of a C'-component necessary to cause lysis of 50 percent of the corresponding intermediate cells under standard conditions; E, sheep erythrocytes; EAb, sheep erythrocytes optimally sensitized with rabbit antiserum to sheep erythrocytes; EAbC'1, EAbC'14, and so forth, EAb that were reacted with C'1, C'1 and C'4, and so forth, and can be brought to lysis by the action of the remaining components.
4. A. G. Osler, H. G. Randall, B. M. Hill, Z. Ovary, *J. Exp. Med.* **110**, 311 (1959).
5. W. Dias Da Silva and I. H. Lepow, *J. Immunol.* **95**, 1080 (1965).
6. R. A. Nelson, Jr., J. Jensen, I. Gigli, N. Tamura, *Immunochemistry* **3**, 111 (1966); R. A. Nelson, Jr., *The Inflammatory Process* (Academic Press, New York, 1965), chap. 25; K. Inoue and R. A. Nelson, Jr., *J. Immunol.* **95**, 355 (1965); ———, *ibid.* **96**, 386 (1966); J. Jensen, R. A. Nelson, Jr., N. Tamura, in preparation.
7. R. A. Nelson, Jr., in *Symposium on Suppression of the Graft Rejection, Little Rock, Ark., 1965*, in press; R. Zarco and R. A. Nelson, Jr., in preparation.
8. M. M. Mayer, in *Experimental Immunochemistry*, E. A. Kabat and M. M. Mayer, Eds. (Thomas, Springfield, Ill., ed. 2, 1961).
9. N. R. Cooper and E. L. Becker, *Fed. Proc.* **24**, 446 (1965).
10. K. D. Friedberg, G. Engelhardt, F. Meineke, *Int. Arch. Allergy* **25**, 154 (1964).
11. R. A. Nelson Jr., *J. Exp. Med.* **108**, 515 (1958).

12. I thank Dr. R. A. Nelson, Jr. and my colleagues for their support of this investigation.

* Present address: Department of Microbiology, University of Miami School of Medicine, Coral Gables, Fla. 33134.

5 July 1966; 2 December 1966