Activation of Human Complement by Immunoglobulin G Antigranulocyte Antibody

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ABSTRACT The ability of antigranulocyte antibody to fix the third component of complement (C3) to the granulocyte surface was investigated by an assay that quantitates the binding of monoclonal anti-C3 antibody to paraformaldehyde-fixed cells preincubated with Felty's syndrome serum in the presence of human complement. The sera from 7 of 13 patients with Felty's syndrome bound two to three times as much C3 to granulocytes as sera from patients with uncomplicated rheumatoid arthritis. The complement-activating ability of Felty's syndrome serum seemed to reside in the monomeric IgG-containing serum fraction. For those sera capable of activating complement, the amount of C3 fixed to granulocytes was proportional to the amount of granulocyte-binding IgG present in the serum. Thus, complement fixation appeared to be a consequence of the binding of antigranulocyte antibody to the cell surface. These studies suggest a role for complement-mediated injury in the pathophysiology of immune granulocytopenia, as has been demonstrated for immune hemolytic anemia and immune thrombocytopenia.

INTRODUCTION

Binding of antibody to surface antigens and fixation of complement to the surfaces of cells are two of a variety of mechanisms that mediate immunologic injury to cells of patients with immune hemolytic anemia and immune thrombocytopenia (1). The role of immunoglobulin-mediated complement activation in cell destruction is well understood in warm antibody autoimmune hemolytic anemia (2), cold agglutinin hemolysis (2, 3), and quinidine purpura (4). Despite the apparent clinical analogy of immune granulocytopenia to immune hemolytic anemia and immune thrombocytopenia, immunologic mechanisms directed against granulocytes are not well defined in immune granulocytopenia. Specifically, the ability of antigranulocyte antibody to activate and fix human complement to granulocytes has not been explored.

Although cell-mediated immunity appears to be involved in some patients with autoimmune granulocytopenia (5, 6), we and others have demonstrated that patients with Felty's syndrome (FS)[1] often have increased serum granulocyte-binding IgG (7–11). By analogy with immune hemolytic anemia and immune thrombocytopenia, one might expect some FS sera to bind complement to granulocytes. Membrane-bound components of complement may modulate the host's interaction with its own granulocytes by involving cells with receptors for those components (12). The ability of antigranulocyte antibodies to fix complement to granulocytes may have implications for the type and subclass of immunoglobulin involved in granulocyte destruction, in addition to providing information about the arrangement of granulocyte surface antigens. Consequently, we sought to determine whether antibodies in the sera of patients with FS fixed complement to granulocytes.

In this study, we describe an assay system to detect and quantitate the third component of human com-

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[1] *Abbreviations used in this paper*: anti-C3, ^{125}I-labeled anti-C3 antibody; C3, third component of human complement; FS, Felty's syndrome; PFG, paraformaldehyde-fixed granulocytes; RA, rheumatoid arthritis; SPA, ^{125}I-labeled staphylococcal protein A; VBS, veronal-buffered saline.

plement (C3) on the granulocyte surface and provide evidence for the ability of serum from patients with FS to activate complement and fix C3 to allogeneic granulocytes. Most FS sera were able to fix greater amounts of C3 to granulocytes than sera from patients with uncomplicated rheumatoid arthritis (RA), and this ability appeared to reside in the monomeric IgG-containing fraction of FS serum. For the majority of patients, the amount of C3 fixed was directly related to the amount of granulocyte-binding IgG present.

METHODS

Patients. Sera from 13 patients with classic FS were examined. The group consisted of nine males and four females. There was no history of blood transfusion in 9 of the 13 patients. Two patients, both males, had undergone splenectomy, and one of them had responded with normalization of his granulocyte count. Both of these patients had been transfused preoperatively and serum for testing was obtained 5 yr after transfusion. Two other male patients had been transfused 4 and 2 yr before testing. All four females were over 55 yr of age and one was multiparous. All patients except one were granulocytopenic (granulocyte count $< 1000/cm^3$) at the time of study.

Sera and complement sources. Control sera from normal volunteers and patients without RA or immune granulocytopenia, and test sera from patients with uncomplicated RA and patients with FS were obtained from peripheral venous blood that had been allowed to clot at room temperature. Following centrifugation at 800 *g*, the sera were stored at −50°C for up to 12 mo. Sera were obtained from healthy volunteers for use in the fresh state as a source of complement. Immediately before use, thawed test sera and fresh sera were centrifuged for 1.5 min in a Beckman Microfuge B miniature centrifuge (Beckman Instruments, Inc., Palo Alto, CA) and then diluted with veronal-buffered isotonic saline pH 7.4 with 0.15 mM calcium chloride and 0.50 mM magnesium chloride (VBS) (13).

Granulocytes. Peripheral venous blood from O blood type normal male volunteers and from one splenectomized nongranulocytopenic patient with FS was drawn into heparinized syringes. Granulocytes were harvested by sedimentation of blood at 1 *g* in 20% (vol/vol) Plasmagel (H.T.I. Corporation, Buffalo, NY) for 45 min at room temperature followed by 400,000-mol wt Ficoll-sodium diatrizoate (Sigma Chemical Co., St. Louis, MO) discontinuous density gradient (specific gravities 1.119 and 1.077) centrifugation of the leukocyte-rich plasma for 20 min at 800 *g* (10, 14). The granulocyte layer was removed, washed once in Alsever's solution (13) to minimize autoagglutination, resuspended, and treated with 1 vol 2% (wt/vol) paraformaldehyde (Fisher Scientific Co., Pittsburgh, PA) in phosphate-buffered saline pH 7.2 for 10 min at room temperature (8, 15). These paraformaldehyde-fixed granulocytes (PFG) were then washed twice and resuspended in Alsever's solution before storage at −50°C for up to 3 mo. Immediately before use, PFG were thawed, washed in VBS, and adjusted to a concentration of 20 million cells/ml. All cell washing procedures were done by centrifugation at 800 *g* for 10 min at room temperature. Thawed PFG preparations were >95% pure and had <1% contaminating erythrocytes by microscopic examination.

^{125}I-Labeled anti-C3 antibody. Monoclonal mouse IgG antibody against purified human C3 was prepared from mouse ascites fluid (Bethesda Research Laboratories, Inc., Gaithersburg, MD) by ammonium sulfate precipitation and DE52 (Whatman, Inc., Clifton, NJ) ion exchange column chromatography according to the method of Parker et al. (16). This antibody reacts with C3, C3b, C3bi, and C3c, but not C3d. Purity of the antibody was established by the presence of only two distinct bands on sodium dodecyl sulfate (SDS) reduced polyacrylamide gel electrophoresis. A single precipitin line was noted upon agarose gel immunodiffusion against goat anti-mouse whole serum and anti-mouse IgG_{2b} (Meloy Laboratories, Inc., Springfield, VA) and against purified human C3 (gift of C. J. Parker) in a 5% (wt/vol) polyethylene glycol and 1% (wt/vol) agarose gel.

The antibody was radioiodinated using the chloramine-T method (17). 1.0 mCi ^{125}I in sodium hydroxide (Amersham Corp., Arlington Heights, IL) was added to 100 μg antibody at 4°C. 10 μl chloramine-T (1 mg/ml in 0.2 M borate buffered isotonic saline pH 8.0 with 10 mg% (wt/vol) sodium azide) was added to initiate the reaction, which was terminated after 30 min by the addition of 10 μl potassium iodide (1 M). ^{125}I-Labeled anti-C3 antibody (anti-C3) was separated from unbound ^{125}I by filtration through a Sephadex G-25M PD-10 column (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) in VBS at room temperature. Greater than 98% of the radioactivity in the initial peak was trichloroacetic acid-precipitable, indicating efficient separation. Specific activities of anti-C3 preparations were 2.4 and 9.5 million cpm/μg. Protein concentration was 20 μg/ml by protein assay (Bio-Rad Laboratories, Richmond, CA). 0.1% (wt/vol) bovine serum albumin (Sigma Chemical Co.) was added to the final solution to minimize autoirradiation damage.

Measurement of surface-bound C3. In the standard assay, 200 μl PFG were added to a dilution of test serum or VBS followed by the addition of a dilution of type-compatible fresh normal serum as a source of complement. The entire 600 μl reaction mixture was incubated at 37°C (incubation 1) and the reaction terminated by ice bath temperatures. Reacted cells were washed twice at room temperature in VBS and resuspended in McCoy's Medium 5A, modified with L-glutamine and 15% fetal calf serum (B & B Research Laboratories, Inc., Durham, NC). An aliquot was removed for the determination of cell number by automatic blood cell counter (Coulter Electronics, Inc., Hialeah, FL) and the remainder was assayed in triplicate for the presence of surface-bound C3. Light microscopic examination of Wright-stained preparations of these reacted cells showed only rare microaggregated PFG and no increase in the number of microaggregates when FS sera were compared with control sera. 50 μl reacted cells were added to 50 μl anti-C3 that had been layered on 100 μl of phthalate ester oil mixture [n-butyl phthalate and *bis*(2-ethylhexyl)phthalate (Fisher Scientific Co.) in a ratio of 6:4 (vol/vol)] in 400-μl size microfuge tubes (Analytic Lab Accessories, Rockville Centre, NY) and the entire system was incubated at 37°C (incubation 2). Efficient separation of cell bound from unbound anti-C3, which remained in the aqueous phase, was accomplished by centrifugation of the cells through the oil mixture in a Beckman Microfuge B for 2.5 min. The cell pellet was removed by excision of the tube tip with a razor blade and its radioactivity was quantitated by a gamma scintillation counter (Beckman Instruments, Inc.). The femtograms of anti-C3 bound per cell were calculated. The level of anti-C3 binding resulting from the incubation of granulocytes with a complement source at 37°C in the absence of FS serum was 12 fg/cell (Fig. 1). This serum-independent binding appeared to be due to a small amount of nonspecific complement activation. Inactivation of both the test serum and complement

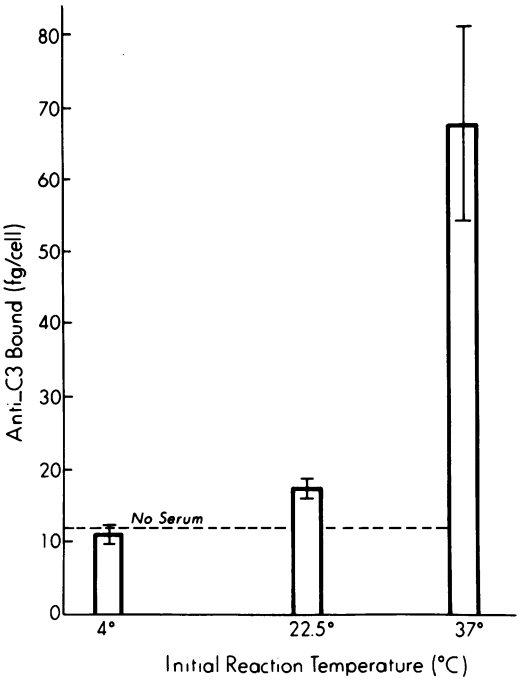

FIGURE 1 Temperature dependence of C3 fixation: anti-C3 binding to PFG incubated at different temperatures with FS sera and complement. The bars represent the mean±SE of three determinations on sera from one patient. Mean anti-C3 binding at 37°C in the absence of serum but in the presence of complement is indicated by the dotted line.

source by heating at 56°C for 20 min before incubation with PFG in the standard assay reduced this level of anti-C3 binding by 80%. In the absence of both test serum and fresh normal serum as a source of complement, 0.2 fg anti-C3 was bound per cell. There was therefore a minimal amount of anti-C3 binding to complement components present on the surface of granulocytes at the time of fixation with paraformaldehyde and of nonspecific anti-C3 binding to Fc receptors on PFG. Results in the standard assay were expressed as an increment in the amount of anti-C3 bound per cell by the complement source and test serum that was above the amount bound by the complement source in the absence of serum. The low levels of nonspecific test serum-independent complement activation by PFG were thereby excluded from our data.

Variation of conditions in incubation 1. The fixation of C3 to granulocytes as a function of the dilution of serum from a patient with FS is depicted in Fig. 2. The amount of anti-C3 bound per cell rose with increasing strength of sera from both a patient with FS and a normal control subject, but was severalfold greater in the case of FS sera, in which it reached a relative plateau at 1:10 dilution of serum. This dilution of test serum was used in all subsequent studies.

The dependence of C3 fixation by FS serum on the amount of complement available is illustrated in Fig. 3. Increasing concentrations of fresh sera in the initial reaction mixture resulted in increasing amounts of anti-C3 bound by cells exposed to both patient and normal control sera. A 1:10 dilution of fresh normal serum appeared to be an adequate source of complement in the standard assay.

To determine the time course of C3 fixation to the cell surface by FS serum, granulocytes were incubated with serum from a patient with FS in the presence of a source of complement for varying time intervals and then assayed for anti-C3 binding, using the standard assay. The results are illustrated in Fig. 4. Both FS and normal control sera led to increased binding of anti-C3 with longer periods of incubation 1. Anti-C3 bound by control sera increased linearly after 15 min, but the maximal difference between FS and contol sera occurred after 45 min. This incubation period was used in subsequent experiments.

Variation of conditions in incubation 2. A 20-min period was found to be the optimum duration of incubation of anti-C3 with reacted cells. When increasing amounts of anti-C3 were added to reacted cells in incubation 2, 100 ng appeared to be adequate to detect differences in C3 fixation to PFG by FS and control sera (Fig. 5). This amount was chosen to economize on monoclonal antibody stock. When the number of sensitized cells exposed to anti-C3 in incubation 2 was varied, optimum binding occurred with 0.5 to 1 million cells. Thus, a result of 50 fg anti-C3 bound/cell by 0.5 million cells represented binding of 25% of the total available anti-C3.

Measurement of granulocyte-binding IgG. In some experiments, granulocytes were incubated with test sera, washed, and the amount of granulocyte-binding IgG determined by a modification of previously described methods, using ^{125}I-labeled staphylococcal protein A (SPA) (Amersham Corp.) binding (9). Specific activity of the SPA was 55 mCi/mg and was diluted 1:100 in VBS. Unbound SPA was separated from cell-bound SPA by centrifugation through a phthalate oil mixture. Results were expressed as the increment of SPA bound by test sera above the amount bound by cells in the absence of serum. Sera from 10 normal, 10 RA, and 13 FS patients bound 0.04±0.02 (mean±SE), 0.05±0.01, and 0.14±0.03 fg SPA/cell, respectively. No difference in the amount of SPA bound was discerned when a fresh complement source was added to the initial reaction mixture of PFG and FS serum.

Statistical methods. Differences between groups were tested for significance by the Mann-Whitney U test. Linear regression was determined by least squares analysis.

RESULTS

Sera were obtained from controls, patients with RA, and patients with FS. Results of the standard assay performed on sera from these 33 individuals are depicted in Fig. 6. Sera from 10 controls bound 15.3±7.0 fg anti-C3/cell as compared with 17.0±3.7 by sera from 10 patients with RA ($P > 0.1$). The amounts of anti-C3 bound by 7 of the 13 FS sera were >2 SE above the means of RA and control sera.

6 of the 13 sera from patients with FS did not fix increased amounts of C3 to granulocytes despite the presence of elevated levels of granulocyte-binding IgG and suggested the existence of two subgroups of FS patients, one whose sera activated complement to a lesser extent than the other. The optimal incubation time for assessing C3 fixation in the first subgroup might have been longer than the 45 min established for the standard assay. This possibility was examined

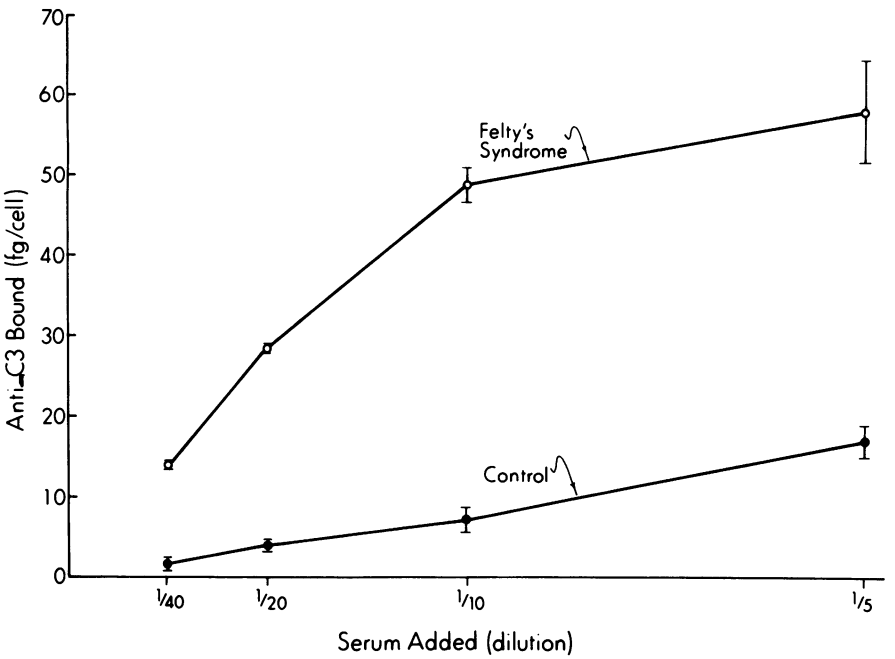

FIGURE 2 Dependence of C3 fixation on dilution of serum: anti-C3 binding to PFG incubated with different dilutions of test sera and complement. 4 million PFG, dilutions of test sera, and a 1:10 dilution of fresh normal serum as a source of complement were incubated together for 45 min in incubation 1 (Methods) and 0.5 million reacted cells were added to 100 ng anti-C3 in incubation 2. The results are expressed as the increment of anti-C3 bound over the amount bound in the absence of serum. The data represent the mean±SE of three determinations on sera from one patient with FS and one control subject.

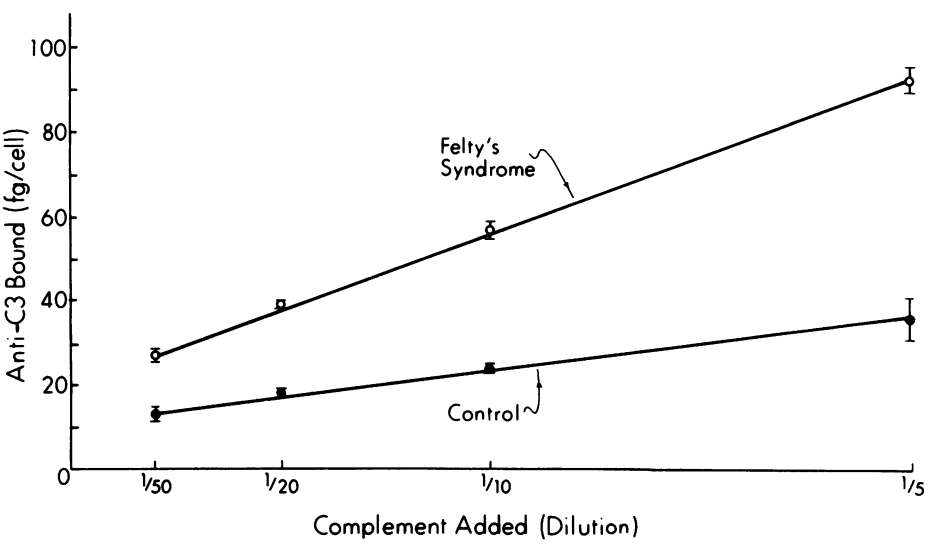

FIGURE 3 Dependence of C3 fixation on amount of complement added: anti-C3 binding to PFG incubated with different dilutions of a fresh complement source and serum. 4 million PFG, a 1:10 dilution of test serum, and a dilution of fresh normal serum as a source of complement were incubated together for 45 min in incubation 1 and 0.5 million reacted cells were added to 100 ng anti-C3 in incubation 2. The results are expressed as in Fig. 2. The data represent the mean±SE of three determinations on sera from one patient with FS and one control subject.

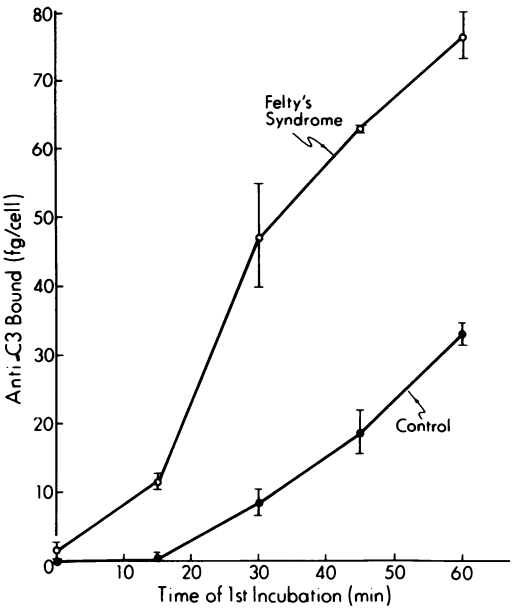

FIGURE 4 Kinetics of C3 fixation: anti-C3 binding to PFG incubated for varying time intervals with sera and complement. 4 million PFG, a 1:10 dilution of test serum, and a 1:10 dilution of fresh normal serum as a source of complement were incubated together for varying time intervals in incubation 1 and 0.5 million reacted cells were added to 100 ng anti-C3 in incubation 2. The results are expressed as in Fig. 2. The data are the mean±SE of three determinations on sera from one patient with FS and one control subject.

by incubating granulocytes with sera from one patient in each of the two subgroups and comparing the time course of C3 fixation. Despite an extension of the incubation period to 3 h, the differences between the amounts of anti-C3 bound by the two sera remained constant and the amount bound remained proportional to the amount bound at 45 min. The possibility that disparate clinical presentations of the patients from whom these sera were obtained might underlie the observed variability in complement-activating ability was also considered, but no notable differences were discovered. Specifically, there were no differences in severity of arthritis, in incidence of infection or of leg ulcers, in spleen size, in degree of granulocytopenia, anemia, or thrombocytopenia, in titer of rheumatoid factor or antinuclear antibody, or in therapy. Of the six patients whose sera activated complement to a lesser extent, all were males, two of whom had received prior blood transfusion. A sufficient number of patients had not undergone splenectomy to allow any conclusions regarding response to that procedure.

The cause of elevated anti-C3 binding in the seven patients who activated complement to a greater extent and the specificity of anti-C3 for fixed C3 molecules were determined by manipulating the activity of the complement source. The effect of temperature on C3 fixation was determined by carrying out incubation 1 (during which granulocytes, test serum, and a fresh complement source were present) at 4°C and at room temperature (22.5°C), in addition to physiologic temperature (37°C). The amount of subsequent anti-C3 binding is depicted in Fig. 1. A minimal amount of binding occurred at 4°C and slightly more at 22.5°C. 67.5 fg were bound by cells incubated with FS serum and complement at 37°C.

The effect of complement inactivation on the amount of anti-C3 binding was determined (Table I). Use of heated normal serum as a source of complement for both FS and control sera in incubation 1 abrogated binding of anti-C3 to sensitized cells in incubation 2. When incubation 1 was carried out in the presence of 50 mM EDTA, binding was reduced by 95 and 91%, respectively.

The temperature dependence of complement activation and the effect of complement inactivation during incubation 1 on subsequent anti-C3 binding during incubation 2 strongly support the contention the C3 is being fixed to the surface of PFG. Immune complexes, which did not bind to granulocytes but caused fluid-phase complement activation in the assay, could explain the observed ability of FS sera to activate complement and fix increased amounts of C3 However, when granulocytes were incubated with test serum and washed before the addition of fresh normal serum as the complement source and completion of the standard assay, FS serum bound 48.4 fg anti-C3 (with the extra wash step), as compared with 48.6 fg anti-C3 (using the standard assay).

IgG antibody or immune complexes bound to granulocyte Fc receptors might activate complement and fix C3 to the surface of granulocytes. We investigated whether complement activation was occurring by nonspecific IgG binding to Fc receptors by examining binding of anti-C3 after incubation of cells with a fresh complement source and the sera of two patients with multiple myeloma. Both these patients had a monoclonal gammopathy by serum electrophoresis with elevated amounts of "M" protein of the IgG_1 type as established by immunoelectrophoresis against specific antigamma immunoglobulins. Granulocytes incubated with these two sera in the presence of a complement source bound only 10.7 and 7.0 fg anti-C3 per cell, respectively. Furthermore, granulocytes incubated with these sera bound 0.01 and 0.07 fg SPA, respectively, indicating that the amount of IgG bound to granulocytes by FS sera was in excess of that which could be accounted for by Fc receptor binding of monomeric IgG. To determine whether granulocyte-bound immune complexes might also be contributing to the activation of complement in the assay, PFG were

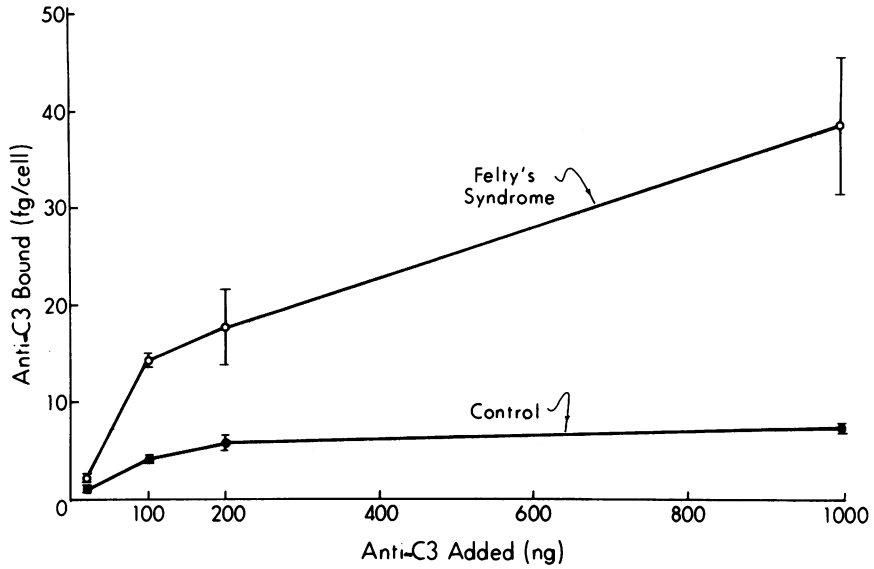

FIGURE 5 Dependence of C3 fixation detectable on amount of anti-C3 available: anti-C3 binding to PFG sensitized by sera when different amounts of anti-C3 were added. 4 million PFG, a 1:10 dilution of test serum, and a 1:10 dilution of fresh normal serum as a source of complement were incubated together for 45 min in incubation 1 and 0.5 million reacted cells were added to varying amounts of anti-C3 in incubation 2. The results are expressed as in Fig. 2. The data represent the mean±SE of three determinations on sera from one patient with FS and one control subject.

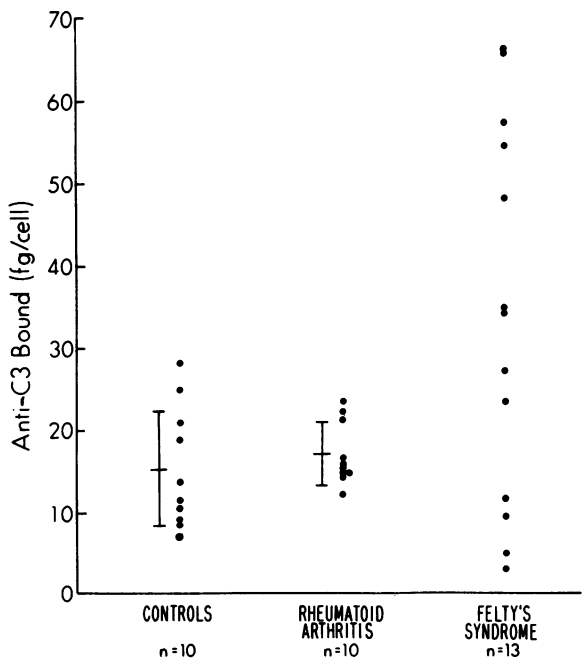

FIGURE 6 C3 fixation by sera from control subjects, patients with RA, and patients with FS. The results are expressed as in Fig. 2. Each data point represents the mean of three determinations on sera from an individual patient. The mean±SE values obtained for 10 control subjects and 10 patients with RA are indicated.

incubated with heat-aggregated IgG_1 and fresh normal serum before measurement of SPA and anti-C3 binding. Purified IgG was obtained by DEAE cellulose (Sigma Chemical Co.) anion exchange chromatography of serum from a patient with IgG_1 multiple myeloma and was heated at 63°C for 20 min. Although SPA binding was substantially increased, only background binding of anti-C3 was demonstrated. These results militate against immune complex-mediated complement activation in our assay system.

To further study the basis for the increased C3-fixing activity, serum protein from a patient with FS was fractionated according to molecular size. 5 ml of serum from one patient with FS was applied at 4°C to a 5 × 100-cm column packed with Sephadex G-200 (Pharmacia Fine Chemicals) in 0.15 M sodium chloride preserved with 10 mg % (wt/vol) sodium azide. An assessment of protein concentration of serial fractions was obtained by measuring absorbance at 280 nm on an ultraviolet spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Three standard protein peaks were obtained (Fig. 7). Aliquots of selected serum fractions were used without further adjustment of concentration in the standard anti-C3 binding assay. The peak of complement activating ability coincided with the monomeric IgG-containing fractions of FS serum, represented by the second protein peak.

TABLE I
Effect of Complement Inactivation on Subsequent Anti-C3 Binding to PFG

Complement source	Anti-C3 bound	
	FS	Control
	*fg/cell**	
Fresh	45.6±6.6	15.8±3.6
Heat-inactivated	0	0
Treated with 50 mM EDTA	2.0±0.4	1.4±1.4

Binding of anti-C3 to PFG that had been incubated with serum from one patient with FS and one control subject in the presence of untreated or treated fresh normal serum as the source of complement.

* Represent mean±SE of three determinations.

To further define the surface elements to which complement-activating IgG in FS might bind, we measured anti-C3 binding after incubation of PFG prepared from each of three normal individuals with complement and with sera from a patient with FS; virtually identical results were obtained. Furthermore, we tested the serum from one patient with FS against his own granulocytes obtained after remission with splenectomy therapy. In the standard assay, using a fresh complement source, his serum caused the binding of 55.5 fg anti-C3/cell with autologous PFG and 48.6 fg anti-C3/cell with allogeneic PFG. These results were consistent with the autoimmune nature of FS.

The question of whether binding of antigranulocyte IgG was related to the fixation of C3 was addressed by the concurrent measurement of SPA and anti-C3 binding following the incubation of granulocytes with test sera. The amount of anti-C3 binding per cell was plotted against the amount of SPA bound per cell for each patient (Fig. 8). For 10 patients with RA, the distribution of values fell within the range of controls. Results from patients with RA were clustered together in the region of low amounts of granulocyte-binding IgG and low amounts of C3 fixation. In contrast, both of these parameters were elevated in most FS patients. Although the values were spread over a large range, increased amounts of granulocyte-binding IgG were positively correlated with an increased ability of FS sera to fix C3 to granulocytes (r = 0.6).

DISCUSSION

The role of complement in modulating the immune destruction of erythrocytes and platelets and the similarity in clinical expression of immune granulocytopenia to immune hemolytic anemia and immune thrombocytopenia suggest a potential role for complement activation in immune granulocytopenia (1). Some antigranulocyte antibody assays detect complement-mediated granulocytotoxicity. Drew and coworkers (18) have developed an antibody-dependent, complement-mediated microgranulocytotoxicity assay, which has found wide application in the screening

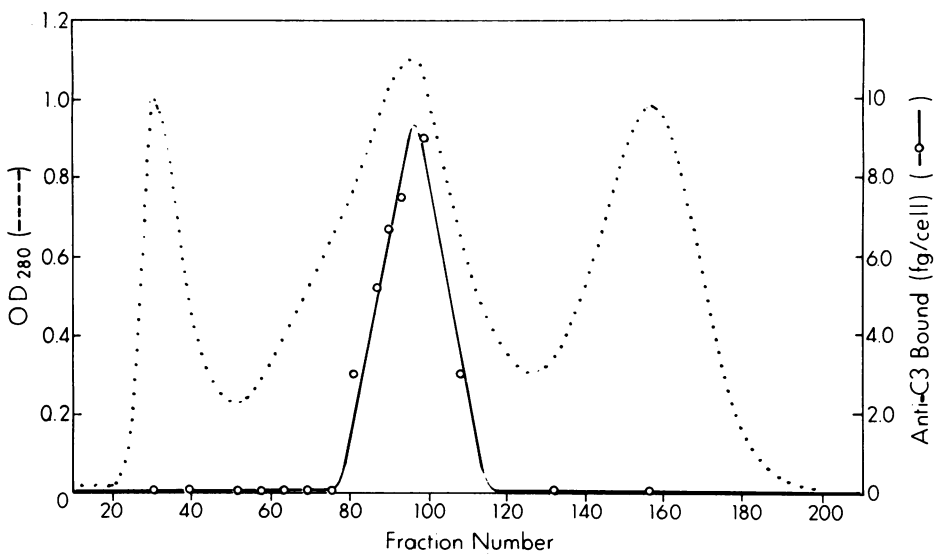

FIGURE 7 C3 fixation by fractionated serum: OD at 280 nm of sequential 3.8 ml fractions obtained by Sephadex G-200 gel filtration of serum from one patient with FS (dotted line). Anti-C3 binding to PFG incubated with complement and aliquots of fractionated serum (solid line). The results are expressed as in Fig. 2. The data points represent the mean of three determinations.

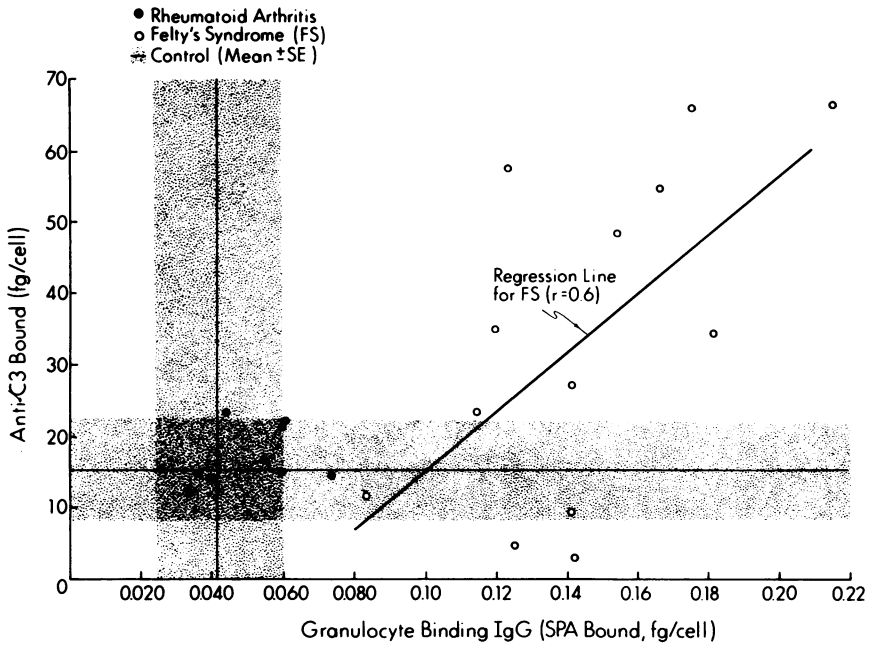

FIGURE 8 Concurrent determinations of C3-fixing ability and granulocyte-binding IgG: anti-C3 binding and SPA binding to PFG incubated with sera from normal subjects, patients with RA, and patients with FS. Results are expressed as in Fig. 2. Each data point represents the mean of three determinations on sera from an individual patient. The shaded areas represent the mean±SE of anti-C3 binding and SPA binding caused by sera from 10 normal subjects. The regression line was derived from a least squares analysis of the data points for sera from patients with FS.

of panels of sera for antigranulocyte antibodies and for defining granulocyte surface antigens (19). This assay is most useful for the detection of cold-reactive antibody, predominantly IgM, and requires rabbit complement for production of lysis. Using a similar assay system, van Boxel et al. (20) reported the presence of circulating cytotoxic autoantibody in patients with FS. Unfortunately, human granulocytes have been found to be resistant to the lytic action of human complement in vitro (21), making human complement-mediated cytotoxicity assays unrewarding (22). Furthermore, activation of sublytic amounts of complement may occur and remain undetected by cytotoxicity assays. In immune hemolytic anemia, fixation of sublytic complement components to the cell membrane, as well as formation of the lytic complex by completion of the terminal complement sequence are involved in the pathophysiology of erythrocyte destruction (12). Because of the limitations of using granulocyte lysis as an indication of complement activation and because of the success of assays that quantitate surface membrane-bound C3 in the study of immune hemolytic anemia (23) and immune thrombocytopenia (24), we developed an assay system that measures fixation of C3 to granulocytes. We took advantage of the increased specificity possible with monoclonal anti-C3 antibody and thereby reduced the problem of nonspecific adsorption of antibody seen with polyclonal preparations (25).

Using the ^{125}I-labeled anti-C3 antibody (anti-C3) binding assay, we found major differences in the ability of sera from patients with FS to fix C3 to granulocytes, as compared with sera from patients with uncomplicated RA and control subjects. Although the C3-fixing activity of sera from patients with RA was indistinguishable from that of normal subjects, sera from 7 of 13 patients with FS had increased C3-fixing capabilities. In these patients, two to three times as much anti-C3 was bound per cell as in patients with RA. The effects of alterations in temperature and of complement inhibition during the incubation of cells with serum in the presence of a complement source support the conclusion that C3 was being fixed to granulocytes by the activation of complement.

The ability of FS serum to activate complement and fix C3 to granulocytes may be derived from the presence of immune complexes. Hurd et al. (26) found elevated levels of complement-fixing circulating im-

mune complexes in FS sera using a radiolabeled C1q binding assay and others have demonstrated the presence of granulocyte-binding IgG and high-molecular weight immune complexes in the sera of patients with FS (27). By interposing a wash step between the time when cells were incubated with FS serum and the time when a fresh complement source was added, we were able to determine whether the anti-C3 binding detected in our assay was due to fixation of C3 to granulocytes after fluid-phase activation had occurred. The complement-activating principle in test serum bound to cells and persisted after washing. In addition, incubation of granulocytes with heat-aggregated IgG_1 and fresh normal serum did not cause augmented anti-C3 binding despite an increase in the amount of granulocyte-bound IgG. This finding diminished the possibility that bound immune complexes were responsible for significant levels of complement activation in our assay system. To further define the immunoglobulin species involved, binding of anti-C3 was measured following incubation of granulocytes and a fresh complement source with fractionated FS serum. Virtually all of the C3-fixing activity resided in the monomeric IgG-containing fraction, whereas no C3 was fixed by the fractions containing pentameric IgM or high-molecular weight immune complexes.

Although autoantibodies to a granulocyte-specific antigen have been described in a patient with idiopathic autoimmune granulocytopenia (28), the antigens against which antigranulocyte antibodies are directed in FS have not been identified. We excluded any potential role of ABO blood group antigens by using granulocytes obtained from group O individuals. Increased fixation of C3 to autologous granulocytes and to granulocytes obtained from different individuals by FS sera suggested that C3 fixation was caused by autoantibodies and not alloantibodies.

In these studies, we measured C3 bound to PFG rather than live granulocytes because methodologic considerations have made it difficult to interpret immunologic phenomena on the surface of metabolically active granulocytes. A cleavage fragment of the fifth component of complement (C5a) has been implicated in the in vitro aggregation of live granulocytes which occurs in the presence of activated complement (29). Weitzman et al. (30) demonstrated that granulocytes process surface antigen-bound antibody and respective antigens by internalization and cycloheximide-resistant regeneration of antigen. This clearance of antigranulocyte antibody opsonic activity was temperature dependent and sometimes failed to occur without autologous serum. Such loss of surface-bound antibody or aggregation of cells might preclude correct correlation of granulocyte-binding IgG and fixation of C3 to the granulocyte surface. Similarly, such loss of surface-bound antibody may preclude reproducible measurement of C3 on a patient's own cells.

To allow isolation of the surface-related events from intracellular metabolic activity in the anti-C3 binding assay, we used fixed cell surfaces as the target for activation of complement. Paraformaldehyde fixation of granulocytes has been shown to preserve potential antigen-binding sites for serum antigranulocyte antibody and to preserve granulocyte-bound IgG (8, 15, 31, 32). This fixation procedure apparently does not disturb the cell surface configuration that is receptive to binding to C3b in nonfixed target cell systems (33), as demonstrated by our ability to detect C3 fixation by binding of anti-C3 to sensitized PFG. Importantly, <2% of anti-C3 binding to unsensitized PFG was a result of nonspecific adsorption of anti-C3, such as to Fc receptors, and PFG by themselves did not activate complement to an appreciable extent in the absence of FS sera.

We investigated the contribution of bound antigranulocyte antibody to the complement-activating ability of FS sera by the concurrent measurement of SPA binding and anti-C3 binding. No serum caused increased complement activation without elevated granulocyte-binding IgG. The antigens involved in the binding of antigranulocyte antibody must therefore be densely distributed on the granulocyte surface since fixation of C1q requires a pair of closely apposed IgG molecules (34). In contrast, pentameric IgM antibody may not require proximity of antigens to activate complement. Six patients with FS had elevated granulocyte-binding IgG without increased fixation of C3. These patients may have had antibodies directed against sparsely distributed antigens, as has been found in the lack of complement fixation by anti-Rh IgG (35) or may possibly have had granulocyte-binding IgG of subclasses that do not react efficiently with C1q, such as IgG_2 or IgG_4 (36), both of which bind SPA (37). Alternatively, such patients may have pathophysiologically less important levels of antigranulocyte antibody and perhaps have suppressor T-cell mediated granulocytopenia (6).

The amount of C3 fixed to granulocytes and the amount of IgG bound were elevated in 7 of 13 patients with FS, but the amount of C3 fixed was proportional to the amount of IgG bound in the entire group. In concert with the results of our other experiments, this correlation is strong evidence that the granulocyte-binding IgG present in the sera of patients with FS activates complement and fixes C3 to the surface of granulocytes when bound to antigens on the granulocyte surface. The fixation of C3 by granulocyte-binding IgG may prove to be critical in our understanding of the pathophysiology of granulocytopenia in FS, as well as in the other clinical syndromes of immune

granulocytopenia, including perhaps drug-induced granulocytopenia (38), by virtue of the opsonizing and chemotactic properties of the C3 oligopeptides.

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