# Granulocyte Aggregometry: A Sensitive Technique for the Detection of C5a and Complement Activation

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**We have previously shown that complement (C) activated plasma causes granulocyte (PMN) aggregation in vitro[1] and that C5a is responsible. The C-induced aggregation of PMNs treated with cytochalasin-B (CB) is markedly enhanced and irreversible, and the magnitude of the response is proportional to the log (concentration of activated plasma), allowing use of this technique to detect C5a and hence C activation. To compare the sensitivity of granulocyte aggregometry to that of more standard methods of detecting C-activation, we produced graded C-activation in vitro by treating fresh serum with varying amounts of zymosan. Aggregometry was the most sensitive index of C-activation, detecting C-activation produced by 0.02 mg zymosan/ml of serum—1/10 that required to produce C-activation detectable by C3 immunoelectrophoresis (the next most sensitive technique). Granulocyte aggregometry may also be used to detect in vivo C-activation. We have found aggregating activity in plasmas from patients with systemic lupus erythematosus, immune vasculitis, transfusion reactions, and other conditions associated with in vivo C-activation, but not in the plasmas of normal subjects.**

COMPLEMENT ACTIVATION, when appropriate, is critical to host defense mechanisms and hence beneficial. Inappropriate or excessive complement activation, however, is frequently a destructive process leading to unwanted tissue damage. Studies of complement activation have long been used to monitor patients with immune diseases. However, the assays in clinical use measure the levels of plasma complement components; since such levels represent a dynamic balance between synthesis and degradation of components, their assay will fail to detect a state of increased complement consumption, if such consumption is balanced by an increased synthesis (as is often the case in inflammatory states). We postulated that a more sensitive approach might be the detection of circulating by-products of complement activation, much as fibrin split products may be detected in abnormal coagulation states before depletion of clotting factors is perceptible. We have previously shown that one of these by-products of complement activation, C5a, causes granulocyte aggregation in vitro[1] and that the aggregation response of cytochalasin-B-treated polymorphonuclear cells (PMNs) is markedly enhanced and irreversible.[2] From studies of serial dilutions of zymosan-activated plasma (ZAP), it was apparent that the amplitude of the aggregation response is a predictable function of the concentration of ZAP (and thus C5a) achieved in the cuvette[1,2] and that the technique may therefore be used to estimate C5a in a test sample. We now report that nephelometric quantification of the aggregation of cytochalasin-B-treated granulocytes is a sensitive technique for the detection of C5a, and hence complement activation, either in vitro or in vivo.

## MATERIALS AND METHODS

### *Zymosan*

Zymosan (ICN Pharmaceuticals, Inc., Cleveland, Ohio) was suspended at 20 mg/ml in 0.15 *M* NaCl, boiled for 30 min, washed twice, and resuspended in 0.15 *M* NaCl at a final concentration of 20 mg/ml.

### *Zymosan-Treated Serum*

Graded alternative-pathway complement activation was produced in fresh normal human serum by incubating 1 ml aliquots of this serum with 0.02, 0.2, 2, 4, 8, 16, 32 mg of zymosan, with tumbling, at 37°C for 30 min. The serum was then cooled rapidly to 4°C, centrifuged for 10 min at 2000 *g*, and decanted from the zymosan button.

### *Graded Classical Pathway Complement Activation*

Antibody-coated erythrocytes (EAs) were prepared by cooling ABO-matched normal erythrocytes in heparinized heat-decomplemented plasma from a patient with a high titer of anti-I cold agglutinin. The suspension was then centrifuged at 500 *g* for 15 min at 4°C, the plasma was decanted, and the red cells were resuspended in an equal volume of Hanks' balanced salt solution (HBSS) at 37°C. This cycle of cooling and washing was repeated three times, yielding a suspension of EAs in HBSS relatively free of patient plasma components other than the cold agglutinin: such cells were agglutinated by anti-IgM Coombs' reagent at a dilution of 1:100, but were not agglutinable by anticomplement Coombs' sera or by commercial anti-C3 antiserum (Behring Diagnostics, Somerville, N.J.). The red blood cell count was adjusted to $10^6/\mu l$, and serial twofold dilutions of this suspension were prepared in HBSS. Control erythrocytes were prepared in like manner, but were cooled initially in autologous plasma, which was free of cold agglutinin.

Incremental activation of serum complement via the classical pathway was accomplished by adding 1 ml of EA suspension (or dilution thereof) to each 1-ml aliquot of normal serum, and incubating with mild agitation for 45 min at 30°C; EAs were then removed from the serum by centrifugation at 1000 *g* for 10 min at 4°C. Serum samples were stored at −70°C, and all complement determinations were done within 2 wk of preparation.

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### *Total Hemolytic Complement ($CH_{50}$)*

This activity was measured on serum stored at −70°C by a slight modification of the tube assay method of Nelson et al.,[3] using reagents obtained from Cordis Laboratories (Miami, Fla.). All test dilutions were made in a buffer that consisted of equal volumes of 5% glucose in water and veronal buffer, with 0.003 *M* calcium, 0.001 *M* magnesium, and 0.1% gelatin (G1-GVB$^{2+}$). One volume was equal to 0.2 ml. Six dilutions were made for each serum: 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160. All dilutions and subsequent steps were performed in an ice bath. The percent hemolysis in each reaction mixture was determined according to Mayer[4] and the hemolytic titer in the undiluted serum expressed in $CH_{50}$ units.

### *Hemolytic Assay for C3*

This assay was performed by a modification of the test tube assay method[3] using the cellular intermediates EAC1 (guinea pig), 4 (human) ($10^9$ cells/ml), lyophilized human complement components C2, 5, 6, and 7, and lyophilized guinea pig C8 and C9 (all reagents supplied by Cordis Laboratories). For the assay the EAC1, 4 cells were diluted to $10^8$ cells/ml in G1-GVB$^{2+}$, and 1 vol was equal to 0.2 ml. The following test dilutions of sera were made: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200.

### *Third Component of Complement*

The third component of complement was also measured by a modification of the radial immunodiffusion method of Mancini et al.,[5] using reagents supplied by Hyland (Hyland Division Travenol Laboratories, Inc., Costa Mesa, Calif.) as described by the "precision" method of Hyland.

### *Immunoelectrophoresis*

Immunoelectrophoresis (IEP) of serum was performed using a Corning ACI (Corning ACI, Palo Alto, Calif.) cassette electrophoresis cell and power supply (Cat. No. 470136). Corning agarose special purpose electrophoresis films and 0.05 *M* barbital buffer, pH 8.6, were used. One-microliter samples were applied to the wells and the films electrophoresed as directed for 60 min. After electrophoresis, 10 μl antiserum to human C3/C3c (lot number 51046 Behring Diagnostics) was added to the troughs, and the films were placed in a humidified chamber at room temperature for 16 hr. Nonprecipitated protein was eluted with several changes of 0.9% NaCl over 12–24 hr. The films were then put through 2 15-min water changes, dried, and stained with amido black.

### *Cytochalasin-B (CB)*

One milligram of CB (ICI Research Laboratories, Alderly Park, Cheshire, England) was added to 20 μl of dimethylsulfoxide (DMSO) (Sigma Chemical Company, St. Louis, Mo.), into which 10 ml of phosphate-buffered saline (PBS) (isotonic, pH 7.4) was immediately mixed. The mixture was allowed to stand for 10 min, an additional 10 ml of PBS added with vortexing, and after standing for an additional 10 min, centrifuged at 12,000 *g* for 10 min. This final solution contained CB 50 μg/ml. A DMSO control solution was identically prepared with the omission of CB. In all studies involving CB, the PMNs were preincubated (15 min, 37°C) with the specified concentration of CB and the aggregation experiment performed with the same concentrations of CB.

### *Cell Preparation*

Leukocyte suspensions were prepared from the heparinized (5 U/ml) venous blood of normal volunteers by dextran sedimentation, osmotic lysis of erythrocytes, and density-gradient centrifugation as previously described.[1,6] The cells were suspended in HBSS containing 1% salt-poor human serum albumin (HBSS-A) at a concentration of 2 × $10^7$ cells/ml. These preparations typically contained 95%–99% viable granulocytes and were free of red cells and red cell ghosts.

### *PMN Chemotaxis*

PMN chemotaxis was studied by a modification of the method of Gallin et al.[8] $^{51}$Cr-labeled PMNs (2 × $10^6$) were placed in the top compartment of a plastic Boyden chamber and the substance to be tested for chemotactic activity in the bottom compartment. Two filters separated the two compartments, a Nucleopore filter of 3 μm mean pore size (Nucleopore Corp., Pleasanton, Calif.) on top of a Millipore filter, also of 3 μm mean pore size (Millipore Corp., Bedford, Mass.). After incubation of the chambers for 2 hr at 37°C, chemotaxis was estimated by determination of $^{51}$Cr counts in the lower filter.

### *PMN Aggregometry*

PMN aggregometry was performed according to our previously published method.[1] One-half milliliter of cell suspension ($10^7$ PMNs) was stirred in a siliconized cuvette in a standard platelet aggregometer (Model 300 BD, Payton Associates, Buffalo, N.Y.) and light transmission recorded on a strip chart recorder. We added 50 μl of the serum or plasma to be tested for aggregating activity to the stirred cell suspension and recorded aggregation as increment in light transmission (ΔT) on an arbitrary scale. The ΔT between the initial cell preparation and a 1:1 dilution thereof with HBSS-A was set at 20 cm (8 mV).

## RESULTS

### *Detection of Graded Complement Activation*

Assays of complement activity and components proved a relatively insensitive index of C-activation, as depicted in Fig. 1. Total hemolytic complement activity ($CH_{50}$) and hemolytic C3 activity (which actually

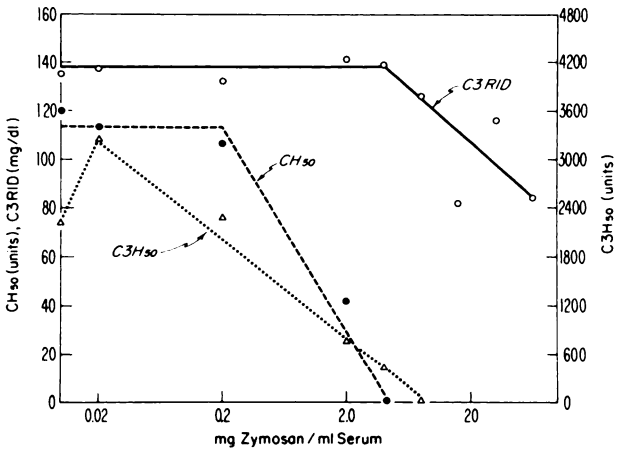


**Fig. 1. Depletion of complement in zymosan-treated serum. Serum incubated with indicated amount of zymosan for 30 min at 37°C. Total hemolytic complement ($CH_{50}$) (closed circles) and hemolytic C3 ($C3H_{50}$) (triangles) activity (which actually increased in serum treated with minimal amounts of zymosan) were decreased in sera treated with 2.0 mg zymosan/ml or more. C3 measured by radial immunodiffusion (C3 RID) was decreased in serum treated with 8.0 mg zymosan/ml or more.**

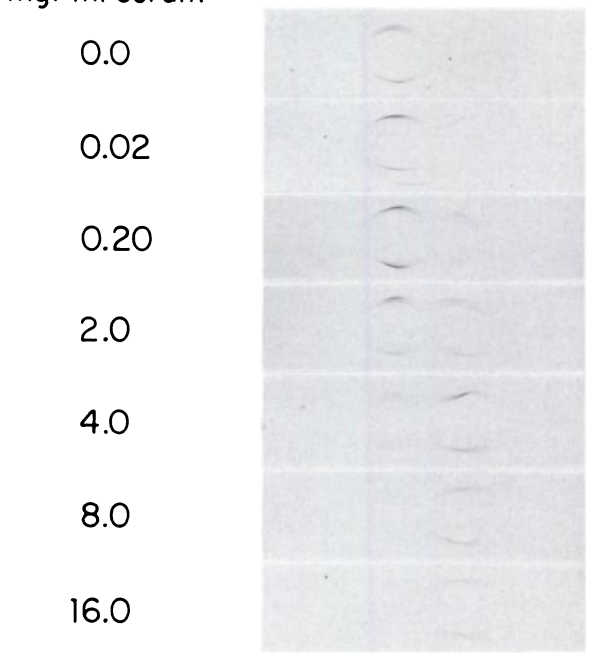


**Fig. 2. C3/anti-C3 immunoelectrophoresis of sera treated with indicated amounts of zymosan for 30 min at 37°C. Anode to the right, serum applied at left, anti-human C3 in troughs. Increased C3 conversion product is demonstrated in sera treated with 0.2 mg zymosan/ml or more. Very small apparent conversion arcs in some untreated serum samples are probably due to C3 cleavage by plasmin generated during clotting.**

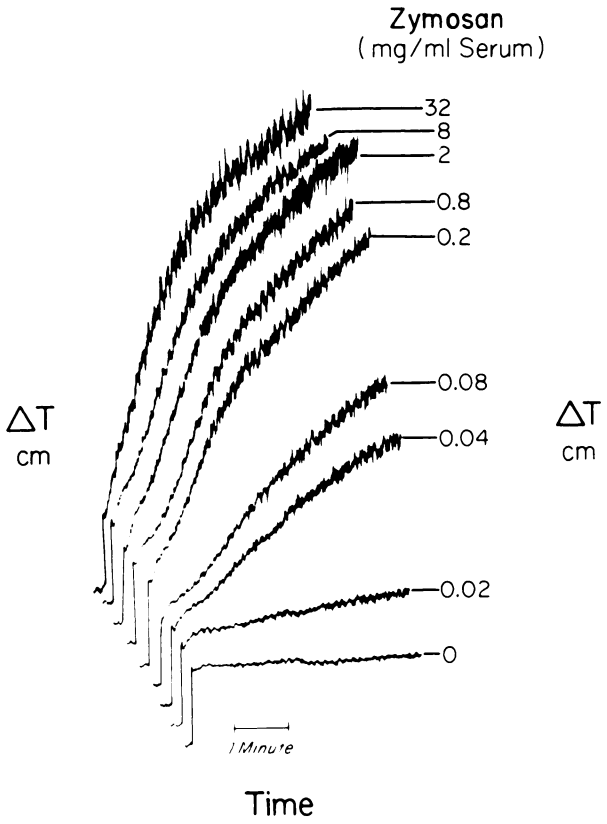


**Fig. 3. Aggregation of cytochalasin-B-treated granulocytes (recorded as increment in light transmission or ΔT) induced by sera treated with indicated amount of zymosan. Fresh normal serum (0 mg zymosan) induces dilution artifact only. Serum treated with 0.02 mg zymosan/ml induces definite aggregation.**

increased at the lowest levels of zymosan treatment) were both appreciably decreased only in sera treated with 2.0 mg/ml or more of zymosan. Radial immunodiffusion assay of C3 remained normal even in serum treated with 4.0 mg of zymosan.

C3 conversion by immunoelectrophoresis was detectable in serum treated with 0.2 mg/ml of zymosan (Fig. 2); that treated with 0.02 mg/ml could not reliably be distinguished from untreated serum (which may show a small arc of conversion products, presumably due to C3 cleavage by plasmin generated during clotting.[9]). This technique, however, was the most sensitive of the standard assays employed.

We were unable to detect complement activation or depletion by any of these techniques in serum treated with erythrocyte–antibody complexes in the range of cell counts tested.

PMN chemotaxis was not a sensitive index of complement activation. Two milligrams per milliliter of zymosan was required to generate detectable chemotactic activity in fresh serum. Subsequent to the performance of the studies reported here, more sensitive modifications of the Boyden technique have been employed in our laboratory and have allowed the detection of chemotactic activity in plasma or serum incubated with as little as 0.25 mg zymosan/ml. While now approaching the sensitivity of C3 IELP, this method is still far less sensitive than PMN aggregometry.

The aggregation response of CB-treated granulocytes on exposure to sera with graded alternative pathway complement activation is depicted in Fig. 3. Serum treated with 0.02 mg/ml zymosan (the smallest amount tested) produced unequivocal granulocyte aggregation. The magnitude of the aggregation response increased with the amount of zymosan used to produce C-activation. As shown in Fig. 4, the aggregation response, plotted as increment in light transmission or ΔT, was proportional to the $\log_2$ of the amount of zymosan used to induce C-activation, without apparent threshold within the range of amount of zymosan used. Not depicted are the results of aggregation studies using EA-treated serum. Serum treated with $0.6 \times 10^8$ EAs/ml produced equivocal aggregation, and that treated with $1.2 \times 10^8$ EAs/ml or more produced definite aggregation. Thus, PMN aggre-

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