Gross and Ultrastructural Observations on Lesions Produced by Intradermal Injection of Human C3a in Man

Irwin H. Lepow, MD, PhD, Kaethe Willms-Kretschmer, MD, Richard A. Patrick, PhD and Fred S. Rosen, MD

THE THIRD COMPONENT of human complement, C3, is cleaved into higher and lower molecular weight fragments by reactions involving prior-acting components or by enzymes unrelated to the complement system, such as trypsin.[1-5] At least in the case of the complement-mediated reaction, the larger fragment, C3b, attaches to the immune complex and directly or indirectly subserves the pathobiologic functions of immune adherence, conglutination, immunoconglutination and enhanced phagocytosis. Attachment of C3b is also requisite for subsequent interaction of the remaining components of complement leading to lesions in cell membranes (immune cytotoxicity).[6,7] However, cleavage of C3 is an uncommonly economical reaction in that not only does the larger fragment play a critically important role in the biologic properties of complement but also the smaller fragment, C3a, possesses pathobiologic activities consistent with a role in the inflammatory response.[2-7]

C3a is a polypeptide with a molecular weight of about 7000 which originates from the N-terminal portion of human C3.[3-5,8] It can be isolated from appropriate reaction mixtures ($C1\bar{s}$ + C4 + C2 + C3; trypsin + C3; etc) by gel filtration on Sephadex G75 at pH 3.5.[3] The peak emerging with the void volume of the column contains C3b; the first retarded peak contains C3a. Preparations of C3a prepared in this manner or by sucrose density gradient ultracentrifugation possess properties compatible with classification as an anaphylatoxin.[3-5] These include contraction of guinea pig ileum with tachyphylaxis, failure to contract rat uterus, enhancement of vascular permeability in guinea pig skin, and degranulation of mast cells in guinea pig mesentery preparations. The lack of identity of C3a with classical anaphylatoxin is indi-

From the Departments of Pathology, Health Center of University of Connecticut, Farmington and the National University of Mexico, Mexico City; and the Department of Pediatrics, Harvard Medical School, Boston, Mass.

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Address for reprint requests: Dr. Irwin H. Lepow, Department of Pathology, Health Center, University of Connecticut, Farmington, Conn 06032.

cated by its ability to release histamine from rat peritoneal mast cells and its failure to cross-desensitize guinea pig ileum to anaphylatoxin prepared by incubation of rat serum with antigen-antibody complexes or agar.[3,4] Classic anaphylatoxin is now known to be a cleavage product of C5.[3,4,9,10]

In attempting to assess the possible pathobiologic function of human C3a in human inflammatory states, it is of direct importance to determine the extent and range of activity of C3a in man. The present investigation was undertaken as an initial step toward this goal. The results to be presented demonstrate that intracutaneous injection of as little as 10 ng of human C3a into human volunteers produces significant enhancement of vascular permeability, as measured by wheal, erythema and extravasation of intravenous dye. This activity is inhibitable by an antihistaminic drug and is associated ultrastructurally with opening of endothelial junctions in postcapillary venules and marked degranulation of mast cells.

Materials and Methods

Preparation of Human C3a

Human $C1\bar{s}$, C4, C2 and C3 were purified, C3 was labeled with ^{125}I, and reaction mixtures were prepared as described previously.[3] In brief, 5 ml of a solution containing 25 units of $C1\bar{s}$, 500 μg of C4, 125 μg of C2 and 2500 μg of ^{125}I-C3 in phosphate-buffered saline (pH 7.4, ionic strength 0.15, 5×10^{-4} M Mg^{2+}) was incubated at 37 C for 5 min. The incubation mixture was then chilled in an ice bath and simultaneously acidified to pH 3.5 with 2 drops of 0.5 N HCl. Complete conversion of C3 to C3b was verified by examining an aliquot of the mixture by immunoelectrophoresis. A 0.5 ml aliquot of the mixture was removed to test its effect on guinea pig ileum; a full contraction followed by tachyphylaxis was observed at a dose of 0.1 ml as described previously.[3] The remaining 4.5 ml of the acidified mixture was layered onto a 1.5 × 80 cm column of Sephadex G-75 equilibrated with glycine-buffered saline at pH 3.5. Appropriate fractions of the eluate containing C3a were pooled and concentrated to 2.3 ml on an Amicon UM2 membrane by ultrafiltration under 50 lb/sq in of N_2. The concentrated C3a had an OD of 0.294 at 210 mμ, equivalent to 14.4 μg of C3a/ml. It was highly active on guinea pig ileum at a dose of 0.1 ml.

Effect of Human C3a in Human Skin

Three healthy male volunteers between the ages of 28 and 44 years served as subjects for this study and were well informed as to the predicted action of C3a before they consented to participate. Two of them were pretreated by intravenous injection of 5 ml of Evans blue, USP. Ten minutes later various amounts of C3a or histamine were injected intradermally in the forearm through a 26-gauge needle in 0.1 ml of sterile barbital buffer diluent at pH 7.4, ionic strength 0.15. Two perpendicular diameters of the resultant wheal and erythema were measured at regular intervals after the intradermal injection and the intensity of bluing of the lesion by extravasated Evans blue was estimated on a scale of 0–4+. All

parameters were maximal within 10 min and results were recorded at that time. The lesions began to subside within 30 min and gradually disappeared thereafter.

In order to test the effect of an antihistamine, one subject ingested 100 mg of Pyribenzamine 1 hr prior to the experiment. As a control, another subject ingested 1200 mg of acetylsalicylic acid 1 hr prior to the experiment.

In one instance, 1% Xylocaine was injected subcutaneously, 5 cm proximal to the area of injection of 50 ng of C3a. When the resultant lesion was maximally developed at 8 min after injection, a punch biopsy of the distal margin of the lesion was obtained. The skin biopsy was completed about 10 min after injection of human C3a and was immediately fixed in 2.5% glutaraldehyde in 0.067 M cacodylate buffer at pH 7.4, postfixed in osmium-collidine, and embedded in Epon 812. Thin sections were prepared on an LKB II Ultrotome, stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope.[11]

Results

Gross Observations

Injection of human C3a into human skin resulted in the appearance of a wheal and erythema response and extravasation of intravenously administered Evans blue dye (Table 1). The dose-response relationship was very narrow; maximal lesions were observed at doses of 50–1000 ng with a sharp decrement in activity at 10 ng. Pseudopodia were observed at several sites of injection and minimal itching was noted by the subjects.

Pretreatment of one subject (C, Table 1) with Pyribenzamine re-

Table 1. Wheal, Erythema and Bluing Response 10 Min after Intradermal Injection of Human C3a and Histamine

	Subject A			Subject B*		Subject C†		
Dose (ng/0.1 ml)	Wheal size (mm)	Erythema size (mm)	Bluing	Wheal size (mm)	Erythema size (mm)	Wheal size (mm)	Erythema size (mm)	Bluing
C3a								
1000	10 × 10	30 × 34	3+	11 × 11	40 × 50	8 × 7	12 × 13	+
500	9 × 10	27 × 24	3+	—	—	6 × 8	9 × 12	±
250	12 × 11	28 × 24	3+	—	—	8 × 7	12 × 11	±
100	10 × 11	24 × 24	3+	8 × 9	35 × 35	8 × 9	15 × 11	±
50	10 × 11	25 × 23	3+	—	—	8 × 8	12 × 12	0
10	8 × 8	8 × 8	0	7 × 6	20 × 15	6 × 5	0	0
Histamine								
500	13 × 12	30 × 28	3+	—	—	12 × 11	16 × 14	3+
100	11 × 10	26 × 25	3+	—	—	7 × 6	13 × 12	+
Diluent	8 × 7	0	0	10 × 9	0	7 × 6	0	0

* Subject received 1200 mg acetylsalicylic acid 1 hr prior to experiment.

† Subject received 100 mg Pyribenzamine 1 hr prior to experiment.

sulted in partial inhibition of his response to C3a, equivalent to the diminution in his response to 100 ng of histamine.

Variable reactivity to C3a was observed in the responses of the 2 subjects not treated with Pyribenzamine, indicating variability in host response to C3a. The diminished response of the subject pretreated with Pyribenzamine was not due solely to this variability for when he was injected with 100 ng of the same preparation of C3a 12 hr later, a more brisk response was observed, consisting of a wheal 8 × 10 mm and erythema 16 × 21 mm in area.

Ultrastructural Observations

A skin biopsy specimen was examined from the margin of a wheal produced by 50 ng of C3a, approximately 10 min after the injection. The C3a preparation was different from that employed in the experiment cited under gross observations. A 12 × 12 mm wheal and 30 × 40 mm of erythema resulted from this injection, along with extensive pseudopod formation, in Subject A.

Postcapillary venules exhibited discontinuities of basement membranes and frequent endothelial gaps. Some electron-dense granules were observed in areas of extravasated plasma, possibly representing lipid droplets (Fig 1).

Mast cells in the biopsy specimen exhibited extensive diminution of electron density in the granules. Only occasional intracellular granules remained intact in mast cells of affected areas (Fig 2A and 2B).

No accumulation of leukocytes was noted in the lesion and no other abnormalities were seen in the biopsy specimen.

Discussion

The multiplicity of potential mediators of the acute inflammatory response presents a challenge in evaluating the qualitative and quantitative participation of a given mediator in a given inflammatory process. In analogy with Koch's postulates for infectious diseases, criteria for defining the role of a mediator of inflammation have been formulated by Miles and Wilhelm.[12] These include mechanisms for the generation or release of the mediator at tissue sites, demonstration of the pathobiologic activity of the mediator *in vivo* and its presence in lesions, and inhibition of the inflammatory state under study by methods that prevent generation, release or action of the mediator. Rigorous fulfillment of these criteria is not only technically difficult but is further beset with the conceptual complexities that may result from concerted action of a variety of mediators in a given process.

Recent investigations have focused interest on the complement system as a mediator of inflammation. With advances in the purification, characterization and understanding of the biochemical mechanism of action of the components of complement, it has been possible to define pathways by which complement may mediate such parameters as enhanced phagocytosis, enhanced vascular permeability, contraction of smooth muscle and chemotaxis.[6,7,9,10,13–17] In most instances, however, assessment of the participation of complement in these phenomena *in vivo* has been mainly inferential and has frequently been complicated by the use of model systems involving mixed animal species.

Human C3a is a reaction product of low molecular weight resulting from interaction of the first four components of complement. The potential for its formation therefore exists at tissue sites in which appropriate complexes of antigen and antibody can trigger the complement system. A very similar product can be formed by nonimmunologic mechanisms involving cleavage of C3 by enzymes unrelated to complement.[3–5,7,9,10] Thus, biochemical pathways have been defined that are capable of leading to generation of C3a. The range of pathobiologic activities of human C3a has been described in animal systems and includes contraction of guinea pig ileum, enhancement of vascular permeability in guinea pig skin, degranulation of mast cells in guinea pig mesentery, and release of histamine from rat peritoneal mast cells.[2–4] The chemotactic response of rabbit polymorphonuclear cells to human C3a is controversial.[3,5] Attempts to demonstrate C3a in inflammatory lesions are compromised by the existence of mechanisms for its rapid inactivation in normal serum.[5,10] Inhibition of the well-documented activities of human C3a can be achieved with antihistaminic drugs.[3,4] A serious omission in this review of the present status of human C3a as a potential mediator of inflammation is information concerning its activity in man. The present study was designed to provide initial data on this critical point.

Intracutaneous injection of very low doses of human C3a resulted in impressive wheal and erythema responses in human skin with extravasation of intravenously administered Evans blue dye. Maximal lesions were observed over a dosage range of 50–1000 ng with a sharp drop in activity at 10 ng. The association of the lesions with pseudopodia and itching and their partial inhibition by Pyribenzamine were compatible with a mechanism involving histamine release.

The activity of human C3a in human skin is markedly greater than its modest activity in guinea pig skin. The response achieved with 10–50 ng of C3a in man was approximately comparable to that previ-

ously observed with microgram quantities in the guinea pig.[3] Thus, man is at least two orders of magnitude more responsive to permeability enhancement by human C3a than the guinea pig. A dose of 10 ng of human C3a corresponds to approximately 10^{12} molecules. Thus, when tested in the homologous species, human C3a is among the most potent permeability factors thus far described.[18]

Ultrastructural observations on skin biopsies were also compatible with a phenomenon of histamine release.[19,20] Gaps between endothelial junctions and discontinuities in the basement membrane were visualized in postcapillary venules. Electron-dense bodies, probably of lipid nature and possibly representing extravasated chylomicra, were found outside of these vessels. The extensive diminution in the electron density of mast cell granules was very striking and was comparable with that to be expected from such potent histamine releasers as Compound 48/80.[21] It appears reasonable to propose that human C3a increases vascular permeability in man by release of histamine from mast cells with resultant opening of endothelial junctions and passage of noncellular intravascular contents through the basement membrane into the perivascular area.

These observations documenting the very marked activity of human C3a in man provide additional impetus for evaluating the role of this mediator in specific inflammatory processes.

Summary

Purified human C3a is a potent enhancer of vascular permeability in human skin, producing wheal and erythema responses with extravasation of intravenously administered dye at doses as low as 10 ng. The characteristics of the gross lesion and its partial inhibition by an antihistaminic drug are compatible with a pathogenesis involving histamine release. This is further indicated by observations on a biopsy of a human skin lesion 10 min after intradermal injection of 50 ng of human C3a. Electron micrographs demonstrated gaps between endothelial cells and discontinuities in the basement membrane of postcapillary venules. A striking finding was the diminution of electron density of mast cell granules, indicating extensive mast cell degranulation.

Human C3a is at least two orders of magnitude more active in human than in guinea pig skin. On the basis of its activity in man, it is among the most potent permeability factors and mast cell degranulators yet described.

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The complement nomenclature used in this publication conforms to the recommendations of an expert committee, as published in the Bulletin of the World Health Organization 39: 935–938, 1968.

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Dr. Patrick's present address is: Department of Microbiology, University of South Dakota, Vermillion, South Dakota 57069. Work was performed during tenure of a Research Fellowship from the Connecticut Heart Association.

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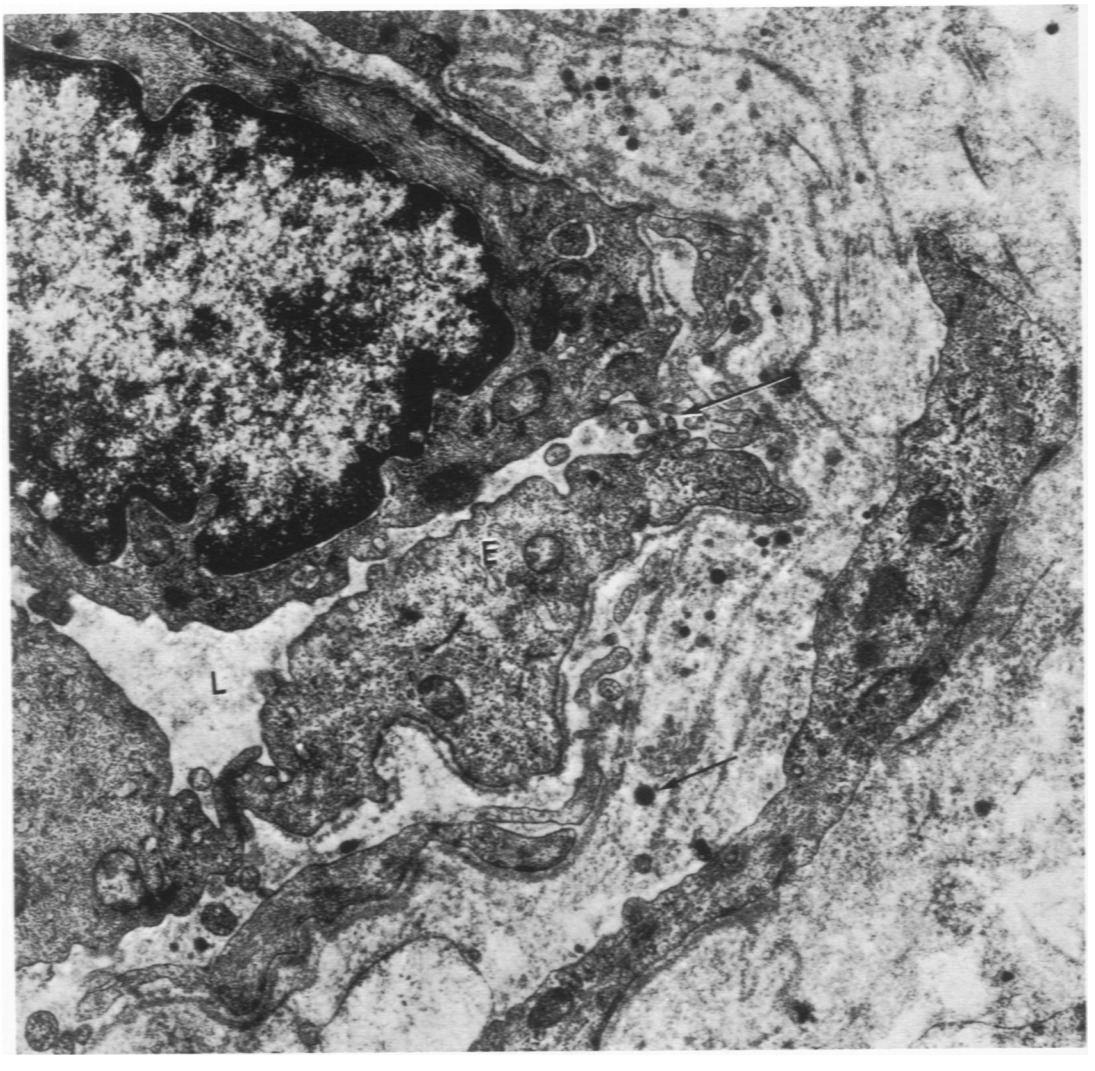

Fig 1. Human forearm skin 10 min after intradermal injection of 50 ng of human C3a. Postcapillary venule with endothelial gap *(long arrow)*, discontinuous basement membrane, and droplets of extravasated material, possibly lipid *(short arrow)*. *E* indicates endothelium; *L*, lumen. Approx × 20,000.

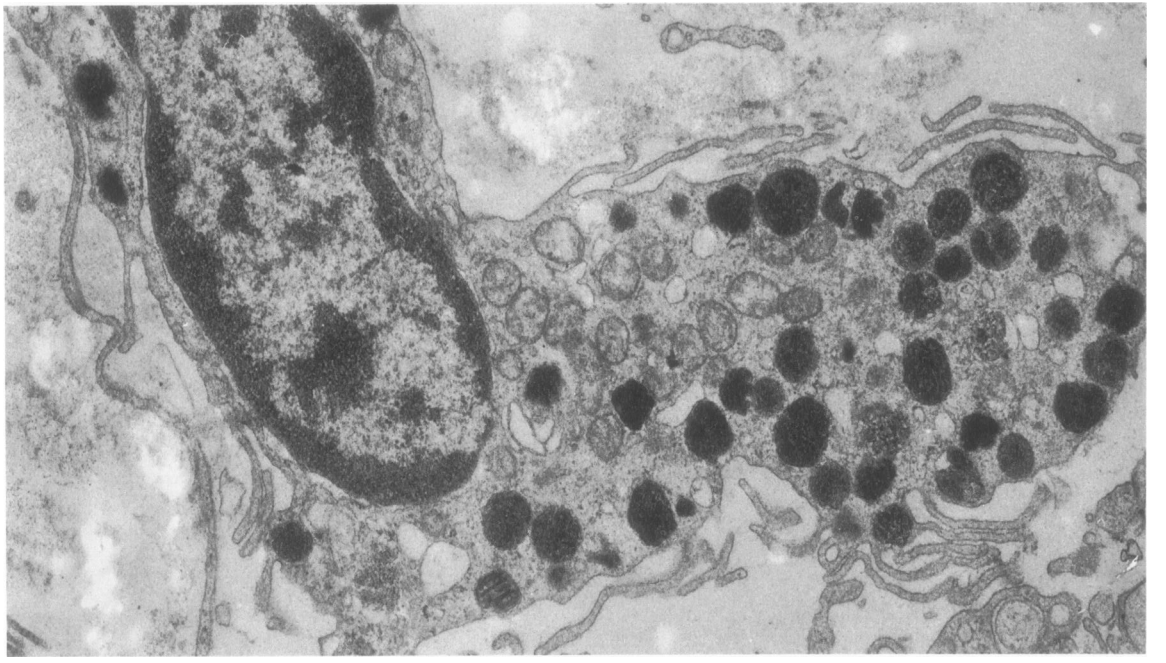

Fig 2. Human forearm skin. **A.** Control site. Section of typical mast cell, showing normal appearance of granules. Approx × 19,000.

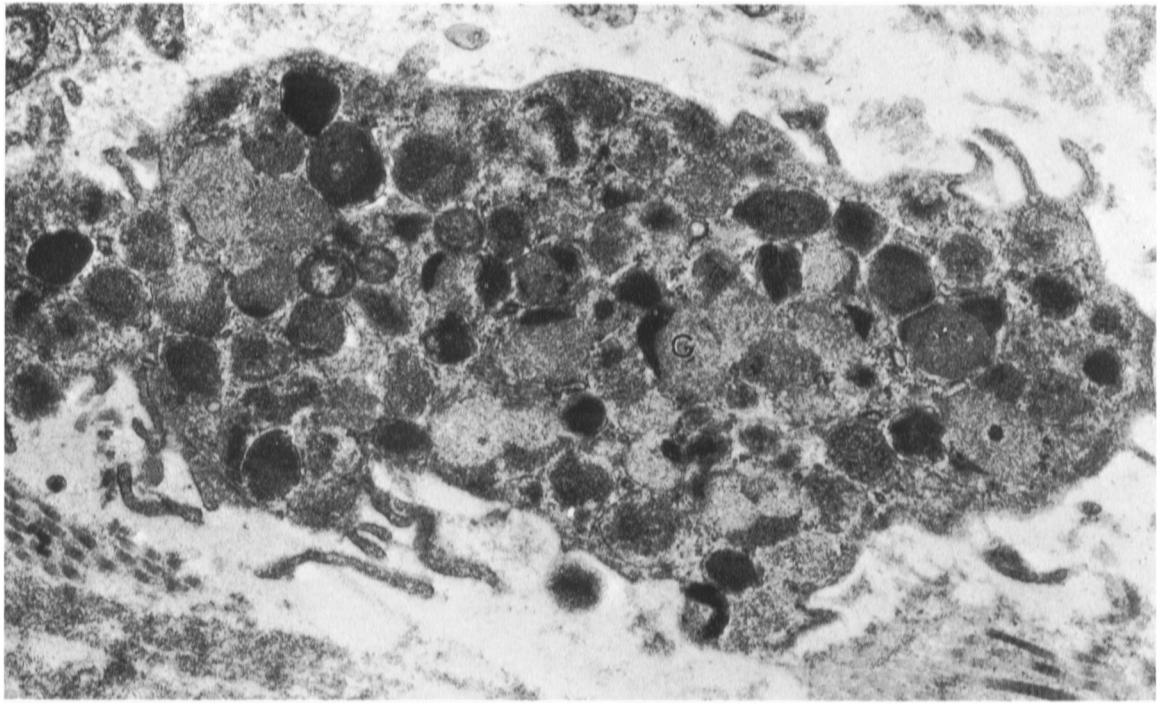

Fig 2B. Ten minutes after intradermal injection of 50 ng of human C3a. Section of typical mast cell, showing almost complete degranulation. Approx × 22,000.

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