Immunoglobulin G Independent Activation of the Classical Complement Pathway by Monosodium Urate Crystals

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ABSTRACT 10 mg of monosodium urate crystals reduced the CH_{50} of 1 ml of human serum by 57% after 30 min at 37°C. C1, C4, and C3 depletion of 52, 68, and 46% were typical of classical pathway activation. C1 binding and activation occurred when urate crystals were incubated with isolated precursor C1, and required the intact macromolecule, C1qrs. Activation of isolated C1 by urate crystals was not diminished by $F(ab')_2$ anti-Fc under conditions in which C1 activation by aggregated immunoglobulin(G) was blocked by the $F(ab')_2$ antibody.

INTRODUCTION

Monosodium urate crystals initiate gouty inflammation. Several lines of evidence have implicated the complement system in this process. For example, prior complement depletion in rats (1) and dogs (2) ameliorates the inflammation of experimentally induced gout. Further, urate crystals activate complement in human serum (3) with concomitant development of mediators of inflammation (4). Thus, activation of complement by monosodium urate crystals may well contribute to the development of acute gout.

The mechanism by which complement is activated by urate crystals has not been fully elucidated. Naff and Byers (3) reported that serum incubated with crystals showed depleted levels of complement components, C2 to C5. These workers suggested that urates activate complement via a novel pathway, because C1 was only slightly depleted. One purpose of this study was to determine a mechanism by which the components of the classical complement pathway might be activated by urate crystals.

It is known that urate crystals avidly bind immunoglobulin(G) when exposed to serum (5). This suggests the possibility that adsorbed immunoglobulin might be responsible for activation of complement by urate crystals in serum. Although Naff and Byers (3) reported that urate crystals also activated complement in immunoglobulin-deficient sera, the presence of minor quantities of contaminating immunoglobulin could not be excluded. The purpose of this study was thus twofold. First, we wanted to determine to what extent urate crystals could activate C1, either in serum or in purified form, and second, whether IgG might be required for the observed C1 activation.

METHODS

Urate crystals. Uric acid crystals (ICN Pharmaceuticals Inc., Cleveland, Ohio) were heated at 200°C for 2 h to destroy possible pyrogens. These crystals were then slowly neutralized under aseptic conditions with 0.1 M NaOH in sterile nonpyrogenic water in glassware that had been heated 200°C for 2 h as described (6). The monosodium urate crystals produced were negatively birefringent needle-shaped, and showed an x-ray powder diffraction pattern typical of monosodium urate monohydrate (7). Culture of the crystal suspension yielded no bacterial growth. Crystals were stored at −20°C at 25 mg/ml, and thawed once before use.

Reagents and proteins. Sodium dodecyl sulfate (SDS),[1] acrylamide, and dithiothreitol were purchased from Sigma

This is publication 1528 from the Department of Immunology and Pathology, Research Institute of Scripps Clinic, La Jolla, Calif.

Dr. Giclas is a recipient of National Institutes of Health Research Fellowship Award 5-F32-AI05739-02. Dr. Ginsberg is a recipient of U. S. Public Health Service Clinical Investigator Award 1KO8-AM 00393-1.

Received for publication 22 September 1978 and in revised form 8 December 1978.

[1] *Abbreviations used in this paper*: SDG, sucrose density gradient; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis.

Chemical Co. (St. Louis, Mo.) and Bio-Rad Laboratories (Richmond, Calif.). Precursor forms of C1q, C1r, and C1s were purified from normal human serum by published methods (8–10). Human immunoglobulin (IgG) was prepared by DEAE cellulose chromatography of dialyzed human serum (0.01 M phosphate buffer, pH 7.4) and was heat aggregated at 63°C for 15 min. C1s was radiolabeled with Na ^{125}I (carrier free, New England Nuclear, Boston, Mass.) using lactoperoxidase-coupled Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N. J.) as described (11). $F(ab')_2$ fragments of goat anti-human Fc antibody were prepared as published (12).

Reconstitution of macromolecular proenzyme C1. C1 was reconstituted from highly purified C1q, proenzyme C1r, and ^{125}I-labeled proenzyme C1s in the presence of calcium as described (11).

SDS polyacrylamide gel electrophoresis (SDS-PAGE). Experiments were performed with a modification (9) of the method of Weber and Osborn (13). After electrophoresis, gels were sectioned at 2-mm intervals and analyzed for radioactivity.

Sucrose density gradient (SDG) ultracentrifugation. SDG studies were carried out in 9–31% linear sucrose gradients in barbital-buffered saline containing 1.5 × 0.1 mM calcium chloride (14) exactly as published (15).

Complement and complement component measurements. Complement and C1, C3 (16), and C4 (17) measurements were carried out by standard hemolytic techniques.

C1 binding assays. Binding of C1 to urate crystals was assessed directly, with SDG ultracentrifugation. This technique was chosen because it permits not only direct measurement of C1 binding but also detects C1 that has been activated and has dissociated from the activator (18). Varying amounts of urate crystals in 5 μl vol were incubated for 10 min at 0°C with 8 μg of C1q and 4 μg each of C1r and ^{125}I-C1s in a total volume of 20–30 μl in 0.05 M Tris, pH 7.5 containing 0.1 M NaCl and 0.005 M calcium resulting in the formation of 5 μg C1 macromolecule plus remaining free subcomponents. The mixtures were then subjected to SDG as described above. The proportion of radioactivity in the 16 S peak and on the bottom of the tubes was taken as total C1 for calculating the percentage bound (bottom) in the control and experimental samples.

C1 activation assays. Determination of C1 activation was carried out by procedures that have been published (11). In brief, reconstituted C1 was further purified by SDG ultracentrifugation and the gradient fractions collected at 0°C into tubes chilled in an ice bath. The 16 S area of the gradient was pooled and frozen in aliquots at −70°C. Activation analyses were carried out by incubating experimental samples with reconstituted ^{125}I-C1s-labeled C1. Each aliquot contained 0.2–1.0 μg of C1, and the total volume of the activation reaction mixture was 25–50 μl. Negative controls lacking activator but containing urate-saturated buffer and positive controls containing 10 μg of aggregated IgG or 5 μg of lipopolysaccharide (strain R 595) (15) were routinely included. After incubation for 20 min at 30°C, SDS, urea, and dithiothreitol were added and the mixtures analyzed by SDS-PAGE. Because all of the radiolabel appears in either the proenzyme 87,000-dalton peak or the 59,000-dalton peak of the heavy chain of $\overline{C1s}$, quantitation of C1 activation by the various activators was achieved by determining the percentage of total radioactivity transferred to the 59,000-dalton peak of the heavy chain of $\overline{C1s}$, after correcting for spontaneous C1s activation in the sample containing urate-saturated buffer. Urate-saturated buffer alone did not activate C1 in comparison to the same buffer without dissolved urate.

RESULTS

Complement activation by urate crystals in serum. To determine the extent to which urate crystals would deplete serum complement, varying amounts of crystals were incubated in serum for 30 min at 37°C. Hemolytic assays for C1, C4, C3, and CH_{50} were performed on the serum after the crystals had been removed by centrifugation. Complement depletion by 20 mg/ml of crystals in serum is shown in Fig. 1. The pattern of depletion observed, with proportionate reductions of CH_{50} and of C1, C4, and also C3 is indicative of classical pathway activation. As shown in the dose-response experiment depicted in Fig. 2, CH_{50}, C1 and C3 activities were progressively depleted as the dose of crystals added to serum was increased. Thus, the CH_{50}, C1, and C3 activities were reduced 57, 52, and 46%, respectively, after incubation of serum with 10 mg/ml of crystals.

C1 binding by urate crystals. The reduction in C1 levels observed in the above studies (Figs. 1 and 2) suggested that C1 bound to urate crystals. To further evaluate this possibility, urate crystals were incubated for 10 min at 0°C with reconstituted macromolecular C1 that had been formed from highly purified immunoglobulin-free C1q, proenzyme C1r, and ^{125}I-labeled proenzyme C1s during 10 min of incubation at 0°C in the presence of calcium as described (11). C1 binding was appraised and quantitated by sucrose density gradient ultracentrifugation. As shown in Table I, urate crystals bound macromolecular C1 in the absence of immunoglobulin. No binding of radiolabeled C1s occurred when C1q, C1r, and C1s were incubated with crystals in the presence of EDTA. Binding also did not occur when C1q was omitted from the mixtures.

C1 activation by urate crystals. Although the

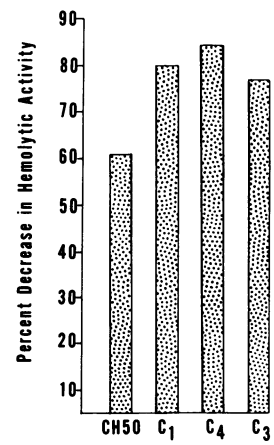

FIGURE 1 Percentage of decrease in complement activity of normal human serum after incubation 30 min at 37°C with 20 mg/ml urate crystals.

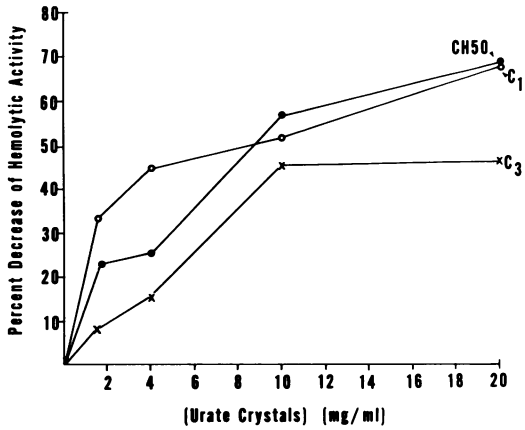

FIGURE 2 Percentage of decrease in CH_{50}, C1, and C3 activities of normal human serum after 30 min at 37°C with increasing amounts of urate crystals.

above studies indicated that C1 could bind to urate crystals without the participation of immunoglobulin, it remained to be determined whether this interaction was productive and resulted in C1 activation. To investigate this question, urate crystals were incubated with macromolecular C1 reconstituted from purified immunoglobulin-free C1q, proenzyme C1r, and ^{125}I-labeled proenzyme C1s. Activation was assessed by SDS-PAGE. As depicted in Fig. 3, urate crystals efficiently activated macromolecular C1. In similar experiments urate crystals produced no direct activation of C1s or C1r-C1s mixtures but did so on addition of C1q, implying that integrity of the C1 macromolecule is required for activation. Dose-response studies showed that urate crystals activated C1 over a very wide dose range with the maximal activation achievable being ≅90% of that obtained with aggregated IgG. In five experiments, the average dose of urate crystals producing 50% of maximal activation of the 0.7 μg of C1 in the test system was 2.7 μg. In addition, however, inhibition of C1 activation at high urate:C1 ratios, as shown in Fig. 4, was observed in each of the five studies of this type. Nevertheless, even at 120 μg of urate crystals, binding studies identical to those described above showed that C1 was bound to the crystals. Thus, the inability of high urate doses to activate C1 was not because of failure of the crystals to bind C1.

Lack of participation of endotoxin in complement activation by monosodium urate crystals. The uric acid crystals were originally heated at 200°C before neutralization with NaOH to destroy possible pyrogens, and then frozen in small aliquots that were thawed once just before use. Several tests were performed to further rule out the participation of lipopolysaccharide in the complement activation observed in these results. The crystals were dissolved in sterile

TABLE I
C1 Binding by Urate Crystals

Dose of crystals*	Percentage C1 bound‡
μg	%
15	46
3	16
0.6	1

Percentage of C1 bound by urate crystals after 10 min at 0°C.
* Amount of crystals incubated with 5 μg of C1.
‡ Corrected for background level of 17% C1 pelleted in absence of crystals.

NaCl at 100°C at a concentration of 0.1 mg/ml and 50 ml was infused intravenously into rabbits. Neither temperature elevation nor neutropenia resulted, indicating that the crystals contained <50 ng of bacterial lipopolysaccharide per 5 mg of urate crystals (19). Also, bacteriologic cultures of the crystals were sterile.

In another approach, sodium urate crystals were heated at 200°C for 2 h to destroy potential pyrogens and then reexamined for complement activating ability. As shown in Table II, the ability of the heated crystals to activate the classical pathway in serum was somewhat decreased but not abolished.

Lack of IgG requirement for complement activation by urate crystals. To investigate a possible requirement for immunoglobulin for C1 activation by urate crystals, C1 activation assays were carried out in the presence of $F(ab')_2$ fragments of a goat anti-human Fc antibody. As shown in Table III, the level of $F(ab')_2$

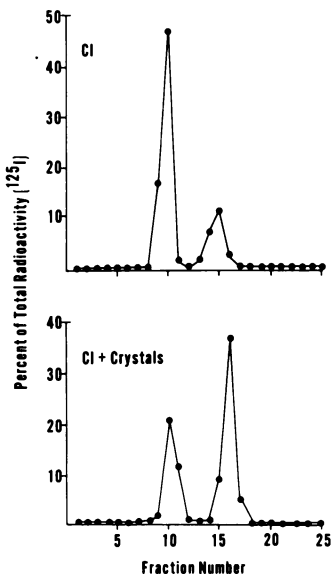

FIGURE 3 Activation of C1 as shown by shift in peak of ^{125}I-C1s from proenzyme 87,000 dalton form to 59,000 dalton activated $\overline{C1s}$ form. Top of SDS-PAGE is at the origin, points represent percentage of total radioactivity of each 2-mm slice.

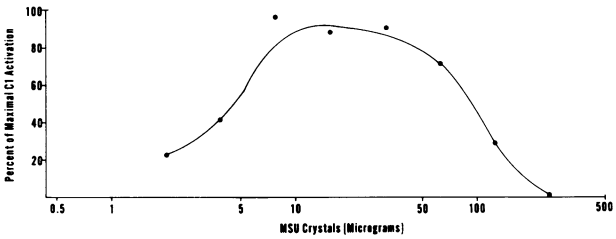

FIGURE 4 Activation of C1 urate crystals over a 100-fold dose range.

anti-Fc antibody used, 15 μg, completely blocked C1 activation by 10 μg of aggregated human IgG. However, the $F(ab')_2$ anti-Fc antibody had no effect on the C1 activating ability of the urate crystals. Because of the biphasic relationship between the dose of urate crystals and C1 activation (Fig. 4), it was important to study the effect of $F(ab')_2$ anti-Fc antibody on doses of crystals producing submaximal activation. As shown in Table III, in this experiment, 3 μg of urate crystals produced only 33% of the maximal amount of C1 activation. 15 μg $F(ab')_2$ anti-Fc antibody had no effect on C1 activation even at this low crystal dose. Thus, we conclude that IgG is not required for C1 activation by urate crystals.

DISCUSSION

These studies indicate that urate crystals can activate the classical pathway of complement when added to serum. In our experiments, 10 mg urate crystals/ml produced 57% reduction of CH_{50}. For comparison, in experimental gout (20), crystal concentrations of ≅7.5 mg/ml induce inflammation in canine stifle joints. Although complement activation by urate crystals has been demonstrated (3, 4, 21), and depression of C4, C2, and C3 levels was described (3), indicating classical pathway activation, the exact mechanism was not resolved. The marked depression of C4, C2, and C3 levels with little alteration in C1 titers observed by Naff and Byers (3) led them to postulate a novel mechanism of complement activation. In addition the possible roles played by immunoglobulin and lipopolysaccharide in the observed complement activation have not been clarified.

TABLE II
Effect of Heating on the Ability of Urate Crystals to Activate Complement in Serum

	Percentage of reduction in complement activity			
	CH_{50}	C1	C4	C3
		%		
Urate crystals untreated	90	94	98	87
Urate crystals, 200°C × 2 h	63	81	88	75

Complement activation in normal human serum incubated 30 min at 37°C with 20 mg/ml untreated crystals or crystals heated at 200°C for 2 h.

TABLE III
Lack of Requirement for IgG for C1 Activation by Urate Crystals

	Percentage of maximal C1 activation
	%
Agg IgG, 10 μg	100
+ 15 μg $F(ab')_2$ Ab Fc*	0
Urate crystals, 10 μg	92
+ 15 μg $F(ab')_2$ Ab Fc	92
Urate crystals, 3 μg	33
+ 15 μg $F(ab')_2$ Ab Fc	33

Effect on C1 activation of adding goat $F(ab')_2$ anti-human IgG Fc to aggregated IgG or to urate crystals.
* anti-Fc antibody.

The pattern of complement component consumption observed in our studies, with marked C1, C4, and C3 depletion is typical of classical pathway activation. We thus have no explanation for the failure of Naff and Byers (3) to observe a reduction in C1 levels proportionate to the reduction in C4, C2, and C3 levels. In addition, in our studies, urate crystals were found to directly activate isolated macromolecular C1, further indicating that urates can activate complement via C1. Furthermore, activation by urate crystals required intact macromolecular C1, as no C1s activation occurred when omitting C1q or C1r or both components. Thus, C1 activation by urate crystals does not involve direct activation of C1r or C1s by the crystals as observed with univalent 2,4-dinitrophenol-polylysine-antibody complexes (22) and certain enzymes such as plasmin (23). It is most likely, therefore, that urate crystals, like most activators, activate C1 via an initial interaction with C1q followed by sequential C1r and C1s activation. We thus find no evidence for another pathway of activation distinct from the classical and alternative pathways.

There has been some concern that possible bacterial lipopolysaccharide contamination of urate preparations might be involved in complement activation by urate crystals. Both Phelps and McCarty (21) and Naff and Byers (3) reported that urate crystals heated at 200°C for 2 h, to destroy possible contaminating pyrogens, consumed less complement than unheated crystals. However, complement consuming activity was regained in Naff and Byers' studies (3) when the heated crystals were dissolved and reformed, providing evidence against lipopolysaccharide mediation of the complement consumption. In our studies, we also found that

crystals heated at 200°C for 2 h could still activate the complement system in serum (Table II). Common contaminating bacterial lipopolysaccharides are smooth, polysaccharide-rich strains that predominantly activate the alternative pathway (24). Contamination with a lipid A-rich rough mutant that selectively activated the classical pathway (15) would be unusual. Measurable complement consumption in our studies was obtained with 16 μg of urate crystals per milliliter of serum that could have contained at most 0.16 ng of lipopolysaccharide per milliliter. This is several orders of magnitude less than the amount of lipopolysaccharide required to produce measurable classical pathway activation (15). Similarly, activation of purified C1 occurred with 3–10 μg of urate crystals that could have contained no >0.03–0.1 ng of lipopolysaccharide. However, C1 activation by lipid A or by lipid A-rich lipopolysaccharide preparations requires 100 ng or more of this material (15). By similar argument, the C1 preparations themselves must have contained <100 ng lipid A-rich–lipopolysaccharide as they were unactivated. Taken together, these data rule out the possibility that lipopolysaccharide alone is responsible for complement activation by urate crystals.

Although not examined in detail, there appeared to be a reasonable relationship between C1 binding and C1 activation at lower crystal:C1 ratios. For example, 15 μg of urate crystals bound 46% of the offered 5 μg of C1, whereas 2.7 μg of crystals produced 50% activation of 0.7 μg of C1. However, at high doses of crystals, C1 activation was inhibited, although parallel binding studies showed that the C1 was bound. This same phenomenon of inhibition of activation at elevated doses has been previously observed with another activator (15). The reasons for this type of inhibition are not clear but are presumably related to steric effects.

Urate crystals avidly bind IgG (5) and are probably coated with immunoglobulin in vivo. Naff and Byers (3) showed that urate crystals could deplete complement activity in most immunoglobulin deficient sera. Our studies show that IgG is not necessary for efficient C1 activation by urate crystals. Not only did urate crystals bind and also activate immunoglobulin-free macromolecular C1, but C1 activation by urate crystals proceeded unimpaired in the presence of $F(ab')_2$ fragments of anti-human Fc antibody. In the same experiments, the $F(ab')_2$ anti-Fc completely blocked C1 activation by aggregated IgG.

The data presented in this paper have defined the mechanism by which monosodium urate crystals, the etiologic agent of gout, activate the complement system. Efficient immunoglobulin-independent activation of the classical pathway supports the hypothesis (3) that activation of complement directly contributes to the acute inflammatory response of gout.

ACKNOWLEDGMENTS

The authors would like to thank Susan Host, Kathy Altman, and Rick Landes for their expert technical assistance; Jonne Stephenson for typing the manuscript, and Michael Miller for the x-ray crystallography of the urate crystals.

This work was supported by U. S. Public Health Service grants GM-24834, AI-07007, CA-14692, 1 SO7-RR05514, and HL-16411.

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