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Anaphylatoxin Release from the Third Component of Human Complement by Hydroxylamine

Abstract. *Treatment of highly purified preparations of the third component of complement (C3) with 0.5*M *hydroxylamine at 20°C for 15 to 30 minutes, followed by acidification, resulted in dissociation of a peptide from the C3 molecule. The isolated fragment (molecular weight, 7600) resembled enzymatically liberated anaphylatoxin (C3a) with respect to size, charge, amino acid composition, and biological activity. Its capacity to contract smooth muscle was inhibitable by antihistamines; it also produced tachyphylaxis and desensitization of the guinea pig ileum to C3a. Thus native C3 probably contains an esterlike bond and hydroxylamine-liberated anaphylatoxin may represent one of the polypeptide chains of the C3 molecule.*

Recently, anaphylatoxin could be identified with two enzymatically produced split products derived from the components of complement C3 and C5. Both fragments, designated C3a and C5a, respectively (*1*), exhibit smooth muscle contracting activity and the capacity to change capillary permeability. They are not only chemically distinct but also biologically in that C3a anaphylatoxin can cause contraction of guinea pig ileum desensitized to the action of C5a anaphylatoxin and vice versa. This field of research has been reviewed (*2*).

The C3a anaphylatoxin has been shown to be liberated from the native C3 molecule by the following enzymes: C3 convertase (*3–5*), C3 inactivator complex (*4*, *6*), trypsin, and plasmin (*5*). The ease with which C3a may be cleaved off its precursor, particularly by trypsin (see below), indicated the presence of a site in the native C3 molecule which is highly susceptible to enzymatic attack. The following experiments were carried out to determine whether a biologically active, C3a-like piece can be liberated from C3 by chemical dissociation.

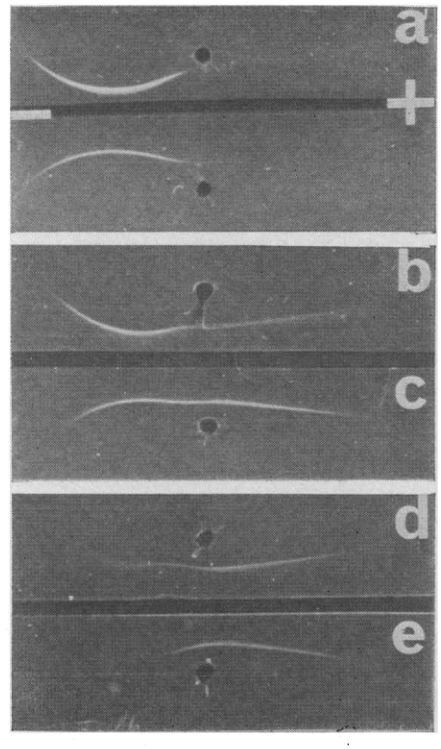

Fig. 1. Change of immunoelectrophoretic appearance of isolated C3 caused by treatment with hydroxylamine for 5 to 60 minutes. Concentration of C3 was 3 mg/ml, that of NH_2OH was 0.5*M*; 20°C, *p*H 7.0. Electrophoresis was carried out for 2 hours at *p*H 8.6 with a potential gradient of 2 volt/cm; patterns were developed with a rabbit antiserum to human C3. The cathode is to the left. (a) Control; (b) 5 minutes; (c) 15 minutes; (d) 30 minutes; (e) 60 minutes.

Samples (30 mg) of highly purified human C3 (*7*) in 10 ml of phosphate buffer, *p*H 7.0, ionic strength 0.1, were treated with 0.5*M* NH_2OH for 15 to 30 minutes at 20°C. The NH_2OH solution was prepared as described by Lipmann and Tuttle (*8*). The reaction between NH_2OH and C3 was stopped by transferring the reaction mixture to an ice bath and by increasing the hydrogen ion concentration with 1*N* HCl to *p*H 3.8; the reaction mixture was then dialyzed at 4°C (overnight) against acetate buffer, *p*H 4.0, ionic strength 0.1. Separation of the C3 products was accomplished at *p*H 4.5 either by filtration on a Sephadex G-100 column or by preparative electrophoresis on a polyacrylamide gel column according to the procedures described for the isolation of C3a (*5*). Enzymatically liberated C3a anaphylatoxin was prepared by treatment of 30-mg batches of C3 in phosphate buffer, *p*H 7.5, with either trypsin [1 percent (by weight), 30 seconds, 20°C, followed by addition of soybean trypsin inhibitor (2 percent by weight)] or C3 convertase (1 to 2 percent by weight), 20 minutes, 37°C (*5*). The maximum yield of C3a in both instances was approximately 1 mg. Anaphylatoxin activity was assayed on segments of guinea pig ileum in a Schultz-Dale bath (*4*).

Figure 1 shows the effect of treatment with 0.5*M* NH_2OH on the immunoelectrophoretic appearance of isolated C3. Within minutes, an electrophoretically faster migrating component appeared which resembled the hemolytically inactive form of C3 (*9*) or its major fragment, C3b (*5*). As judged by the immunoelectrophoretic patterns, the reaction with NH_2OH was complete after 30 to 60 minutes at 20°C, and there was no evidence for a second reaction product which would have indicated cleavage of the C3 molecule. However, analytical disc electrophoresis at *p*H 4.5 (Fig. 2) revealed in NH_2OH-treated C3 a minor component which migrated toward the cathode with the same mobility as C3a produced either by trypsin or C3 convertase (*5*). This fragment could not be liberated by exposure of C3 to acid *p*H alone,

nor by overnight treatment of C3 with 1*M* KCNS which causes conversion (*10*) of C3 to a product resembling, on immunoelectrophoresis, the fast-migrating component shown in Fig. 1. Further, isolated human C4, immunoglobulin G, and albumin did not yield detectable cleavage products when treated with NH_2OH under conditions identical to those used for treatment of C3.

The similarity of the NH_2OH-produced fragment to C3a prompted examination of this material for biological activity. As shown in Fig. 3, NH_2OH-treated C3 had smooth muscle contracting activity, and it rendered the guinea pig ileum unresponsive to C3a but not to C5a. Similarly, enzymatically derived C3a desensitized the muscle to the action of NH_2OH-treated C3, but the muscle retained its responsiveness to C5a anaphylatoxin. The NH_2OH-induced activity was inhibitable by antihistamines (not shown in Fig. 3). The newly described C3 piece is tentatively referred to as NH_2OH-produced C3a.

Comparison of physical and chemical properties of C3a and the NH_2OH-produced C3a disclosed a high degree of similarity. Their molecular weights were 7200 and 7600, respectively. These values represent the mean of the minimum molecular weight calculated from amino acid analyses and of determinations by the polyacrylamide gel electrophoresis method (*11*, *12*). The amino acid compositions in terms of number of amino acid residues per molecule of protein were almost identical, except that NH_2OH-produced C3 contained the following additional residues: one serine and two glycine. The ratio of basic to acid amino acid residues was 1.3 for both fragments.

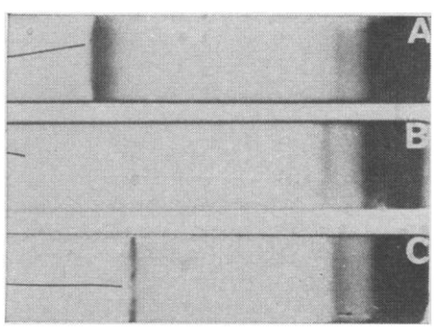

Fig. 2. Demonstration by disc electrophoresis of a C3 piece liberated upon treatment of native C3 with 0.5*M* hydroxylamine for 15 minutes at 20°C. Electrophoresis was performed at *p*H 4.5; the cathode was at the left. The position of the buffer interface was used as reference point and is indicated by the tip of the wire which was inserted into each gel from the cathodal end immediately after electrophoresis and prior to staining. The NH_2OH-produced piece (C) has a mobility comparable to that of C3a anaphylatoxin liberated from C3 by treatment with trypsin (A) (1 percent by weight, 30 seconds, 20°C); (B) control.

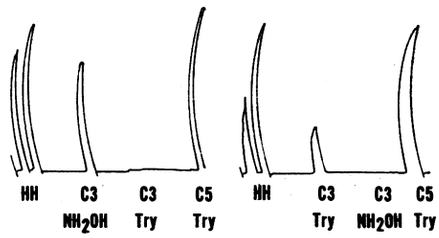

Fig. 3. Demonstration of smooth muscle contracting activity generated by treatment of C3 with hydroxylamine (0.5*M*, 15 minutes, 20°C). The patterns represent recordings of contractions of isolated guinea pig ileum in a Schultz-Dale bath. H denotes the response to 0.2 μg of histamine. After a contraction caused by hydroxylamine-treated C3 the muscle was unresponsive to anaphylatoxin formed by brief treatment of C3 with trypsin (C3 Try) and vice versa. In both instances, the ileum retained susceptibility to anaphylatoxin generated by treatment of human C5 with trypsin (C5 Try). In the recording of the contractions 0.05 cm of the base line corresponds to 1 second. The second peak of the first recording obtained with 0.02 μg of histamine corresponds to a 5-cm contraction.

Our data indicate that treatment of C3 with NH_2OH under mild conditions results in the dissociation of a small fragment and in the generation of smooth muscle contracting activity which, biologically, is identical with the activity of enzymatically produced C3a anaphylatoxin. The NH_2OH-produced C3a closely resembles C3a anaphylatoxin in size, charge, and amino acid composition. The small differences in size and composition suggest that the NH_2OH-liberated piece contains, in addition to the C3a peptide, a few more amino acid residues. Accordingly, the bond susceptible to NH_2OH appears to be different from the bond cleaved by C3 convertase and by trypsin upon formation of C3a anaphylatoxin, although the respective bonds must be located in the same narrow region of the C3 molecule. Present evidence indicates that trypsin and C3 convertase liberate anaphylatoxin from C3 by cleaving peptide bonds (*13*). The nature of the NH_2OH-susceptible bond in the C3 molecule is unknown. That NH_2OH-produced C3a constitutes a noncovalently bound subunit of C3 is improbable in view of the failure of KCNS and low *p*H to dissociate the piece. Hydroxylamine is known as a reagent capable of splitting ester and thioester bonds (*8*), and it cleaves ester-like bonds in collagen (*14*, *15*). The possibility is raised, therefore, that it acts on C3 by cleaving an ester-like bond, the NH_2OH-liberated piece of the biologically active piece. Should the NH_2OH-susceptible bond prove to be ester-like and not an unusual peptide bond, the NH_2OH-liberated piece would constitute one of the polypeptide chains of C3 rather than a fragment of such a chain. Although more work is needed to define the nature of the NH_2OH-susceptible bond, we have demonstrated the liberation of an anaphylatoxin-like piece from C3 by a nonenzymatic, chemical mechanism.

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