HUMAN PLATELET ACTIVATION BY C3a AND C3a des-arg*

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We have previously demonstrated that certain platelet functions are modulated in the presence of complement. Thrombin-mediated platelet aggregation and release were significantly increased in the presence of the complement components C3, C5, C6, C7, C8, and C9. (1) Further, when platelets were stimulated by thrombin in the presence of these complement components, C5b-9 dimers were visualized ultrastructurally on the platelet membranes (2). Since the complement effect was inhibited by known inhibitors of platelet cyclo-oxygenase such as aspirin and indomethacin it was postulated that the complement-mediated effect was mediated via the arachidonic acid transformation pathway (2). In further studies (3) it was found that arachidonate-mediated release of serotonin and of Thromboxane B_2 was significantly enhanced in the presence of the human complement components C5, C6, C7, C8, and C9. Dimers of the C5b-9 complex were similarly visualized ultrastructurally on the platelet surface subsequent to stimulation with arachidonate in the presence of C5-C9.

It is the purpose of the present study to demonstrate an additional role for complement in platelet physiologic processes. Human C3a was found to induce platelet aggregation and release of serotonin. At concentrations below which it no longer induced these effects directly, the peptide showed significant synergism with ADP in inducing platelet aggregation and serotonin release. Removal of the C-terminus arginine from C3a did not alter this effect on platelet function and thus it was possible to localize the platelet-stimulating activity to the C3a des-arg peptide.

Materials and Methods

Preparation of Gel-filtered Platelets. The method used was a variation of several different methods (4–8) with modifications as follows: Blood (usually 100 ml) was drawn into a syringe that contained 1/6 volume acid citrate dextrose (ACD)[1] (9). In some experiments prostaglandin E_1 (PgE_1) (Sigma Chemical Co., St. Louis, MO) to a final concentration of 100 nM (5) was also present in the syringe. The whole blood in ACD was centrifuged at 90 *g* for 15 min; the supernatant plus buffer layer were removed and recentrifuged at 500 *g* for 6 min. The supernatant platelet-rich plasma (PRP) was removed and was labeled

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[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; ADP, adenosine diphosphate; ANOVA, analysis of variance; $\overline{C4b^{oxy}2a}$, fluid phase intermediate complex prepared from human C1 esterase, C4 and oxidized C2; CPB, carboxypeptidase B; EAC$\overline{1}$, $4b^{\overline{oxy}2a}$, erythrocyte intermediate complex prepared from sheep cells sensitized with rabbit anti-sheep cell antibody, and human complement components C1, C4 and oxidized C2; Hepes, N-2 hydroxyethylpiperazine-N-2-ethane sulfonic acid; LDH, lactic dehydrogenase; MTHB, modified Tyrode's buffer, pH 6.5 containing Hepes; PgE_1, prostaglandin E_1; PRP, platelet-rich plasma.

with ^{14}C serotonin when experiments were to be performed to measure the release reaction. The PRP either labeled with ^{14}C serotonin, or unlabeled, was centrifuged at 1,200 *g* for 10 min, and 2 ml modified Tyrodes's buffer, pH 6.5 containing 0.01 M N-2 hydroxyethylpiperazine-N-2-ethane sulfonic acid (Hepes) (Sigma Chemical Co.) was added to the platelet button. This buffer (MTHB) contained per liter: 8 g sodium chloride, NaCl); 0.2 g potassium chloride (KCl); 0.057 g sodium phosphate ($NaH_2PO_4 \cdot H_2O$); 0.1 g sodium bicarbonate ($NaHCO_3$); 1.0 g dextrose, and 2 g bovine serum albumin. (Bovine serum albumin was obtained from Miles Laboratories, Inc., Elkhart, IN). The platelet preparation was allowed to stand at 37°C for 15 min, then was applied to a 28 cm × 1.8 cm column of Sepharose CL-2B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) equilibrated with MTHB. The platelets emerged from the column as a sharp peak; the fractions containing the platelets were pooled, then the suspension was adjusted to the appropriate concentration ($2.0–4.0 \times 10^5/\mu l$) and the pH was adjusted to 7.3 with 1 N sodium hydroxide (NaOH).

Comparison of Gel-filtered Platelets with Washed Platelets

A single donor was bled 200 ml into syringes containing ACD and PGE_1 at the same concentration as those used when the platelets were to be gel-filtered alone. The whole blood was divided into two equal aliquots. Each aliquot was processed to a platelet button as described above. To the first aliquot was added 2 ml MTHB and the platelets were subjected to gel-filtration as described above. Platelets in the second aliquot were washed with the modified Ardlie Buffer system as previously described (1). The platelet concentration of both preparations was adjusted to $3.0 \times 10^5/\mu l$.

Purification of Complement Components. C1 (10), $C1\bar{s}$ (11), C2 (12), C3 (13), C4 (14), C5 (15), C6, C7 (16), C8 (17), and C9 (18) were prepared from human serum by methods described earlier. In addition, in some experiments, purified human complement components C6, C7, C8, and C9 prepared by Cordis Laboratries, Inc., Miami, FL were utilized.

Preparation of Fluid Phase C3 Convertase ($\overline{C4b^{oxy}2a}$). 40 μg C4 and 30 μg ^{oxy}C2 (19) were incubated at 32°C for 10 min with 9 μg $C1\bar{s}$.

Preparation of Erythrocyte-bound C3 Convertase ($EAC\bar{1}$, $\overline{4b, {}^{oxy}2a}$). The erythrocyte intermediate $EAC\bar{1}$, $\overline{4b, {}^{oxy}2a}$ was prepared as described earlier. (20)

Preparation of C3a. 2 mg of C3 were incubated for 30 min at 37°C with either the $\overline{C4b^{oxy}2a}$ fluid phase complex prepared as above or with 2×10^{10} packed $EAC\bar{1}$, $\overline{4b, {}^{oxy}2a}$. The third method used for the preparation of C3a was treatment of C3 with trypsin (21). C3 at 1 mg/ml was treated for 60 s at room temperature with 0.8% (wt/wt) trypsin (Worthington Diagnostic Systems, Inc., Freehold, NJ) (The trypsin was treated with L-(tosylamido 2-phenyl) ethyl chlormethyl ketone to inhibit contaminant chymotryptic activity). Soybean trypsin inhibitor (Calbiochem-Behring, La Jolla, CA) was added at twice the trypsin concentration. In each case the preparation was immediately acidified to pH 3.5, and subjected to molecular sieve chromotography on Sephadex G-100 (Pharmacia Fine Chemicals) equilibrated with acetate-buffered saline, pH 3.5 (21).

Preparation of C3e

C3 at 1 mg/ml was treated with 2% (wt/wt) trypsin for 2 h at 37°C. Soybean trypsin inhibitor was added at twice the trypsin concentration (22).

Preparation of C5a

C5 at 500 μg/ml was treated with 2% (wt/wt) trypsin for 12 min at 37°C. Soybean trypsin inhibitor was added at twice the trypsin concentration (23).

Platelet Aggregation

Assays for platelet aggregation were performed in a Payton dual channel aggregometer equipped with a Riken Denshi recorder (Payton Associates, Inc., Buffalo, NY). Gel-filtered platelets were suspended at $2.0–4.0 \times 10^5/\mu l$ in MTHB. C3a and ADP (Sigma Chemical Co.) were added singly or together at various concentrations to 0.4 ml of platelets

containing 1.2 mM $CaCl_2$ and fibrinogen at 900 μg/ml (Kabi Group, Inc., Greenwich, CT). The platelet mixtures were stirred for 5 min while aggregation responses were recorded, and when release measurements were to be made, the samples were centrifuged and the supernatants were removed. ~80% of the gel-filtered platelet preparations showed suboptimal aggregation with 10 μM ADP. Only one in five preparations aggregated optimally with 10 μM ADP alone. Fur the purposes of the present study, gel-filtered platelets that reacted minimally to ADP alone were used to study the effects of complement on aggregation. To quantitate platelet aggregation, the area under the curve of light transmission was determined by tracing the curve 5 min after the addition of the stimulus with a planimeter No. 123A (Dietzgen Corp., Des Plaines, IL) three times and calculating the average (24).

In some experiments platelets were stimulated with thrombin (kindly supplied by Dr. J. Fenton II, New York State Department of Health, Albany, NY).

Release of ^{14}C Serotonin. Blood was drawn into ACD as described earlier, but no PGE_1 was present in the syringe. Platelets suspended in plasma were labeled with ^{14}C-serotonin by the method of Valdorf-Hansen and Zucker (25). The radiolabeled platelets were gel-filtered in the usual way. Various reagents were added to 0.4 ml of the gel-filtered platelet preparation and aggregation recorded for 5 min. Then the tube was rapidly centrifuged and the supernatant was transferred to another tube. ^{14}C was counted in both the supernate and the cell button in a Packard liquid Scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) and the percentage of release was calculated.

Removal of Ca^{++} and Mg^{++} from Platelet-suspending Buffer (MTHB). Trace amounts of Ca^{++} and Mg^{++} contained in MTHB were removed by addition of Chelex 100 (Bio-Rad, Rockville Centre, NY) followed by filtration through a Chelex 100 column as previously described (8).

Release of Lactic Dehydrogenase (LDH). LDH was determined by the method of Wroblewski and LaDue (26). 1U was expressed as decrease in OD at 340 μm of 0.001/min per ml. A control sample giving 100% lysis was obtained by the addition of 0.1% Triton X-100.

Removal of C-Terminus Amino Acid Arginine from C3a. 22 nmol of C3a were incubated at pH 7.6 for 30 min with various concentrations of carboxypeptidase B(2.5 nmol, 12.5 nmol, 25 nmol) (CPB). As a control, bradykinin at the same molar concentration as C3a was treated with similar amounts of CPB.

The effect of CPB on C3a was analyzed on a flat plate high voltage apparatus (Savant Instruments, Inc. Hicksville, NY) at 1 kV for 60–70 min using 20 cm × 20 cm cellulose coated thin-layer chromatography plates (EM Laboratories, Elmsford, NY) and a pyridine acetic acid buffer of pH 6.4 (27). For analytical purposes 15 nmol of C3a or 15 nmol of C3a treated with CPB were applied. As a control 15 nmol of bradykinin or 15 nmol of bradykinin treated with CPB were also applied. Arginine was used as a marker. After drying the plates were stained with a solution of 0.5% ninhydrin in acetone.

C3a Anaphylatoxin Activity. Anaphylatoxin activity was measured on segments of isolated guinea pig ileum as previously described (23).

Statistical Analysis

Statistical analyses were performed on a Hewlett-Packard 9815A computer using prepared programs of the Hewlett-Packard statistics library (09815-15001) for one- and two-way analysis of variance (ANOVA). Although the data are displayed in tables as percentages of control, analyses of variance were performed using the nontransformed data. (24)

Immuno-ultrastructural Studies

Preparation of $F(ab')_2$ fragments of anti-C3a and anti-C3c. Monospecific antiserum to human C3a was prepared in rabbits by injection of the purified protein into the popliteal lymph nodes followed a month later by intramuscular injections of the same protein (28). The antiserum was extensively tested immuno-chemically for specificity and was found to be monospecific for C3a. The γ-globulin fraction was prepared from the antiserum as

described earlier (29). The γ-globulin fraction of monospecific antibody to C3c was purchased from Dako Corp., Santa Barbara, CA. The purified γ-globulin fraction of each antibody was digested with pepsin and the $F(ab')_2$ fragments were isolated by molecular sieve chromatography on Sephadex G-150 as described earlier (29).

The $F(ab')_2$ fraction of each antibody was conjugated with gold by the method of Frens (30) as modified by Handley et al. (31).

Reaction of Gel-filtered Platelets with C3a

To each of eight 1-ml aliquots of gel-filtered platelets at 300,000/μl containing 1.2 mM $CaCl_2$ and fibrinogen (900 μg/ml) was added 1.2 μg C3a. Duplicate samples were allowed to incubate at 37°C with stirring for periods of 30 s, 60 s, or 5 min, or at 0°C for 5 min. At the end of the incubation period the samples were immediately cooled to 0°C. The platelets in each sample were washed three times with ice-cold sterile saline and then were resuspended to 1 ml. To one aliquot of platelets from each incubation period was added 10 μg of gold-conjugated $F(ab')_2$ fragment of anti-C3a, and to the second aliquot was added 10 μg of gold-conjugated $F(ab')_2$ fragment of anti-C3c. The mixtures were allowed to stand at 2–4°C for 16 h, then the platelets were washed three times with saline.

Preparation of Platelet Membranes for Electron Microscopy

The washed platelets were further washed once in a 1:15 dilution of saline and then alternatively frozen at −70°C and thawed three times. The resultant platelet membranes were washed three times in the diluted saline and then were applied unstained to copper grids coated with collodion and carbon. The grids were viewed under a Philips 301 Electron Microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) at 80 kV.

Results

Effect of C3a on Aggregation of Gel-filtered Platelets. C3, activated by fluid phase $\overline{C4b2a}$, EACl, $\overline{4b2a}$ or by trypsin was fractioned by molecular sieve chromatography on Sephadex G-100 into two major protein peaks (a typical protein profile is shown in Fig. 1). C3a was localized to fractions #78–92 as measured by anaphylatoxin activity using isolated guinea pig ileum segments and immunologic reactivity with a monospecific antibody to C3a. A known C3a marker of mol wt 9,000 emerged in the same fractions. Each fraction of the column was tested in addition for its ability to aggregate gel-filtered platelets. The fractions containing C3a caused marked aggregation of the gel-filtered platelets. (Fig. 1). This response was not inhibited by prior exposure of the platelets to aspirin (100 μM). Addition of C3 alone; C3b; C3e; C5 alone; C5a; C3, C5, C6, C7, C8, and C9 or C5, C6, C7, C8, and C9 did not induce the aggregation of gel-filtered platelets.

C3a-mediated aggregation of gel-filtered platelets was found to be Ca^{++} dependent. Optimal aggregation was obtained at Ca^{++} concentrations of 0.6–2.0 mM. When Mg^{++} was added at similar concentrations, the platelets aggregated less well than they did in the presence of Ca^{++}.

The method of platelet preparation significantly influenced the responsiveness of platelets to the complement peptide. Platelets isolated from one donor were split in two separate aliquots, one was processed as above by gel filtration; the other aliquot was processed by the multiple washing procedure (1). The gel-filtered platelets responded to C3a while the washed platelets were refractory (Fig. 2).

C3a alone at low concentrations (2×10^{-12} M) failed to aggregate platelets. ADP alone at 10 μM also failed to induce platelet activation. When platelets were stimulated with both agents together, significant aggregation occurred (Fig.

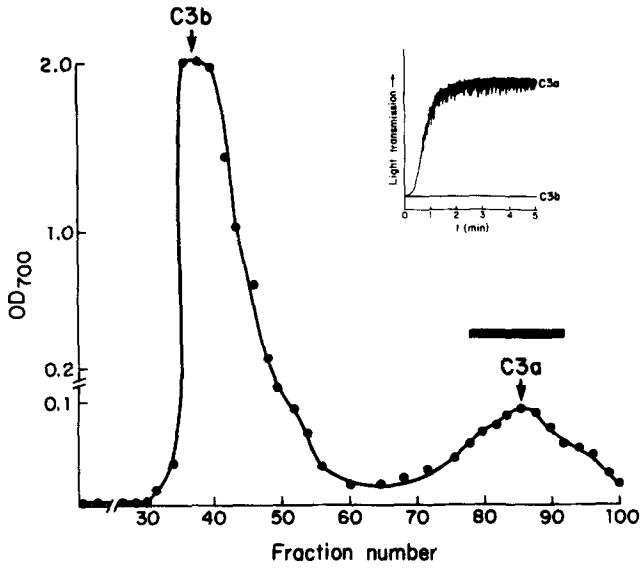

FIGURE 1. Isolation of C3a and C3b by molecular sieve chromatography on Sephadex G-100 and correlation of the distribution of C3a with platelet-stimulating activity. 50 mg of C3 was applied to the column after treatment with 0.8% trypsin (wt/wt) for 60 s at 20°C. The column was equilibrated with 0.15 M acetate-buffered sodium chloride, pH 3.5. The solid bar indicates the only fractions from the column that mediated platelet aggregation. Inset shows platelet aggregometer tracings obtained when platelets were stimulated with C3a and C3b. In the experiment shown, to 0.4 ml gel-filtered platelets at 300,000/μl was added at time 0 either 100 ng C3a or 38 μg C3b in the presence of 1.2 mM $CaCl_2$ and fibrinogen at 500 μg/ml.

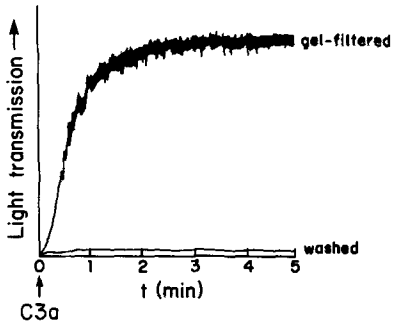

FIGURE 2. Platelet aggregometer tracings obtained when platelets from the same donor that had been separated from plasma proteins by either gel-filtration or by the "washing" procedure (See Materials and Methods) were stimulated with C3a at 2×10^{-11} M.

3). To demonstrate that C3a and ADP synergistically induced platelet aggregation, gel-filtered platelets were incubated with ADP alone, C3a alone, and combinations of ADP and C3a at various concentrations. The data in Table I show that when ADP and C3a were combined the resultant platelet aggregation was greater than the sum of the aggregations generated by each agent alone; this type of result defines synergism. The interaction (synergy) was highly significant ($P < 0.0001$ by two-way ANOVA).

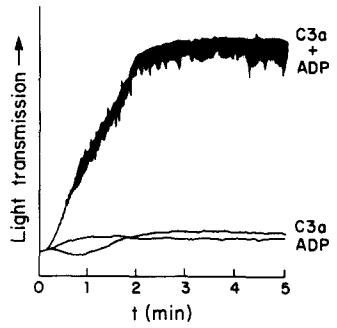

FIGURE 3. Platelet aggregometer tracings obtained when platelets were stimulated with C3a (2×10^{-12} M) or ADP (10 μM) singly or combined at the same concentrations.

TABLE I

Synergy between C3a and ADP in Platelet Aggregation

ADP (μM)	C3a (M)			
	0	10^{-12}	2×10^{-12}	5×10^{-12}
	% Platelet aggregation*			
0	0	0	5 ± 0.7	13 ± 1.4
10	7 ± 0.7	56 ± 2	83 ± 4	100

* Gel-filtered platelets were stimulated with C3a alone, ADP alone, or combinations of C3a and ADP at the concentrations indicated. Aggregation was allowed to proceed for 5 min, then the area under the curve was measured planimetrically. The results are expressed as a percentage of the value obtained when platelets were stimulated with ADP (10 μM) and C3a (5×10^{-12} M) together.

Statistical analysis was performed by two-way analysis of variance to assess the significance of the effects of each agent alone and their combination, with the following results: P_{C3a}, P_{ADP}, $P_{Synergy}$ all <0.0001.

Platelet-stimulating Activity of C3a des-arg

C3a was treated with CPB using conditions previously shown to remove the C-terminal arginine (27). Treatment of C3a by CPB resulted in the liberation of free arginine as demonstrated by high voltage electrophoresis. The resultant C3a des arg had no anaphylatoxin activity but retained full platelet-stimulating activity (Table II). When combined, C3a des-arg and ADP exhibited a synergism on platelet aggregation ($P < 0.0001$) that was identical to the synergism exhibited by C3a and ADP.

Effect of C3a and C3a des-arg on Platelet Serotonin Release

C3a and C3a des-arg at identical concentrations induced similar degrees of serotonin release from stimulated platelets (Fig. 4*A*). There was no difference in the release-inducing effect of C3a or C3a des-arg when compared singly or together with ADP. (Fig. 4*B*). To demonstrate that C3a des-arg together with ADP synergistically induced platelet serotonin release, gel-filtered platelets were incubated with ADP alone, C3a des-arg alone, and combinations of ADP and C3a des-arg at various concentrations. The data in Table III show that when ADP and C3a des-arg were combined, the resulting serotonin release was greater

TABLE II

Anaphylatoxin and Platelet-stimulating Activity of C3a and C3a des-arg

	Anaphylatoxin activity	Platelet aggregation
	%	%*
C3a	100	100
C3a des-arg	0	98 ± 4

* Gel-filtered platelets were stimulated with C3a or C3a des-arg at the same concentration (5×10^{-11} M). Aggregation was allowed to proceed for 5 min, then the area under the curve was measured planimetrically and the data were expressed as a percentage of the value obtained when platelets were stimulated with C3a.

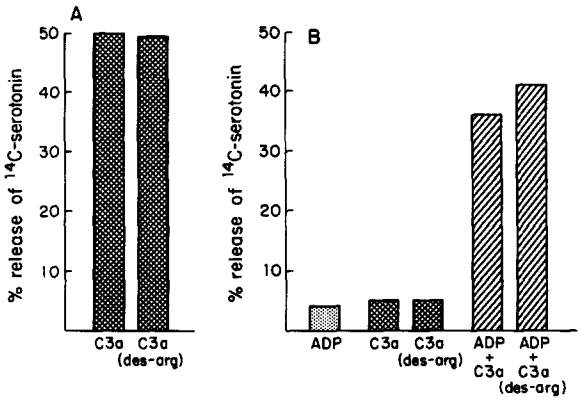

FIGURE 4. (*A*) Release of platelet serotonin mediated by C3a and C3a des-arg at 10^{-11} M. (*B*) Release of platelet serotonin mediated by ADP (10 μM); C3a or C3a des arg (2×10^{-12} M) singly; or ADP together with C3a or C3a des arg at the same concentrations.

TABLE III

Synergy between C3a des-arg and ADP in Platelet Secretion

ADP (μM)	C3a des-arg (M)		
	0	2×10^{-12}	6×10^{-12}
	% ^{14}C serotonin release*		
0	0	12 ± 5	33 ± 2
1	6 ± 2	57 ± 10	67 ± 4
10	13 ± 4	56 ± 3	69 ± 3

* Gel-filtered platelets were stimulated with C3a des-arg alone, ADP alone, or combinations of C3a des-arg and ADP at the concentrations indicated. Aggregation was allowed to proceed for 5 min, then the percentage of release of ^{14}C serotonin was determined as described in Materials and Methods.

Statistical analysis was performed by two-way analysis of variance to assess the effects of each agent alone and in combination, with the following results: P_{C3a}, P_{ADP}, $P_{synergy}$ all <0.001.

than the sum of the release generated by each agent alone. The synergy was highly significant ($P < 0.001$ by two-way ANOVA). Identical results were obtained with C3a and ADP. No release of LDH was measurable at any concentration of C3a or C3a des-arg tested alone or in combination with ADP.

Lack of Synergism between C3a and Thrombin in Platelet Aggregation

Gel-filtered platelets were incubated with C3a alone, thrombin alone, and combinations of C3a and thrombin at various concentrations. As indicated in Table IV, when thrombin and C3a were combined, the resultant platelet aggregation was not significantly greater than the sum of the aggregation generated by each agent alone. This finding indicated lack of synergy between C3a and thrombin.

Immuno-ultrastructural Localization of C3a on the Platelet Membrane Subsequent to C3a Stimulation

As demonstrated in Fig. 5*A* C3a was present on the platelet membrane subsequent to C3a stimulation at all incubation periods tested as evidenced by visualization of the ultrastructural probe, gold, conjugated to monospecific antibody to C3a. No such gold probe was seen on the platelet surface before C3a stimulation. (Fig. 5*B*). No native C3 was present on the platelet surface either before or after C3a stimulation as demonstrated by the absence of gold on the platelet membrane when the ultrastructural probe was conjugated to anti-C3c (Fig. 5*C* and *D*).

Discussion

The present studies demonstrate a new biologic activity that is associated with the human complement system. C3a, the small fragment (M_r 9000) liberated from C3 by its specific activating enzyme C3 convertase (or by trypsin), induced

TABLE IV
Lack of Synergy between C3a and Thrombin in Platelet Aggregation

Thrombin (U)	C3a	
	0	1.5×10^{-11} M
	% platelet aggregation*	
0	0	48 ± 12.7
0.002	4 ± 3.1	57 ± 12.8
0.004	10 ± 2.3	64 ± 2
0.005	33 ± 12	73 ± 6
0.01	74 ± 8.1	100

* Gel-filtered platelets were stimulated with C3a alone, thrombin alone, or combinations of C3a and thrombin at the concentrations indicated. Aggregation was allowed to proceed for 5 min, then the area under the curve was measured planimetrically. The results are expressed as a percentage of the value obtained when the platelets were stimulated with thrombin (0.01 U) and C3a (1.5×10^{-11} M) together.
Statistical analysis was performed by two-way analysis of variance to assess the significance of the effects of each agent alone and their combination. The results indicated lack of significant synergism between C3a and thrombin ($P_{synergy} > 0.45$).

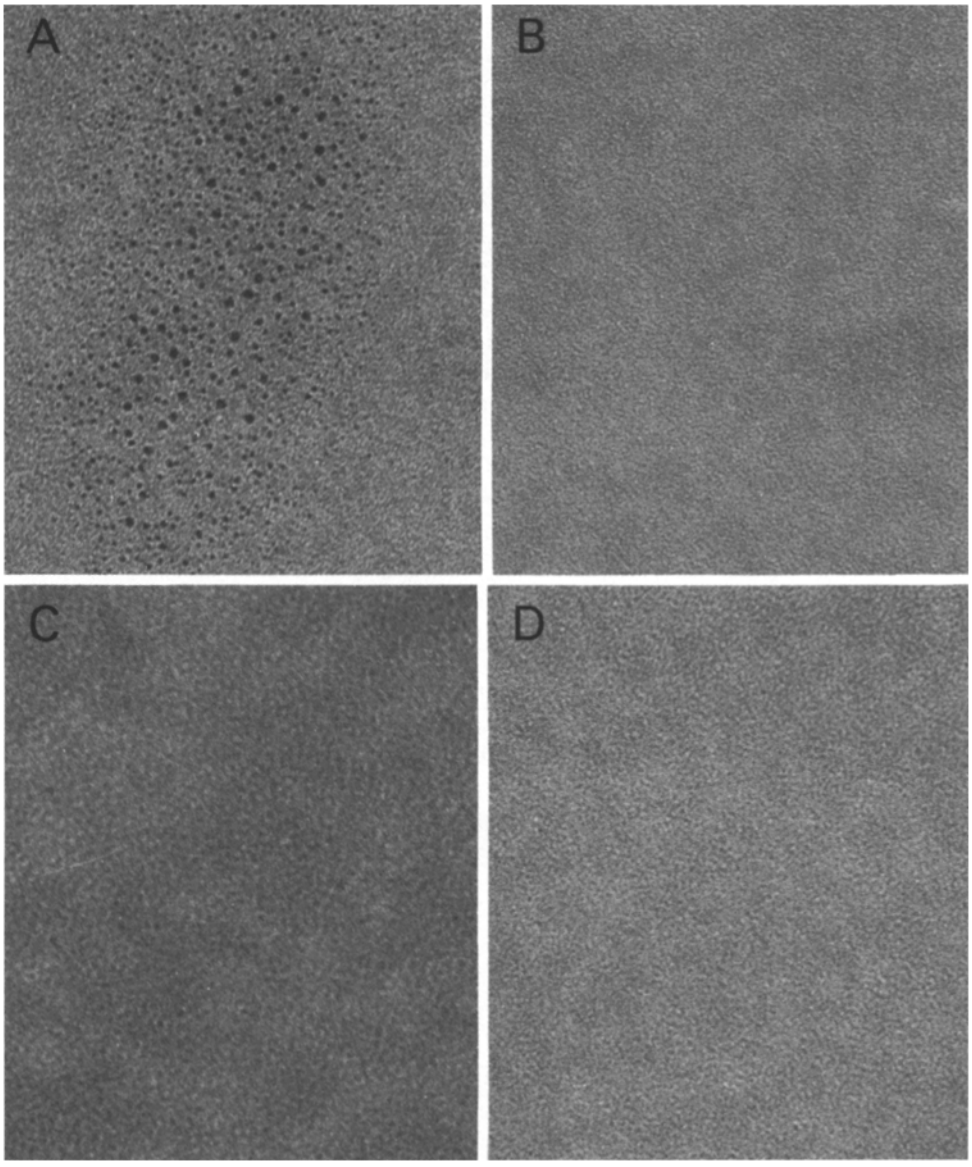

FIGURE 5. Immunoelectron microscopy of C3a on the platelet surface. (*A* and *B*) Platelets treated with gold-conjugated anti-C3a. (*A*) C3a-treated platelets. (*B*) Untreated platelets. (*C* and *D*) Platelets treated with gold-conjugated anti-C3c. (*C*) C3a-treated platelets. (*D*) Untreated platelets. All preparations were unstained. The gold probe was only seen in *A*, which indicated the presence of C3a on the platelet membrane only subsequent to C3a stimulation. The absence of native C3 on the platelet membrane either before or after C3a stimulation was indicated by the absence of the gold marker when anti-C3c was employed. × 264,000.

aggregation and serotonin release from gel-filtered human platelets. At concentrations of 10^{-10} M to 5×10^{-12} M, C3a induced aggregation when added alone to the preparation of gel-filtered platelets. However, at lower concentrations (2×10^{-12} M) C3a did not aggregate platelets directly, but exhibited highly

significant synergism ($P < 0.0001$) with ADP in mediating platelet aggregation and serotonin release. Previous studies by others (32, 33) have shown that human and guinea pig C3a activate guinea pig platelets but not human platelets. In these previous studies the platelets were subjected to extensive washing procedures to free the cells from plasma proteins. In our studies the platelets were separated from the plasma proteins by a more gentle gel filtration procedure. Our studies (Fig. 2) clearly demonstrate that the method used to prepare the platelets significantly influenced the responsiveness of the platelets to the complement peptide. It is not clear at present why the C3a-mediated activation of platelets should be significantly affected by the technique of platelet isolation. However, our data suggest the possibility that human platelets may have a receptor for C3a. If this turns out to be the case, it is possible that the washing procedure induces sufficient trauma to the platelet so that it affects the receptor sterically or otherwise, in such a way that it precludes its eventual acceptance of C3a in an active form.

The synergy exhibited between C3a and ADP, but not between C3a and thrombin, is of interest. Most, if not all of platelet agonists act synergistically (34), and platelet activation in vivo probably involves a combination of agonists acting in concert. These particular relationships may have potential relevance in the micro environment of vascular damage, where sub-optimal amounts of ADP released from activated platelets can interact with adjacent platelet surfaces preconditioned by contact with C3a generated in the vicinity of damage by complement-activating mechanisms, including thrombin generation at the platelet surface (1). The synergistic interaction of ADP and C3a in these circumstances may be important factors in modulating platelet membrane responses to physiologically activating stimuli.

The significance of the lack of synergy between C3a and thrombin remains to be fully clarified, however the observation is compatible with our previous suggestion that complement may function in these circumstances to regulate the fine tuning of the membrane to subtle rather than maximal stimulation (3). Huang and Detwiler (34) have suggested that different combinations of platelet agonists will elicit variable degrees of synergy depending on the mode of action and the concentrations of the individual agonists. The specific mechanisms involved in C3a-induced as well as thrombin-induced trans membrane signal generation remain to be clarified.

As demonstrated in the present study, C3a activated platelets at concentrations higher than 5×10^{-12} M and at 2×10^{-12} M exhibited highly significant synergism with ADP in mediating platelet aggregation and serotonin release. These concentrations involve relatively small numbers of molecules per platelet (10,000 and 4,000 respectively), and suggest the possibility of a C3a receptor on human platelets. This premise is strengthened by the demonstration ultrastructurally of the presence of C3a on the platelet membrane surface subsequent to stimulation with C3a (Fig. 5). Studies are currently underway to isolate and characterize a specific C3a receptor, on the human platelet surface.

It is of considerable biologic interest that the C-terminus arginine of C3a was not required for its platelet-stimulating activity. C3a and C3a des-arg were equally reactive in mediating platelet aggregation and release of serotonin. Further, both

exhibited synergism with ADP of equal significance in both aggregation and release. In vivo the C3a anaphylatoxin inactivator cleaves the C-terminus arginine from C3a, thereby ablating its anaphylatoxin activity (27). Previously no biologic activity was known to be associated with C3a des-arg. Recently (35) it was reported that this peptide inhibits in vitro natural killer cell activity. In the present study, a second biologic activity has been described for C3a des-arg, namely, that it is a peptide with potent platelet-stimulating activity. The biologic importance of circulating C3a des-arg in various clinical states following complement activation remains to be determined.

We have previously reported that human complement plays an important role in platelet physiologic processes. Thrombin-mediated platelet activation was significantly enhanced in the presence of C3-C9 (1) and transformation of arachidonate into thromboxane B_2 via the cyclooxygenase pathway was much increased in the presence of C5-C9 (3). The present study describes a further role for complement in platelet physiology. The significance of the present observation is related to the fact that C3, the parent molecule from which C3a is derived is a key complement component. Activation of the complement system by the classical pathway, alternative pathway, or by the thrombin-platelet pathway (1) all lead to cleavage of the C3 molecule and thereby liberation of C3a. Since C3 is present in plasma at the highest concentration of any of the complement components ($\sim$1.3 mg/ml) activation of the complement system by any of the known pathways can lead to the production of amounts of C3a (C3a des-arg) that may play an important role in regulating platelet function in hemostatic as well as thrombotic events.

Summary

C3a liberated from C3 by treatment with C3 convertase (or by trypsin) induced aggregation of gel-filtered human platelets and stimulated serotonin release. At concentrations of 10^{-10} M to 8×10^{-12} M, C3a induced aggregation when added alone to platelets. However, at lower concentrations (2×10^{-12} M) C3a did not aggregate platelets directly but exhibited highly significant synergism (two-way analysis of variance $P < 0.0001$) with ADP in mediating platelet aggregation and release of serotonin. Removal of the C-terminus arginine from C3a abolished anaphylotoxin activity but did not affect the platelet-stimulating activity of the peptide. C3a and C3a des-arg were equally reactive in mediating platelet aggregation and release of serotonin. Further C3a and C3a des-arg exhibited synergism with ADP of equal significance in both aggregation and the release reaction. The concentrations of C3a required for the platelet-stimulating activity involve relatively small number of molecules per platelet (4,000–10,000 for the synergistic reaction with ADP). These data suggest the possibility of a C3a (C3a des-arg) receptor on human platelets. This premise is strengthened by the demonstration ultrastructurally of C3a on the platelet membrane subsequent to C3a stimulation.

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