THE DERIVATION OF TWO DISTINCT ANAPHYLATOXIN ACTIVITIES FROM THE THIRD AND FIFTH COMPONENTS OF HUMAN COMPLEMENT*

By C. G. COCHRANE,‡ M.D., and H. J. MÜLLER-EBERHARD, M.D.

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

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The relationship of anaphylatoxin to the complement system has received increasing attention in the past few years. Osler et al. (1) observed a positive correlation between the activity of the third component of complement in rat serum and the capacity of the serum to generate anaphylatoxin. Recently, a direct relation of anaphylatoxin with the complement system has been demonstrated through the use of purified components of complement. Dias da Silva and Lepow (2) have presented clear evidence that an agent with the full spectrum of anaphylatoxin activities is dissociated from C'3 on interaction of C'1 esterase with C'2, C'3, and C'4 in free solution. When C'5 was added to this system, anaphylatoxin activity was neither augmented nor diminished, suggesting that C'5 was not essential for anaphylatoxin formation. In contrast to these findings, Jensen (3), working with functionally purified complement components of guinea pig serum, related anaphylatoxin activity to C'5, and not to C'3. This conclusion was drawn primarily from experiments in which anaphylatoxin was generated by treatment of functionally purified C'5 either with trypsin or EAC'1a, 4, 2a, 3, or with an anticomplementary factor of cobra venom.

In view of these disparate conclusions as to the identity of anaphylatoxinogen in the guinea pig and human complement systems, we undertook experiments to examine if C'5 as well as C'3 could serve as parent molecules of anaphylatoxin. Advantage was taken of the availability of highly purified components of human complement in this laboratory and of the possibility of introducing a radioactive label into these proteins. In addition, use was made of an anti-

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‡ Established Investigator, The Helen Hay Whitney Foundation.

complementary enzyme which constitutes a complex of a cobra venom protein and a β-globulin of human serum, and which will be described in detail later. It was found that a low molecular weight fragment with anaphylatoxin activity could be derived from both C'3 and C'5 and that the two chemically distinct types of anaphylatoxin differed biologically and with respect to the mode of their production.

Materials and Methods

Nomenclature.—The nomenclature of complement components is that recommended at the complement workshop of 1966 (4). Numerical designations are given for each component. Complexes of erythrocytes (E), antibody (A), and components of complement (C') are designated according to the reaction step achieved; e.g., EAC'1, EAC'1,4, etc. Components requiring activation before becoming hemolytically active are given the small letter "a", such as C'2a, while components that have been inactivated are designated with "i", such as C'3i. C'2 that has been treated with iodine resulting in a 10-fold enhancement in activity is represented as ^{oxy}C'2 (5).

Purified Components of Human Complement.—C'1 esterase was kindly supplied by Dr. Irwin Lepow. C'2 was isolated from human serum and oxydized with iodine by the method previously described (5). The preparation of C'3, C'4, and C'5 was the same as that previously reported (6, 7, 8). Labeling of C'3 and C'5 with ^{125}I was performed by the chloramine T method (9), as described earlier (8, 10).

Preparation of C'3 Inactivator Complex.—The C 3-inactivating principle of cobra venom was isolated from crude *Naja naja* venom as described (11). In brief, venom is first separated by Pevikon block electrophoresis in barbital buffer, pH 8.6, and the active material is then further fractionated by filtration on Sephadex G-100 in tris buffer pH 7.6, 0.1 M, and 1 M NaCl. The active protein was shown to be homogeneous by polyacrylamide gel electrophoresis and by immunodiffusion analysis using a potent antiserum to whole snake venom (Behringwerke A. G., Marburg-Lahr, West Germany). Since the C'3 inactivator function of cobra venom factor depends upon the formation of a protein-protein complex with a human serum β-globulin (11), this protein was also isolated.[1] Starting material constituted the pseudoglobulin fraction of human serum which was fractionated in succession by DEAE-cellulose chromatography, Pevikon block electrophoresis, and carboxymethyl (CM)-cellulose chromatography. The final product is homogeneous by polyacrylamide gel electrophoresis. To form the C'3 inactivator complex, equimolar amounts of cobra venom factor (CoF) and β-globulin are mixed in the presence of 0.0005 M Mg^{++} and held for 10 min at 37°C. for inactivation of C'3, incubation at 37°C for 20 min with a small amount of complex, usually less than 5% w/w, is sufficient.

Generation of C'3-Converting Activity on Sensitized Erythrocytes and in Free Solution by C'1, C'2, and C'4.—To obtain EAC'1a,4, ^{oxy}2 cells, sheep erythrocytes were sensitized by antibody and exposed to C'1, C'4, and ^{oxy}C'2 as described earlier (10). C'(4,2)a in free solution was prepared as outlined previously (12).

Preparation of EAC'4, ^{oxy}2,3 and of C'3i.—Usually, 20 ml of a 2.5% suspension of EAC'1a,4, ^{oxy}2a cells were treated with EDTA (final concentration 0.01 M) for 5 min at 0°C to dissociate C'1a. The cells were centrifuged and resuspended in 2 ml NaCl-Veronal buffer containing 0.01M EDTA. They were then incubated at 32°C for 20 min with 1 mg C'3 or C'3^{125}I, followed by sedimentation by centrifugation. Under these conditions, approximately 10% of the C'3 became cell-bound and 90% became converted to fluid phase C'3i. The

[1] Fjellström, C. E., and H. J. Müller-Eberhard. Data to be published.

cells were washed three times with physiological saline to remove all unbound C'3, and they were then used for the treatment of C'5. The supernatant containing approximately 450 μg C'3i/ml was immediately acidified to pH 3 using 0.5 N HCl and was then subjected to Sephadex filtration or to anaphylatoxin activity studies.

Detection of C'3 Conversion.—Conversion of C'3 to C'3i was detected by immunoelectrophoresis employing a specific antibody to C'3 (10).

Measurement of C'5 Activity.—C'5 activity was quantitated using EAC'4, ^{oxy}2a,3 cells and a 1:40 dilution of human serum treated with 1 M KSCN to which purified C'3 was added. The reaction was carried out in the presence of 0.01 M EDTA (8).

Chromatography on Gels.—Separation of proteins was carried out on a column of Sephadex G-100 employing 0.01 M sodium acetate buffer containing 0.15 M NaCl at pH 4.0. Chromatography was performed at 4°C. Samples were layered between the buffer and the gel bed by first mixing the sample with sucrose at a final sucrose concentration of 10%.

Density Gradient Ultracentrifugation.—Linear gradients of sucrose were prepared on a Buchler automatic density gradient device. Gradients of 7–31% sucrose were employed and linearity was verified by carbohydrate (anthrone) analysis of fractions. The sucrose contained 0.15 M NaCl and was buffered at pH 4.0 with 0.01 M sodium acetate. Centrifugation was performed at 50,000 rpm and 4°C with an SW 50 rotor, using a Spinco model L ultracentrifuge. Fractions were obtained dropwise from the bottom of each tube through a needle piercing the centrifuge tube.

Contraction of Guinea Pig Ileum.—Tests of the capacity of various agents to evoke a contraction of smooth muscle were assessed on the terminal ileum of guinea pigs. The data presented represent examples of at least two and, most often, of five or more tests. Guinea pigs weighing 250–300 g were sacrificed, generally by cervical separation, and the terminal ileum carefully dissected from the mesentery. Only the lower 25 cm of ileum were employed. After thorough washing by passage of Tyrode's solution through the lumen, sections of approximately 4 cm were cut and fixed by thread to a glass rod at one end and a kymograph lever at the other. The ileal strip was thus suspended in an 8 ml bath of Tyrode's solution maintained at 37°C with a gas mixture of 95% O_2 and 5% CO_2 passing through in fine bubbles. Atropine was added to a final concentration of 10^{-7} M. Each section employed was stimulated with histamine, generally starting with applications of 1 μg (base) and terminating with 0.2 μg in order to achieve a state of replicable contractions to a standard stimulating concentration of histamine (1×10^{-7} M). The ileum was found to lose its capacity of responding to active preparations of anaphylatoxin (human, guinea pig, or rat in origin) after about 1.5–2 hr time of removal. Hence all tests were performed on sections within 1.5 hr of sacrifice of the guinea pig.

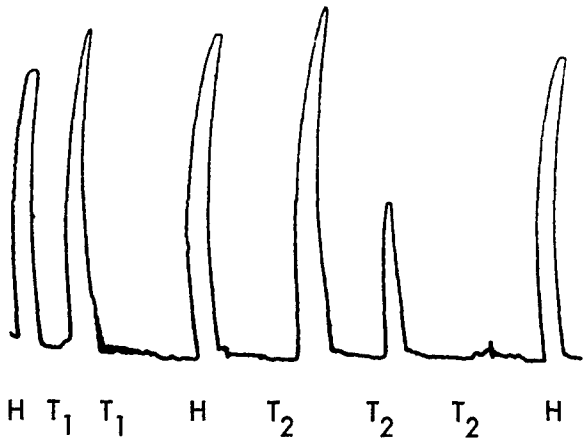
Mast Cell Preparations.—Rat mast cells were obtained from the peritoneal cavity of 200 g rats by modification of the technique of Norton (13). 6 ml medium 199 (Microbiological Associates, Albany, Calif.) was injected intraperitoneally into rats within 15 sec of cervical dislocation. The fluid was mixed in the cavity for 1–1.5 min and immediately aspirated. By diluting the cell suspension obtained in the aspirated fluid with 5 ml of medium 199, clotting was avoided and an anticoagulant was not necessary. The cells were immediately centrifuged and washed four times in 5 ml volumes of medium 199 containing 1% purified gelatin. The resulting washed cell pellet was resuspended in 1.0 ml of medium 199–1% gelatin and cell counts obtained with a hemocytometer. Generally, 2×10^7 total cells were obtained from each rat, about 4% of which were mast cells.

Tests of Vascular Permeability.—These tests were performed in 300–350 g male Hartley strain guinea pigs. Evan's blue, 0.5 ml of a 1% solution in isotonic saline, was injected intravenously prior to testing. Testing samples were injected through No. 27 needles intradermally and the result was determined 20 min later. Maximal and minimal diameters on the outer skin surface were measured and an average of these values was used as the determination. The

same guinea pigs were employed within 30 min of the first intradermal injections for evaluation of antihistamine inhibition of the permeability effect of tested solutions. Chlorpheniramine maleate, 250 μg, was infused intravenously 10 min before further skin tests were performed. The absence of blueing caused by the intradermal injections of 1.0 μg histamine verified the effectiveness of antihistamine inhibition.

Pharmacologic Reagents.—Histamine hydrochloride was obtained from Eli Lilly and Co., Indianapolis, Ind. Measurements are given according to the weight of histamine base. Chlorpheniramine maleate (Chlor-Trimeton) was obtained from Schering Corp., Bloomfield, N. J., and bradykinin from Sandoz Co., Hanover, N. J.

Anaphylatoxin Prepared from Guinea Pig Serum.—Fresh guinea pig serum (1.0 ml) was incubated with 10 μg CoF for 10 min at 37°C. The resulting active serum was maintained at room temperature and used for tests within 2 hr. The same serum batch untreated by CoF failed to induce the various reactions reported below.



TEXT-FIG. 1. Tracing produced by contraction of guinea pig ileum after two applications of 150 μg C'5 treated with trypsin. H, histamine 1×10^{-7} M; T_1, trypsinized C'5; T_2, guinea pig anaphylatoxin, 0.15 ml.

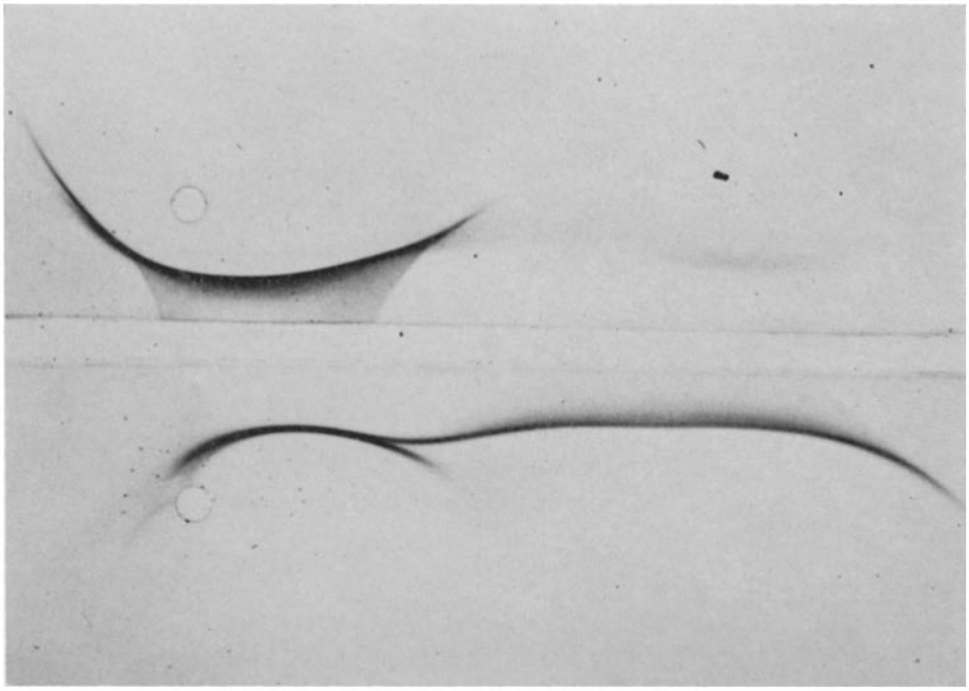
RESULTS

The Formation of a Factor from the Fifth Component of Human Complement Having Anaphylatoxin Activities

Generation of Smooth Muscle-Contracting Capacity by Treatment with Trypsin.—150 μg C'5 was incubated with 3 μg trypsin at 32°C for 5 min at pH 7.8. Soya bean trypsin inhibitor was added and the pH of the mixture adjusted to 3.5 with 0.5 N HCl and the total volume applied to the guinea pig ileum. A strong contraction resulted (Fig. 1). Similarly, 80 μg C'5 after trypsinization as above evoked a contraction of moderate intensity. When a second application of the same dose of freshly trypsinized C'5 was added to the ileum, a marked reduction or complete absence of response resulted (Fig. 1). Application of as much as 180 μg untreated C'5, trypsin, or trypsin inhibitor failed to yield a

contraction. A time-course experiment revealed that activity was not detectable after 2.5 min of trypsin treatment and that maximal activity yield was obtained after 5–10 min of treatment, after which time the yield decreased. Immunoelectrophoretic analysis of trypsin-treated C'5 showed partial conversion of C'5 to an electrophoretically faster migrating component (Fig. 2).

Inhibition of Trypsinized C'5 Activity by Antihistamine.—Exposure of the



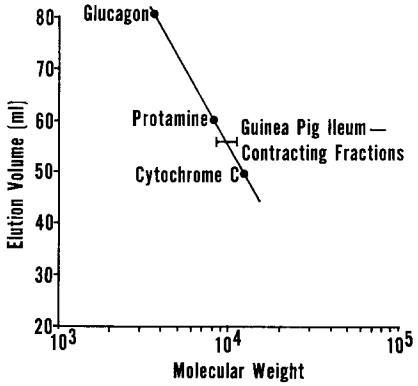
TEXT-FIG. 2. Immunoelectrophoretic demonstration of effect of trypsin on isolated human C'5. Top, native C'5; bottom, C'5 treated with 2% (W/W) trypsin for 5 min at 37°C. Patterns were developed with rabbit anti-human C'5.

guinea pig ileum to the antihistamine chlorpheniramine maleate 9.1×10^{-7} M for 20 sec, followed by removal of the antihistamine and thorough washing, completely inhibited the capacity of 100 μg trypsinized C'5 and of 2×10^{-8} M histamine to evoke a contraction. Bradykinin, 2.2×10^{-8} M, however, was able to induce contractions equal in amplitude to those obtained prior to treatment with antihistamine.

Permeability Effect of Trypsinized C'5 in Guinea Pig Skin.—C'5 (125 μg/0.5 ml) was treated with trypsin (2.50 μg) for 12 min at 32°C. At the end of the incubation time, soya bean trypsin inhibitor (SBTI) (5.0 μg) was added, and

the mixture, along with control solutions, was injected intradermally into two guinea pigs previously given Evan's blue dye intravenously. Injections of 0.1 ml of the incubated mixture (25 μg trypsinized C'5) induced sites of blueing averaging 13 mm diameter, while the equivalent quantity of trypsin and SBTI gave sites of 3 mm diameter, and untreated C'5 gaves sites of 2 mm diameter. When injections of the trypsinized C'5 which was kept in an ice bath were repeated over a period of 1 hr, the permeability inducing activity was found to diminish so that by 1 hr only a trace of blueing was observed. Injections of histamine (1 μg) during this period remained unchanged.

Evidence That the Smooth Muscle-Contracting Principle of C'5 is a Cleavage Product of the Parent Molecule.—In order to find if trypsin treatment of the C'5



TEXT-FIG. 3. Elution volume of trypsinized C'5, glucagon, protamine, and cytochrome *c* from a column of G-100 Sephadex, plotted as a function of molecular weight. The fraction of the trypsinized C'5 having the capacity of contracting guinea pig ileum is found in the molecular weight range of 9,000–11,000.

resulted in a cleavage product having the capacity of contracting guinea pig ileum, 3 mg C'5 was treated with 60 μg trypsin for 5 min at 32°C. The pH of the mixture was then adjusted to 4.0 and the solution was applied to a column of G-100 Sephadex. Elution was effected with 0.01 M sodium acetate buffer, pH 4.0, in 0.15 M NaCl. The fractions obtained were applied directly to the atropinized guinea pig ileum. Smooth muscle-contracting activity was present in a single zone eluting immediately after the eluting volume (Ve) of cytochrome *c*. When the Ve of the active principle and several other proteins used as markers were plotted as a function of the logarithm of molecular weights as shown in Fig. 3, the smooth muscle-contracting moiety of trypsinized C'5 was found to lie in the molecular weight range of 9,000–11,000.

Generation of Smooth Muscle-Contracting Activity after Incubation of C'5 with EAC'4,$^{1\,xy2a,3}$.—In order to find if smooth muscle-contracting activity could

be derived from C'5 by exposure to the preformed washed intermediate complex EAC'4,^{oxy}2a, 3, 180 μg C'5 were treated with 2.5 $\times$ 10^9 cells in a total volume of 1 ml at 32°C for 15 min. The supernatant of this mixture was brought to pH 3.5 and tested on guinea pig ileum that had been made insensitive to C'3 anaphylatoxin (see below). A contraction of moderate intensity followed. Tests of the supernatant revealed that only 15 % of the added C'5 had been inactivated by treatment with EAC'4,^{oxy}2a, 3. When such a supernatant prepared identically was further treated with 3.6 μg trypsin for 5 min and then tested, greater smooth muscle–contracting activity was observed. C'5 (180 μg) alone, as found previously, and C'5 (180 μg) treated with EAC'4,^{oxy}2a were unreactive

TEXT-FIG. 4. Tracing produced by contraction of guinea pig ileum after two applictions of 200 μg C'3 treated with C 3 inactivator complex (see text for details). H. histamine 1 $\times$ 10^{-7} M; T_1, C'3 inactivator complex–treated C'3.

In addition, incubation of washed EAC'4,^{oxy}2a, 3 with saline rather than C'5 failed to yield anaphylatoxin activity.

The Formation of Anaphylatoxin from the Third Component of Human Complement

Formation of Anaphylatoxin from C'3 by Cobra Factor–β-Globulin Complex (C'3 Inactivator).—Generation of anaphylatoxin from C'3 was attempted with C'3 inactivator complex (see Materials and Methods). After generation of the active complex, 200 μg C'3 was added and the mixture was held at 32°C for 20 min before a sample was removed for immunoelectrophoresis and the pH was adjusted to 4.0. The sample was then applied to a fresh guinea pig ileal strip; a strong contraction was observed (Fig. 4). Application of an identically prepared sample after washing of the ileal strip revealed complete desensitization, although the muscle was still fully reactive to histamine. Application of

untreated C'3 or the C'3 inactivator complex failed to induce a contraction. Immunoelectrophoresis indicated the treated C'3 was completely converted to the more rapidly migrating component, C'3i. Treatment of 100 μg of C'5 with C'3 inactivator complex under similar conditions failed to produce anaphylatoxin activity.

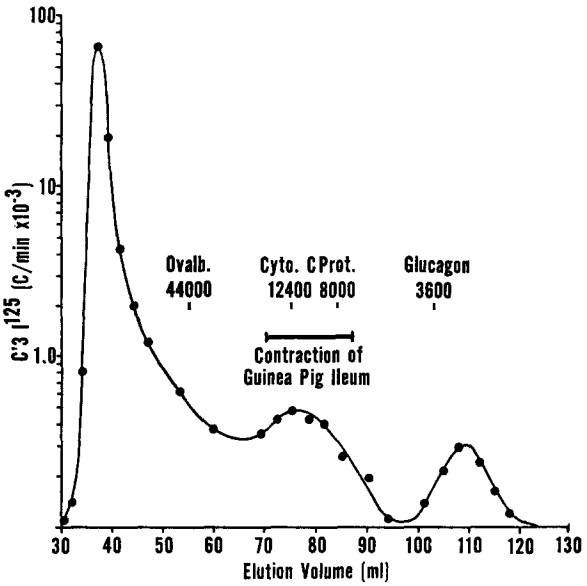
Formation of Anaphylatoxin from C'3 by C'(4,^{oxy}2)a Complex.—C'3 (200 μg) was incubated with preformed C'(4,^{oxy}2)a for 10 min at 37°C. At the end of this period, the pH of the solution was immediately adjusted to 3.0 with 0.5 N HCl, and the entire contents of the tube added to the atropinized guinea pig ileum in a Schultz-Dale bath. A strong contraction followed the first application but did not occur on subsequent additions. Administration of C'3 alone or an identical amount of C'(4,^{oxy}2)a as noted above failed to induce a contraction. This confirmed the previous findings of Dias da Silva and Lepow (2) that C'2 and C'4 in the presence of C'1 esterase can generate anaphylatoxin from C'3 in free solution. In a similar experiment, 1.0 mg C'3 was incubated with 5×10^9 EAC'4,^{oxy}2a cells for 20 min at 32°C. The cells were removed by centrifugation, the pH of the supernatant adjusted to 3.5, and aliquots added to a fresh strip of guinea pig ileum. Samples containing 100 μg treated C'3 yielded nearly maximal contraction, while aliquots containing 60 μg C'3 after incubation evoked a contraction equal to one-third the maximal amplitude and 40 μg treated C'3 produced only a small deflection of the recording needle. Each aliquot was tested on a fresh ileal strip. Immunoelectrophoresis indicated complete conversion of the C'3 to the more rapidly migrating C'3i.

Inhibition of Contraction by Antihistamine.—The contraction brought about by C'3 inactivator complex–treated C'3 (200 μg) along with the response to rat anaphylatoxin was abolished when the ileum was exposed to 9×10^{-7} M chlorpheniramine maleate (antihistamine) for 20 sec and then washed repeatedly with Tyrode's solution. Full reactivity to 2.2×10^{-8} M bradykinin was still observed.

Histamine Release from Rat Mast Cells by C'3 Treated with C'3 Inactivator Complex.—Preparations containing C'3 (350 μg) were each treated with a suitable amount of C'3 inactivator complex and placed on 2×10^6 rat peritoneal mast cells. Incubation was carried out for 1, 15, 30, 45, 60, and 90 min at 37°C. The cells were centrifuged and the supernatant tested for histamine activity on guinea pig ileum. The ileum had previously been made tachyphylactic to C'3 anaphylatoxin. Histamine release was detectable after 1 min incubation and reached a maximum between 45 and 60 min of incubation. Samples containing 2×10^6 cells and C'3 (350 μg) alone or the C'3 inactivator complex did not possess measurable histamine.

Permeability Reaction in Guinea Pig Skin by C'3 after Treatment with EAC'4, ^{oxy}*2a.*—C'3 was treated with EAC'4,^{oxy}2a, the cells were removed by centrifugation, and the supernatant adjusted to pH 3.5. Immediately before injec-

tion into the skin, the pH was brought to 7.0. Guinea pigs that previously had received Evan's blue intravenously were injected intradermally with 50 and 100 μg treated C'3. Blueing at the injection sites of 4 and 7 mm diameters, respectively, occurred within 20 min in each of two guinea pigs. Administration of C'3 alone in an equal amount failed to induce increased permeability. In addition, pretreatment of the guinea pigs with 250 μg antihistamine (chlor-

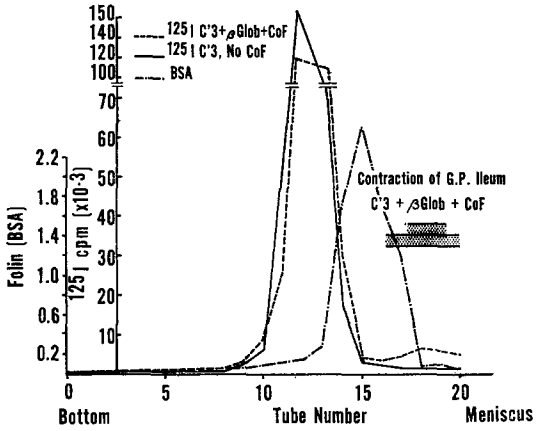


TEXT-FIG. 5. Elution of ^{125}I C'3 treated with C'3 inactivator complex from a column of Sephadex G-100. The fractions containing the factor capable of contracting guinea pig ileum are noted. Comparison of the eluting volume of the active material is compared with the eluting volumes of ovalbumin (Ovalb.), cytochrome *c* (Cyto C), protamine (Prot.), and glucagon. The molecular weight of each is noted.

pheniramine maleate) 10 min before injection prevented development of the permeability reaction induced by treated C'3.

Demonstration of a Fragment of C'3 Responsible for Smooth Muscle Contraction after Treatment with C'3 Inactivator Complex.—Attempts were made to find if the C'3 inactivator complex cleaved a fragment from C'3 that possessed the capacity to induce contraction of guinea pig ileum. Accordingly, 5 mg C'3 labeled with ^{125}I was treated with the β-globulin–CoF complex. After 20 min of incubation at 32°C, the pH was adjusted to 3.0 with 0.5 N HCl and the reaction mixture added to a column of Sephadex G-100 (1.3 × 28 cm) equilibrated with 0.10 M NaCl in 0.01 M Na acetate buffer of pH 4.0. Elution was carried out with the same buffer and fractions were tested directly on guinea pig ileum and the

results appear in Fig. 5. As noted, three peaks of ^{125}I activity were eluted, one in the void volume (Vo), a second eluting just after cytochrome *c* (molecular weight 12,400), and a third immediately prior to the internal volume of the column. Application of the various fractions to guinea pig ileum revealed that the smooth muscle–contracting principle was contained in fractions eluting with the second peak of ^{125}I activity (Fig. 5). This represented about 5% of the total C'3. An estimate of the molecular weight of the smooth muscle–contracting principle was achieved by plotting the volumes of elution (Ve) of this column



TEXT-FIG. 6. Ultracentrifugation of ^{125}I C'3 treated with C'3 inactivator complex in a density gradient. The pattern of treated ^{125}I C'3 is compared with that of untreated ^{125}I C'3 and bovine serum albumin (BSA) that were centrifuged simultaneously. Fractions containing the material capable of inducing release of histamine from rat mast cells is noted by the contraction of guinea pig ileum. Histamine released from the mast cells was detected only in fractions obtained from C'3 inactivator complex–treated C'3. Conditions of centrifugation noted in Materials and Methods.

as a function of the logarithm of molecular weights of various materials applied to it. By this procedure, the active material was detected in the range of molecular weights of from 6,000 to 17,000 using bovine albumin, hen egg albumin, cytochrome *c*, protamine, and glucagon as markers. ^{125}I C'3 not treated with C'3 inactivator complex and containing no smooth muscle–contracting activity was also passed through a G-100 Sephadex column. A single peak of ^{125}I activity was obtained in the void volume of the column, the second and third peaks appearing after C'3 inactivator complex treatment did not appear.

Density gradient ultracentrifugation of 200 μg untreated ^{125}I C'3 is shown in Fig. 6. A rapidly sedimenting symmetrical peak of ^{125}I activity was observed in both preparations, but only in the treated sample was ^{125}I activity found in the slowly sedimenting fractions. Assays were carried out on 0.1 ml volumes of each fraction for its capacity to release histamine from rat peritoneal mast cells.

As noted in Fig. 6, histamine-releasing activity was found in only the slowly sedimenting fractions of the treated C'3.

When labeled C'3 (500 μg) was treated with the C'(4,^{oxy}2)a complex in free solution and the pH adjusted to 5.0, an almost identical pattern of sedimentation of the ^{125}I C'3 was found. Again, histamine-releasing activity was associated with the C'3 sedimenting slowly in the gradient. Immunoelectrophoresis revealed that the C'3 was entirely converted to C'3i by C'(4,^{oxy}2)a.

Comparison of the Two Anaphylatoxin Activities with Each Other and with Guinea Pig Anaphylatoxin

Tests of Cross-Desensitization of Smooth Muscle by the Active Materials Derived from C'5 and C'3.—A strip of guinea pig ileum was exposed twice to C'3 (200 μg) treated with CoF–β-globulin complex. The first strong contraction was followed by unresponsiveness with the second application. Exposure then to a minimal dose of C'5 (40 μg), treated as before with trypsin, resulted in a contraction that was equal in intensity to that obtained by exposure of a fresh strip of ileum to the same dose of trypsinized C'5. Similarly, when a fresh guinea pig ileum segment was made unresponsive to trypsinized C'5 (two exposures to 150 μg trypsinized C'5), the muscle was found capable of responding fully to 150 μg C'3 treated with CoF–β-globulin complex. The response to histamine remained constant throughout.

Tests of Cross-Desensitization with the Two Smooth Muscle–Contracting Agents Derived from C'3.—Guinea pig ileum was made unresponsive to anaphylatoxin prepared from 200 μg C'3 by treatment with CoF–β-globulin complex as noted above. C'4,^{oxy}2a–treated C'3 (200 μg) anaphylatoxin was then applied and no response followed. This unresponsiveness, or crossed tachyphylaxis, also was observed when the ileal strip was made unresponsive in the reversed order of application.

Relationship of the Smooth Muscle–Contracting Capacity of C'5 and C'3 to Guinea Pig Anaphylatoxin.—With the finding of Jensen that guinea pig C'5 serves as anaphylatoxinogen (3), it was essential to find if a relationship exists between guinea pig and human C'5 anaphylatoxin. The data presented represent the results of duplicate or triplicate experiments. Guinea pig anaphylatoxin was prepared and added to guinea pig ileum. A strong contraction followed and complete desensitization was noted after three additions to the ileal strip. When a minimal amount of trypsinized human C'5 (40 μg) was then applied, a moderate contraction resulted, the same magnitude as that obtained by addition of 40 μg of trypsin-treated C'5 to a fresh ileal segment. In a second, similar, experiment after the ileum was made unresponsive to guinea pig anaphylatoxin, addition of trypsin-treated C'5 (100 μg) brought about a maximal contraction. In the reverse order of application, 140 μg C'5 after treatment with trypsin was added to a new segment of ileum and brought about a full contraction and

complete desensitization to a second dose of identically prepared C'5. Subsequent addition of the CoF-treated guinea pig serum then resulted in a maximal contraction, followed by desensitization after three applications as before (Fig. 1). Each ileal segment that had been desensitized to the CoF-treated guinea pig serum and trypsinized human C'5 reacted fully to human C'3 (200 μg) that had been treated with EAC'4,^{oxy}2a.

The ileal-contracting capacity of the CoF-treated guinea pig serum was completely inhibited by antihistamine (9×10^{-7} M chlorpheniramine maleate). The CoF-treated serum failed to contract rat uterus and induced release of histamine from rat peritoneal mast cells; this latter activity did not occur with equal quantities of CoF alone or untreated guinea pig serum. The histamine assays were performed on guinea pig ileum previously made unresponsive to guinea pig anaphylatoxin (CoF-treated serum).

DISCUSSION

The data presented in this paper indicate that both C'3 and C'5 may serve as parent molecules of anaphylatoxin. They also show that the two anaphylatoxins generated by complement differ from each other and from the guinea pig counterpart in biological reactivity.

Treatment of C'5 with trypsin formed a product that induced contraction of guinea pig ileum and with repeated application to the ileum brought about a state of unresponsiveness (tachyphylaxis). The capacity to induce a contraction was inhibitable by antihistamine. Injection of the material intradermally in guinea pigs resulted in increased vascular permeability. The activity was also obtained by reacting C'5 with the intermediate erythrocyte-complement complex EAC'4,^{oxy}2a, 3. As reported previously (8), this reaction results in binding to the cell surface of a small proportion of C'5 molecules and in loss of hemolytic activity of the majority of the molecules which remain in the fluid phase. That in the present experiments only 15% of the C'5 became inactivated is fully explained by the fact that the amount used constituted a large excess, relative to the selected number of cells. Subsequent treatment with trypsin was expected to liberate more anaphylatoxin, as was observed, due to the availability of C'5 which had not reacted with the cells. Although not yet demonstrated conclusively, it is probable that anaphylatoxin formation from C'5 coincides with abolition of hemolytic activity of C'5 by either trypsin or EAC'4,^{oxy}2a, 3 cells. Apparently this cell-complement complex and the proteolytic enzyme exert a similar, if not identical, effect on the C'5 molecule. In fact, peptidase activity has been demonstrated to reside in cell-bound C'4,2a, 3 sites and is believed to depend upon a critical spatial arrangement between the C'4,2a complex and C'3 (14, 15). In the cell free system employed by Dias da Silva and Lepow (2), addition of C'5 to C'1 esterase, C'4, C'2, and C'3 neither augmented nor inhibited the anaphylatoxin activity derived from C'3. It may be inferred from

the present study that the activity which resides in C'4, 2a, 3, and appears to be responsible for cleavage of C'5 is generated in much greater yield on a relatively stable surface such as a cell membrane than in free solution.

As demonstrated above, brief treatment of C'5 with trypsin leads to cleavage of the molecule into at least two fragments. The major fragment, readily detected by immunoelectrophoresis, is electrophoretically faster than native C'5 and relative to the latter it is antigenically deficient. The minor fragment, detected by gel filtration through its biological activity, is of low molecular weight (approximately 10,000) and constitutes C'5 derived anaphylatoxin. In agreement with the prevailing principles of complement nomenclature, the active minor fragment will henceforth be denoted F(a)C'5 and the inactive major fragment F(b)C'5. Although not investigated thus far, it may be assumed that cell-bound C'4,2a, 3 produces the same changes of the C'5 molecule. In contrast, the C'3 converting enzyme (C'4,2a) and the C'3 inactivator complex, both capable of acting on C'3, appear to have no effect on C'5.

Anaphylatoxin activity could also be derived from purified C'3 by either of two different mechanisms. It was produced by incubation with the C'3 inactivator complex which consists of a cobra venom protein and a thermolabile human serum β-globulin which is distinct from any complement component (11). It was also produced upon treatment of C'3 with the C'3 converting enzyme generated from C'2 and C'4 either in cell-free solution or on the surface of sensitized erythrocytes. Both principles had previously been shown to cause cleavage of the C'3 molecule (11, 16, 12) and it was found in this study that anaphylatoxin activity resides in the low molecular weight fragment (6,000–15,000) liberated from C'3 by either of the two enzymes. That the two active products are similar is suggested in the findings that desensitization of guinea pig ileum to anaphylatoxin produced by C'3 treated with the cobra factor inactivator complex resulted in loss of reactivity to anaphylatoxin derived from C'4,2a–treated C'3. As will be reported later,[2] a similar low molecular weight fragment having anaphylatoxin activity could be obtained by treatment of C'3 with trypsin for 2 min at room temperature. This fragment also cross-desensitizes the guinea pig ileum to C'3 anaphylatoxin prepared by the other two methods. Thus, it appears that cleavage of C'3 by several enzymes yields active fragments which so far are indistinguishable from each other by their biological properties. Following a suggestion made by Dr. Irwin Lepow, the active, low molecular weight fragment which can be derived from C'3 will henceforth be called F(a)C'3 and the residual, larger portion of the molecule F(b)C'3.

The results reported in this paper confirm and extend pertinent observations made by others and resolve the existing discrepancy as to the identity of anaphylatoxinogen. Jensen (3), working with guinea pig complement, attrib-

[2] Bokisch, V., H. J. Müller-Eberhard, and C. G. Cochrane. Data in preparation.

uted the capacity to donate anaphylatoxin activity solely to C'5. He was able to generate the activity both by trypsin and EAC'1a, 4, 2a, 3 cells. In addition, he found formation of the activity following treatment of serum fractions with partially purified CoF and again invoked C'5 as the responsible component. Jensen's work appeared to preclude C'3 as potential donor of anaphylatoxin. On the other hand, Dias da Silva and Lepow (2) clearly demonstrated that human C'3 can serve as anaphylatoxinogen, and that the activity arises upon treatment of C'3 with C'1 esterase, C'2, and C'4 in free solution. In this cell-free system, as discussed above, addition of C'5 had no further effect on anaphylatoxin activity.

It may be safely concluded at this point that Jensen and Dias da Silva and Lepow worked with two distinct anaphylatoxinogens and that both C'3 and C'5 in human serum are capable of donating anaphylatoxin. The activities of human F(a)C'3 and F(a)C'5 appear to be quite different in view of their lack of cross-desensitization of guinea pig ileum as shown in the present studies. In addition, both failed to cross-desensitize to guinea pig anaphylatoxin, which has, in unpublished observations, a similar molecular weight as determined by elution from Sephadex G-100. These data suggest that small but distinct structural differences exist in the three types of anaphylatoxin studied. Stegemann et al. (17, 18, 19) isolated anaphylatoxin from whole rat and pig serum and described properties that resemble those of the human anaphylatoxins. The identity of the parent molecule in these two cases, however, is unknown.

Jensen's results with CoF, indicating that C'5 is affected by this agent, are inconsistent with the finding reported here that the C'3 inactivator complex acts on human C'3 but not on human C'5. Species differences may account for this discrepancy which remains to be explained.

Of great interest is the question of how the various anaphylatoxin molecules bring about the release of histamine from mast cells. Studies are currently in progress to determine whether different cells are attacked by each agent, whether different sites are occupied on the same cells by the different anaphylatoxins, or whether different biochemical routes are selected in mast cells to bring about the release of histamine.

SUMMARY

Anaphylatoxin activity was derived from both human C'5 and C'3 molecules. This was achieved in the case of C'5 by interaction with trypsin or with EAC'4, ^{oxy}2a, 3. The smooth muscle–contracting material obtained from the treated C'5 was found to be a fragment of approximately 9,000–11,000 molecular weight. Its action was inhibited with antihistamine. The trypsinized C'5 also increased vascular permeability in guinea pig skin.

When human C'3 was incubated with C'3 inactivator complex, which consists of a cobra venom protein and a β-globulin of human serum, anaphylatoxin

activity was observed. The activity was associated with a fragment cleaved from the C'3 molecule, having a molecular weight of between 6,000 and 15,000 as determined by gel filtration techniques. Similar activity was derived from C'3 by the C'3-converting enzyme in free or in cell-bound form.

The C'5 anaphylatoxin failed to cross-desensitize guinea pig ileum to the contracting capacities of C'3 and guinea pig anaphylatoxin and vice versa. Anaphylatoxin prepared from C'3 by all methods mentioned above caused cross-desensitization to the other C'3 derivatives, but failed to desensitize to guinea pig anaphylatoxin.

Note Added in Proof.—Dias da Silva, Eisele, and Lepow have published studies describing the formation and isolation of a cleavage product of human C'3, F(a)C'3. The F(a)C'3 was split from the native molecule by the action of C'1 esterase, C'2, and C'4. The isolated F(a)C'3, having a molecular weight of 6800 or less, exhibited all the properties of anaphylatoxin previously recorded by these authors (2). (W. Dias da Silva, J. W. Eisele, and I. H. Lepow. 1967. Complement as a mediator of inflammation. III. Purification of the activity with anaphylatoxin properties generated by interaction of the first four components of complement and its identification as a cleavage product of C'3. *J. Exptl. Med.* **126**:1027.)

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