CONCISE PUBLICATIONS

Complement (C5a)-Induced Granulocyte Aggregation in Vitro

A POSSIBLE MECHANISM OF COMPLEMENT-MEDIATED LEUKOSTASIS AND LEUKOPENIA

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ABSTRACT Activated plasma complement will induce biphasic aggregation of human granulocytes detectable by standard nephelometric techniques. The responsible active component was suggested to be C5a by molecular weight and heat-stability assays; moreover, aggregating activity was ablated by anti-C5 but not anti-C3 antibodies. C5a prepared by trypsinization of purified C5 reproduced the aggregating activity of whole activated plasma, whereas plasma from a C5-deficient donor did not support aggregation. Embolization of granulocyte aggregates might be a previously unsuspected cause of leukostasis and pulmonary damage in various clinical situations where intravascular complement activation occurs.

INTRODUCTION

The acute granulocytopenia which occurs during the 1st h of hemodialysis results from pulmonary vascular sequestration induced by complement which has been activated by dialyzer cellophane membranes (1). The complement component(s) involved are of molecular weight 7,000–20,000 daltons (1), similar to those responsible for the leukopenia resulting from cobra venom infusion (2). However, the precise nature of the molecule(s) has not been characterized, and the mechanism of sequestration remains undefined. Leukostasis could result from embolization of granulocytes primarily aggregated in the venous circulation by activated complement component(s), or from complement-mediated adhesion of these cells to the pulmonary vascular endothelium. The present studies indicate that activated complement will induce granulocyte aggregation in vitro, and that C5a is the component responsible. Although the role of complement-mediated adhesion of granulocytes to endothelium has not yet been explored, this C5a-induced aggregation and aggregate embolization might be one mechanism of complement-mediated pulmonary leukostasis.

METHODS

Granulocyte preparation. Normal human venous blood, anticoagulated with heparin U. S. Pharmacopeia (5 U/ml), was mixed with half its volume of 6% (wt/vol) dextran-75 (Gentran 75, Travenol Laboratories, Deerfield, Ill.), and allowed to

A preliminary report of this work was presented to the Annual Meetings of the Central Society for Clinical Research in November 1976, and the American Society of Hematology in December 1976, and appears in abstract form in 1976. *Clin. Res.* **24**: 540A and 1976. *Blood.* **48**: 961.

Received for publication 6 December 1976 and in revised form 21 March 1977.

sediment at 20°C for 90 min. The supernatant, leukocyte-rich plasma was centrifuged at 400 *g* for 5 min, and the cell button resuspended in 1 ml of Hanks' balanced salt solution (Microbiological Associates, Bethesda, Md.) containing human serum albumin (Buminate 25%, Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) at a concentration of 0.5 g/100 ml (HBSS/A).[1] After exposure to hypotonic lysis, by the addition of 12 ml distilled water for 25 s and restoration of isotonicity, the cells were centrifuged out at 400 *g* for 5 min, resuspended in 5 ml HBSS/A, and then applied to the upper surface of 5-ml Ficoll-Hypaque solution (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) (3). This gradient preparation was centrifuged at 400 *g* for 35 min at 4°C. The resulting cell button, which contained 95–99% pure granulocytes free of other blood cells, was washed once in 10 ml HBSS/A, counted, and finally resuspended in HBSS/A at a concentration of 10,000–15,000 granulocytes/mm³. The overall granulocyte yield was 40–60%, and the cells retained normal morphology and chemotactic function.

Preparation of plasmas. Heparinized (2 U/ml) plasma was prepared from normal human venous blood of group AB, and complement was activated with either zymosan or dialyzer cellophane as previously described (1). C5-deficient plasma was obtained from the proband of the family described by Rosenfeld et al. (4). All plasmas were rendered particle-free after complement activation by centrifugation at 20,000 *g* for 10 min. Plasma was decomplemented by heating at 56°C for 30 min (5). After complement activation, plasmas were fractionated by filtration through a Sephadex G-75 ascending column (Pharmacia Fine Chemicals Inc.) (5 × 90 cm, void volume 600 ml, bed volume 1,200 ml) at 4°C using isotonic phosphate-buffered saline, at pH 7.4, which contained 0.001% sodium azide (wt/vol) and 10 mM EDTA to prevent artefactual complement activation by the gel.

Granulocyte aggregometry. A standard platelet aggregometer/recorder system (models 300BD and Pf10HO-D, Payton Associates, Buffalo, N. Y.) was used. To a siliconized cuvette containing a siliconized stirring bar revolving at 900 rpm, was added 1.0 ml of granulocyte suspension. After a 2-min delay to allow warming of the cells to 37.4°C, 0.1 ml of plasma, column fraction, or trypsinized component was added, and the resulting changes in light transmission recorded. To provide the amplification necessary for recording of a well-defined aggregation wave, the aggregometer/recorder system was calibrated with fresh granulocyte suspension diluted 80% (vol/vol) in HBSS/A.

Electron microscopy. Granulocytes undergoing apparent aggregation were harvested from the cuvette after fixation, prepared, and then examined by thin-section and scanning electron microscopy as previously described (6, 7).

Granulocyte chemotaxis. Chemotaxis was evaluated in Boyden chambers by a modification of the ^{51}Cr radioassay described by Gallin et al. (8). Pure granulocytes, prepared by the method described above, were incubated with 20 μCi ^{51}Cr at 37°C for 60 min. After five washes in HBSS to remove unbound ^{51}Cr, 0.2-ml aliquots of labeled cells were added to the chambers, separated from the fraction under study by an upper Nuclepore membrane of mean pore size 3 μm (Nuclepore Corp., Pleasanton, Calif.) and a lower Millipore filter of mean pore size 3 μm (Millipore Corp., Bedford, Mass.). The chambers were incubated for 3 h at 37°C, at the end of which the chemotactic activity of the plasma fractions was estimated by determination of lower filter ^{51}Cr counts.

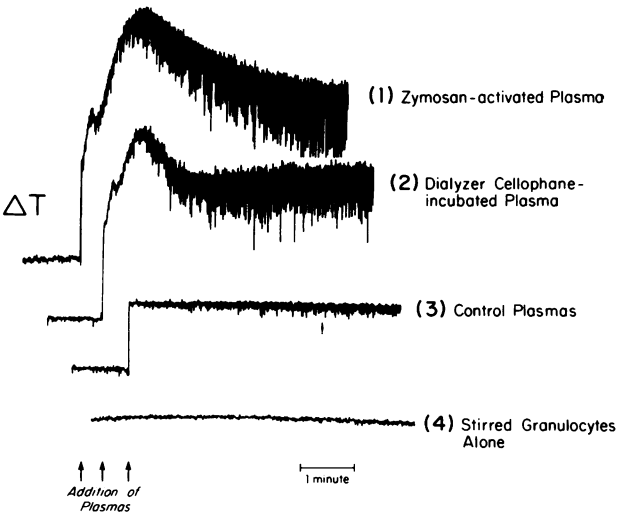

FIGURE 1 Granulocyte aggregation induced by zymosan- (1) and cellophane-activated (2) plasma complement. The addition of control plasmas (3), which included fresh plasma, or plasmas decomplemented by heat at 56°C for 30 min before incubation with zymosan or cellophane, produced dilution artefacts only. Cells stirred alone (4) remained stable.

Characterization of aggregating activity. The aggregating activity in the Sephadex fractions of zymosan-activated plasma was shown to be C5a-related by heating (56°C/30 min) and antibody incubation studies (9, 10). Sera containing anti-human C5 (lot 3980E, Behring Diagnostics, American Hoechst Corp., Somerville, N. J.) and anti-human C3 (lot 2852B, Behring Diagnostics) were decomplemented by heating at 56°C for 30 min, and 50 μl of each was incubated with 0.5 ml of each fraction for 30 min at 37°C. The aggregating activity of the fractions so treated was compared to that of incubated controls. Specific involvement of C5a was further defined by the addition of tryptic digests of purified C5 (11) and purified C3 (12) to granulocytes (13). To 25 μg of each component in 0.5 ml 0.04 M phosphate-buffered saline at pH 7.8 was added 1 μg trypsin (TRL100S/FA, Worthington Biochemical Corp., Freehold, N. J.) in 0.1 ml phosphate-buffered saline. Digestion proceeded for either 10 or 30 min at 31°C, and was stopped by the addition of 2 ug soybean trypsin inhibitor (T9003, Sigma Chemical Co., St. Louis, Mo.) in 0.1 ml phosphate-buffered saline. Negative control for tryptic activity was achieved by treating C5 and C3 with similar amounts of trypsin which had been inactivated by incubation with twice its weight of soybean inhibitor for 30 min at 37°C.

RESULTS

The addition of 0.1 ml zymosan-activated plasma to normal granulocytes stirring in an aggregometer produced an initial, rapid increase in light transmission due to dilution, followed by biphasic increments in light transmission and oscillation amplitude (Fig. 1). Cellophane-incubated plasma induced a qualitatively identical response (Fig. 1). The addition of fresh plasma or plasmas which had been heat decomple-

[1] *Abbreviations used in this paper*: HBSS/A, Hanks' balanced salt solution containing human serum albumin at a concentration of 0.5 g/100 ml.

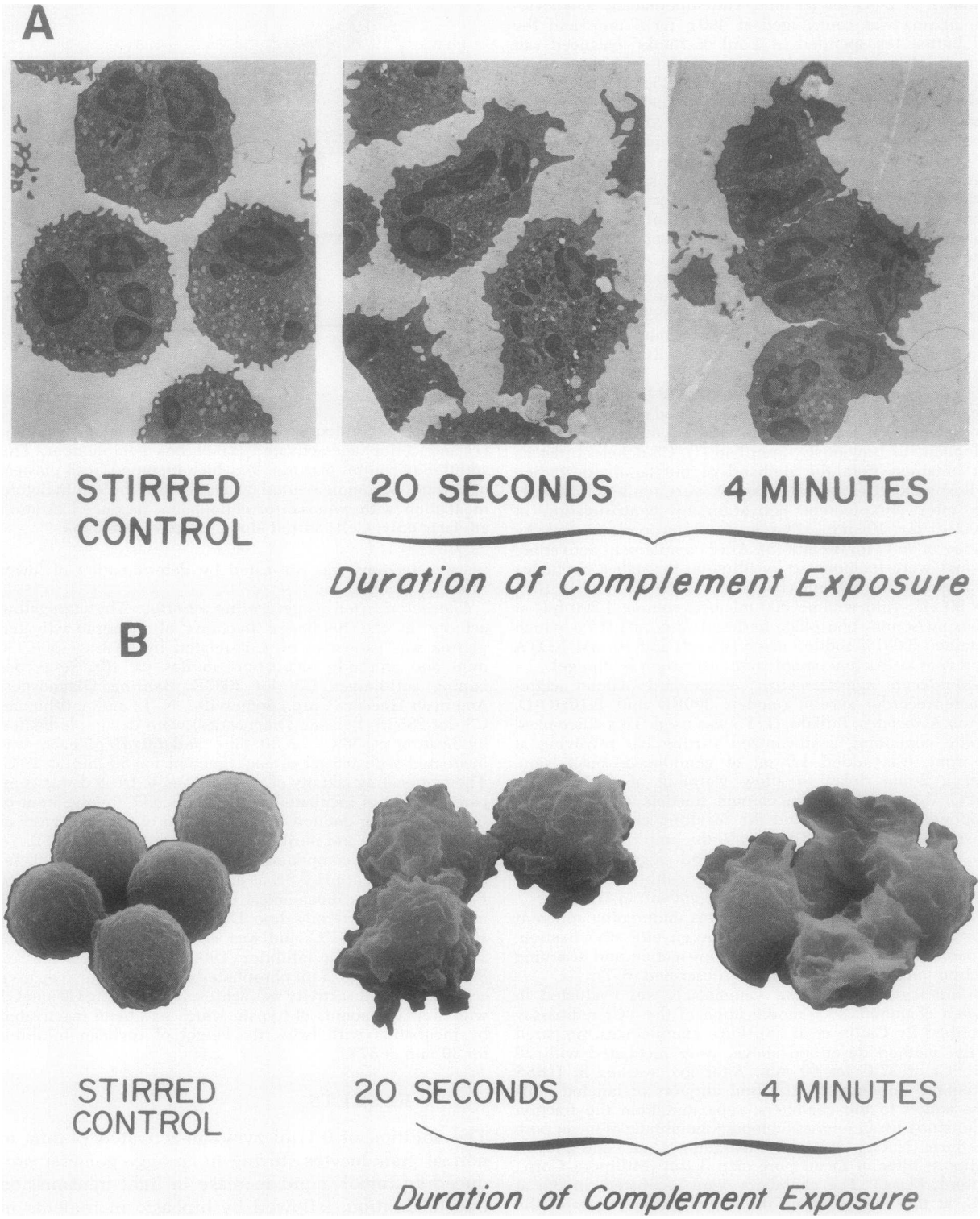

FIGURE 2 (A) Thin-section electron photomicrographs revealed granulocyte plasma membrane ruffling and pseudopod formation within 20 s of addition of activated-plasma complement (center), and cell-cell association within 4 min (right). Cells stirred alone retained normal morphology (left). (Magnification ×2,600.) (B) Identical changes in cell shape and aggregation were apparent with scanning electron microscopy. (Magnification ×1,800.)

mented before zymosan or cellophane incubation produced dilution artefact only, and granulocytes stirred alone remained stable (Fig. 1). Lymphocytes and myeloblasts harvested from the upper surface of the Ficoll-Hypaque layer were unreactive, but identical tracings were produced by granulocytes from all of nine normal adults and zymosan-activated plasma from all of five normal adults.

Examination of complement-treated cells by thin-section and scanning electron microscopy confirmed the occurrence of granulocyte aggregation (Fig. 2). Within 20 s of activated complement addition, granulocytes developed plasma membrane ruffling followed by frank cell-cell association in 4 min. Control cells stirred for similar periods manifest no such ultrastructural changes. The amplitude of the aggregation wave induced by activated complement was dose-related. Addition of serial dilutions of zymosan-activated plasma in HBSS/A produced diminishing

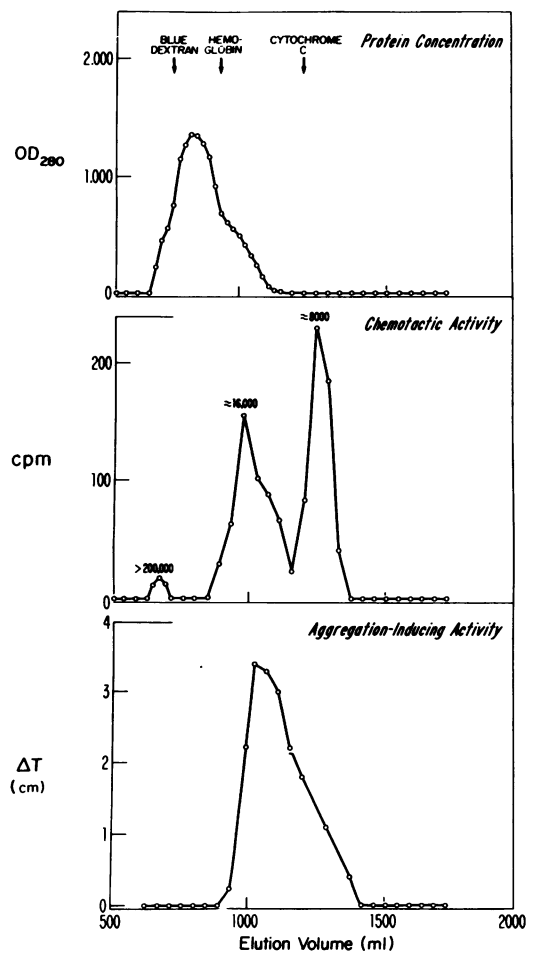

FIGURE 3 The chemotactic (center panel) and nephelometric (lower panel) activities of zymosan-activated plasma fractionated by filtration with Sephadex G-75.

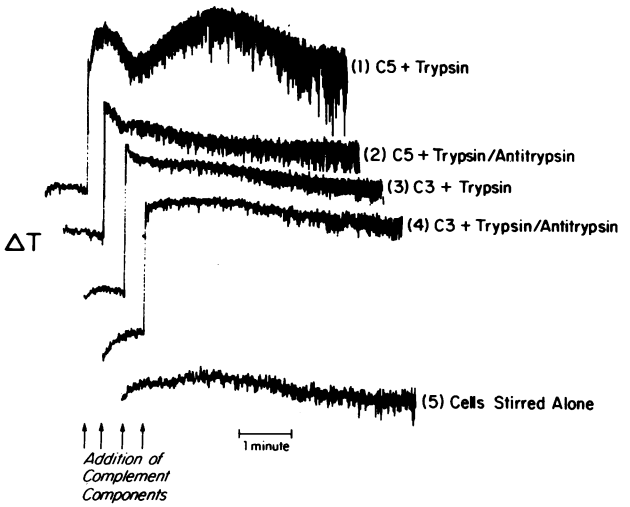

FIGURE 4 Aggregating activity induced in purified C5 by tryptic digestion for 10 min at 31°C (1). C5 incubated with inactive trypsin (2), and C3 incubated with intact (3) or inactive trypsin (4) produced dilution artefacts alone.

increments in both light transmission and oscillation amplitude (not shown).

When zymosan-activated plasma was fractionated with a calibrated Sephadex G-75 column, three peaks of chemotactic activity were defined (Fig. 3). In contrast, only one peak of aggregating activity, coinciding closely with the chemotactic peak of molecular weight approximately 16,000 daltons, was apparent (Fig. 3). All fractions from this peak of aggregating activity retained their potency despite heating at 56°C for 30 min. These molecular weight and heat stability characteristics suggested that C5a is the aggregating component. Indeed, the aggregating activity of these fractions was completely inhibited by preincubation with anti-C5 antibody; incubation with anti-C3 antibody was noninhibitory (not shown).

Furthermore, C5a generated by tryptic digestion of purified C5 for either 10 or 30 min produced granulocyte aggregation which was blocked by the simultaneous presence of antitrypsin during the trypsin incubation (Fig. 4, upper two curves). Conversely, trypsinization of purified C3 produced no aggregating activity (Fig. 4).

Finally, plasma from a genetically C5-deficient donor was incapable of producing granulocyte aggregation after incubation with zymosan (Fig. 5).

DISCUSSION

Light transmission measurements have been widely used to quantitate platelet aggregation (14). The addition of agents such as ADP to platelets stirring in an aggregometer induces biphasic aggregation manifested by increments in light transmission and oscillation amplitude. Activated complement induced similar

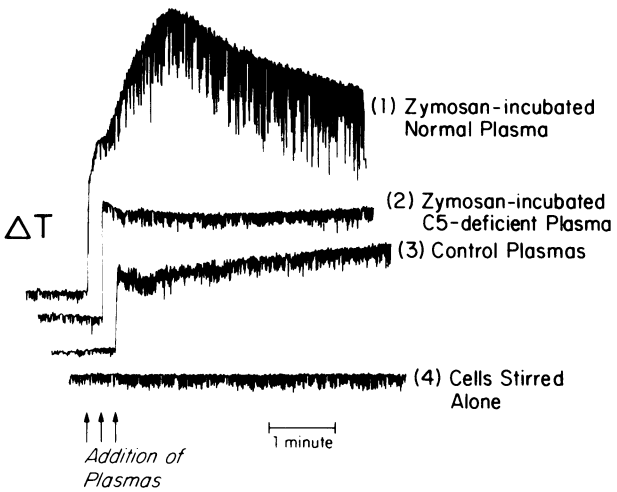

FIGURE 5 The addition of zymosan-incubated C5-deficient plasma to stirring granulocytes (2) failed to produce the increments in light transmission and oscillation amplitude induced by zymosan-incubated normal plasma (1). Fresh C5-deficient plasma and normal plasma were equally inactive (3).

shape change and biphasic aggregation of granulocytes in the same system. The single peak of complement aggregating activity defined by Sephadex filtration of zymosan-activated plasma was of molecular weight approximately 16,000 daltons, chemotactic for granulocytes, stable to heating at 56°C for 30 min, and was inactivated by anti-C5 but not anti-C3 antibodies. Aggregating activity was generated by tryptic digestion of purified C5 but could not be generated in C5-deficient plasma by incubation with zymosan. It is likely therefore that C5a is the complement component predominantly responsible for granulocyte shape change and aggregation in this system (9, 10, 13).

C5a reduces the surface charge on granulocytes (10), and induces the plasma membrane perturbations associated with granulocyte chemotaxis (13). C5a may induce granulocyte aggregation by simply coating and neutralizing negative charges on the cell surface, thereby facilitating cell-cell apposition. However, the gross ruffling and pseudopod formation which precede aggregation suggest an active process is involved. It is likely that this membrane ruffling is an important antecedent to aggregate formation. The inability of nonchemotactic cells to undergo C5a-induced shape change and aggregation suggest that granulocyte activation is specifically required for C5a-mediated aggregation.

These data strongly support the concept that C5a is the complement component responsible for the leukostasis and leukopenia associated with hemodialysis, and perhaps other clinical situations where leukopenia and pulmonary leukostasis are associated with intravascular complement activation.

ACKNOWLEDGMENTS

The authors wish to thank Doctors Stephen Rosenfeld and John Leddy for providing C5-deficient plasma from their patient, and Georgia Huard, Beatrice Heagan, and Marlys Krumweide for their excellent technical assistance.

This work was supported by U. S. Public Health Service grants CA15627, HL11880, AM15317, HL06314, CA12607, CA08832, HL16833, and AM15730, and a grant from the Veterans Administration.

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