

Cryoprecipitogogue from normal serum: Mechanism for cryoprecipitation of immune complexes

(cryoglobulins/immune complexes/fibronectin/inflammation)

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ABSTRACT We designed this study to examine the relationship of cryoglobulins to immune complexes in sera of patients with rheumatic or infectious diseases. Polyethylene glycol was used to precipitate large proteins from normal serum and then was dialyzed away. The precipitated proteins were soluble in warm phosphate-buffered saline but at 4°C they reversibly reprecipitated. As they reprecipitated, they selectively coprecipitated cold-soluble immune complexes. By NaDodSO₄/polyacrylamide gel electrophoresis, these normal, nonimmunoglobulin cryoproteins were similar to the nonimmunoglobulin constituents of washed cryoimmunoglobulins from patients with rheumatic or infectious diseases. These findings suggest that cryoprecipitability is a property not of immune complexes themselves but of a group of large normal serum proteins. In inflammatory diseases, the concentrations of some of these proteins, responding as acute-phase reactants, may increase to the point where intermolecular attractive forces become prominent, particularly in the cold. Cold-augmented molecular aggregation between these nonimmunoglobulin proteins and immune complexes could then act to decrease their collective solubility and result in the cryoprecipitation of otherwise cold-soluble immune complexes.

Serum cryoglobulins, described by Lerner and Watson in 1947 (1), are generally thought to derive directly from circulating immune complexes (2, 3), but the mechanism by which they precipitate in the cold is not known. Classical immunoprecipitation of protein antigens involves three-dimensional antigen-antibody lattice structures that generally are larger than those found in most cryoprecipitable sera and whose solubility is relatively independent of temperature (4).

In previous studies (5) of how cryoglobulins are related to immune complexes, it was observed that sera from some patients formed cryoprecipitates even though they did not contain material reactive in the ¹²⁵I-C1q binding assay for circulating immune complexes. In contrast to cryoproteins that were associated with circulating immune complexes, these cryoprecipitates usually lacked detectable quantities of immunoglobulin. When examined by NaDodSO₄/polyacrylamide gel electrophoresis, they often contained many nonimmunoglobulin peptides. These findings led to consideration of nonimmunoglobulin serum components as possible initiators of cryoprecipitation. This report describes a group of proteins from normal serum that are capable of acting as cryoprecipitogogues (CPGs) for immune complexes.

METHODS

Isolation of Putative CPG. Blood was collected from normal donors and allowed to clot in sterile glass tubes for 2 hr at 37°C. For fractionation of proteins according to size, aliquots (5 ml)

of the serum were combined with equal volumes of 6000 *M*, polyethylene glycol (PEG) dissolved at concentrations ranging from 2% to 12% in phosphate-buffered saline (P_i/NaCl; 0.01 M PO₄/0.15 M NaCl, pH 7.4) and incubated for 1 hr at 4°C. Resulting precipitates were washed three times in P_i/NaCl/PEG at the same final concentrations used in the initial precipitation step. The precipitates were suspended in P_i/NaCl at a volume 1/10th or 1/5th that of the starting serum and incubated for 1 hr at 37°C. At the end of this step, any material that remained insoluble was sedimented at 10,000 × *g* for 10 min and discarded. The supernates containing putative CPG were dialyzed against two changes of P_i/NaCl for 48 hr at 4°C to remove any remaining traces of PEG. During this last step a white precipitate formed that dissolved upon rewarming.

Isolation of Cryoprecipitates. Cryoglobulins were isolated from eight patients, two each with systemic lupus erythematosus, rheumatoid arthritis, Lyme disease, and bacterial endocarditis. Blood (usually 50 ml) was obtained by venipuncture and kept at 37°C for 2 hr. The clotted blood was centrifuged at 1000 × *g* for 10 min at room temperature and the serum was kept at 4°C for 48-96 hr. Any precipitate that formed was sedimented at 1000 × *g* for 20 min at 4°C and washed three times with 10 ml of ice-cold P_i/NaCl. Washed cryoprecipitates were resuspended in 1 ml of P_i/NaCl and incubated at 37°C for 1 hr. The suspension was centrifuged at 10,000 × *g* for 10 min, and any remaining insoluble material was discarded. When cryoglobulins were to be examined electrophoretically, washed and redissolved cryoprecipitates were allowed to reprecipitate at 4°C and the analysis was performed on the redissolved material.

Cryoprecipitation of Immune Complexes with CPG. Immune complexes of known composition were formed in human serum containing hemagglutinating antitetanus toxoid antibodies at a titer of 1:1200. Tetanus toxoid, previously radiolabeled with ¹²⁵I by the lactoperoxidase technique (6), was combined with 400-μl aliquots of this serum and incubated for 18 hr at 4°C. The mixtures were centrifuged at 15,000 × *g*; the supernates were divided into two equal portions and combined with an equal volume of either warm P_i/NaCl alone or P_i/NaCl containing dissolved putative CPG at a concentration 10 times that in normal serum. Each aliquot was kept at 4°C for 8 hr before being centrifuged at 8000 × *g* for 10 min to remove any cryoprecipitates that formed. An aliquot (15 μl) of each sample was fractionated on continuous 10-40% sucrose density gradients (4.5 ml) spun in a 50.1 Ti rotor at 25,000 rpm for 14 hr. Gradient tubes were punctured at the bottom, and individual fractions were collected dropwise. The distribution of radiolabeled tetanus toxoid in each gradient was determined in a crystal scintillation spectrometer. In some experiments, immune complexes

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Abbreviations: CPG, cryoprecipitogogue; PEG, polyethylene glycol; P_i/NaCl, phosphate-buffered saline.

composed of ^{125}I -labeled human albumin-rabbit anti-human albumin were formed in fetal calf serum. These complexes were treated with putative CPG and analyzed in the same way as for complexes based on tetanus toxoid, except that sucrose density gradients were centrifuged at 35,000 rpm for 18 hr.

Other Analytical Methods. Protein preparations were quantified by the Lowry method and compared electrophoretically in NaDodSO₄/10% polyacrylamide slab gels. IgG, IgA, and IgM constituents were measured by radial immunodiffusion in Hyland plates. Fibronectin was detected by immunodiffusion in 1% agarose toward specific antiserum kindly supplied by Frederick Grinnell (7). Putative immune complexes in patients' sera were quantified by means of the ^{125}I -C1q binding assay described by Zubler *et al.* (8). Serum from normal donors was used as a CPG source only if it gave a negative reaction in this assay.

RESULTS

It was postulated that, when the concentrations of certain of the larger serum proteins were increased, their collective solubility would become temperature sensitive. Therefore, potentially cryoprecipitable proteins were isolated from 5-ml aliquots of normal serum to which various concentrations of PEG had been added. The proteins precipitated under these conditions were redissolved in 1 ml of P_i/NaCl at 37°C and tested for their propensity to precipitate spontaneously at 4°C. In general, with increasing concentrations of PEG, increasing amounts of protein were recovered in the 1 ml of warm P_i/NaCl (Table 1). Most of the protein recovered with 1% PEG was cryoprecipitable. Relative amounts of cryoprecipitable protein decreased in preparations made with higher concentrations of PEG, especially those above 3%. Upon warming, cryoprecipitated proteins redissolved. When this experiment was repeated with serum heated to 56°C for 1 hr, the amounts of reversibly cryoprecipitable proteins obtained were similar to those shown. Thus, complement components must comprise at most only a minor portion of these proteins.

It had been observed (9) that putative immune complexes (i.e., ^{125}I -C1q reactive material) are incorporated into cryoglobulins that form in patients' serum. Thus, it was of interest to determine whether the reversibly cryoprecipitable proteins recovered from normal serum could effect the cryoprecipitation of antigen-antibody complexes—i.e., act as a CPG. In the examples shown in Fig. 1, soluble tetanus toxoid-antitoxoid complexes or human albumin-rabbit antialbumin complexes were incubated for 8 hr at 4°C in the presence or absence of putative CPG (made with 3% PEG). Those proteins that cryoprecipitated during this incubation were removed and the remaining soluble immune complexes were analyzed on sucrose density gradients in an ultracentrifuge. The radiolabeled antigens sedimented in two peaks: free molecules sedimented near the 7S

marker, and antibody-bound molecules peaked beyond the 19S marker (Fig. 1). A substantial loss of both types of antigen-antibody complexes occurred in those samples treated with the putative CPG. This loss cannot be accounted for by nonspecific trapping of radiolabeled molecules in the cryoprecipitate or by dissociation of immune complexes because the radioactivity marking the unbound proteins changed little. I conclude that the added cryoprecipitable proteins were responsible for coprecipitating these immune complexes.

The redissolved proteins from three normal sera treated with 3% PEG were complex mixtures. On NaDodSO₄/10% polyacrylamide slab gels stained with Coomassie blue, at least 17 polypeptide bands ranging from 100,000 to about 14,000 in molecular weight were observed. Proteins that migrated along with γ and μ heavy chain markers were present but IgG and IgM made up no more than 20% of the total protein as measured by immunodiffusion. This composition was compared with that of cryoglobulins from eight patients, two each with systemic lupus erythematosus, rheumatoid arthritis, Lyme disease, and bacterial endocarditis. Even after having been washed three times, redissolved in warm buffer, and cryoprecipitated again, these precipitates also contained complex assortments of polypeptides, many of which had molecular weights matching those found in the 3% PEG fractions of normal serum. Patients' cryoprecipitates differed from PEG-derived precipitates by often containing relatively more IgG or IgM. Fibronectin was detected in all of the 3% PEG precipitates and in five of the eight cryoglobulins.

DISCUSSION

Serum cryoglobulins occur in three forms (2). Type I are monoclonal immunoglobulins that precipitate in the cold. Type II are mixed cryoglobulins that contain polyclonal immunoglobulin and another monoclonal component. Type III are cryoglobulins that contain polyclonal immunoglobulins only. Cryoglobulins of this last type are typical of those found during inflammatory illnesses. This study demonstrates that experimentally produced, cold-soluble immune complexes behave like cryoimmunoglobulins in the presence of increased concentration of certain normal serum proteins that can be isolated with low concentrations of PEG. These latter proteins are reversibly cryoprecipitable by themselves, and their polypeptide constituents are similar to the nonimmunoglobulin components of cryoglobulins isolated from patients with rheumatic or infectious diseases. These findings imply that type III cryoglobulins, and possibly other types as well, depend on nonimmunoglobulin components for their cold-insoluble properties.

We found fibronectin both in 3% PEG fractions of normal serum and in cryoglobulins isolated from patients' sera. This protein has several interesting properties that relate it to cryoprecipitation. At 4°C, it binds to fibrinogen to form soluble complexes, but this binding is reversible and does not occur in the warm. In certain disease states, fibronectin acts as a nucleus around which circulating cold-soluble fibrinogen-fibrin complexes bind to form cold-insoluble cryofibrinogen. Indeed, the removal of fibronectin from synovial fluid of patients with rheumatoid arthritis substantially reduces the formation of cryoglobulins (10). However, one can deduce that cryoglobulin formation is not related solely to fibronectin. First, this protein by itself is soluble in the cold (11). Second, we found type III cryoglobulins to be complex mixtures of peptides that remain relatively constant even when obtained from patients with different diseases and even when washed and recryoprecipitated. Thus, it appears that fibronectin and other larger proteins act in concert to initiate cryoprecipitation. Indeed, the first com-

Table 1. Cryoprecipitability of PEG fractions of serum

Final PEG, %	Amount of protein redissolved, mg*	Amount of redissolved protein cryoprecipitated, mg†
1	1.40	1.05
2	4.34	2.74
3	4.14	2.29
4	5.42	1.72
5	7.49	3.14
6	12.45	4.96

* Amount of precipitated protein that was dissolved in 1 ml of P_i/NaCl at 37°C.

† At 4°C.

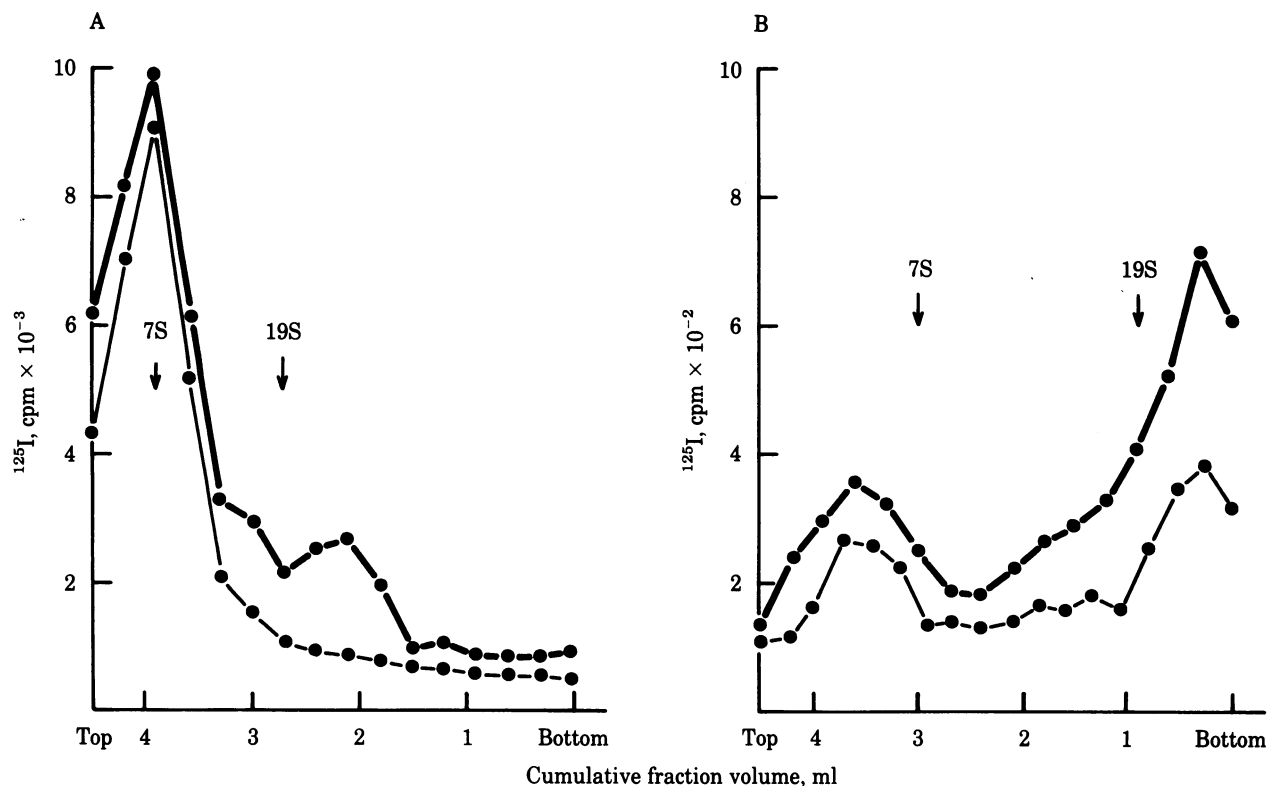


FIG. 1. Sucrose density gradient analysis of immune complexes with (light line) and without (heavy line) exposure to putative CPG and removal of any resulting precipitate. (A) Immune complexes were formed by incubation of 7.5 μg of ^{125}I -labeled tetanus toxoid with 400 μl of human immune serum at 4°C for 24 hr. No visible precipitate formed. The sample was centrifuged and divided into equal parts that were treated with either putative CPG or P_1/NaCl . Aliquots (20 μl) of each sample were fractionated on 10–40% sucrose density gradients. A portion of the ^{125}I -labeled tetanus toxoid appeared in complexes that sedimented ahead of the 19S marker. These complexes were absent from the sample treated with the putative CPG. (B) As in A, immune complexes were formed by addition of 1 ng of ^{125}I -labeled human albumin to fetal calf serum containing rabbit anti-human albumin at a dilution of 1:100 and treated with either putative CPG or P_1/NaCl . In the former sample, the total radioactivity in the rapidly sedimenting peak of radioactivity (antibody-bound ^{125}I -labeled albumin, the bottom 2 ml of the gradient) was decreased by 60%; the slowly sedimenting peak (unbound ^{125}I -labeled albumin, the upper 2.5 ml of the gradient) was decreased by only 17%.

plement component (C1) is necessary for the formation of cryoprecipitate in sera from some patient with systemic lupus erythematosus (12). It recently was observed that fibronectin augments C1q precipitation in the ^{125}I -C1q binding assay for immune complexes, presumably by binding to this molecule (unpublished data). Such binding would not be surprising because fibronectin binds collagen avidly, and collagen and C1q share similar structural regions (13, 14). It would be interesting to know to what extent the interaction of fibronectin with C1q is augmented by cold.

The behavior of macromolecules in solution can theoretically explain the reversible cryoprecipitation of large proteins and the ability of these molecules to facilitate the cryoprecipitation of immune complexes. In very dilute solutions, macromolecules tend to behave as independent units and effectively occupy spherical volumes equal to their diameter (15). With increasing concentrations, effective spherical volumes are reduced because of crowding, and molecular interactions involving intermolecular attractive forces become important. These interactions are temperature dependent; low temperatures augment them and enhance molecular aggregation. Thus, decreasing temperature favors insolubility of macromolecules, especially at higher solute concentrations.

In the present study, the large serum proteins in question were studied at a 5-fold increase in concentration compared to that of their native state within the circulation. Many acute-phase reactants respond to inflammation with rapid increases

in concentration and could approximate the levels examined in this study. On the other hand, fibronectin levels do not appear to vary much except in circumstances, such as trauma or sepsis, in which enhanced function of the reticuloendothelial system leads to depressed levels (16). It seems reasonable that molecular interactions of the type described above might be enhanced during acute-phase responses and thus provide a mechanism for the formation of larger, less-soluble, molecular aggregates. These aggregates might interact with fibronectin or immune complexes to account for the formation of cryoprecipitates *in vitro*. It is conceivable that these molecular interactions also occur *in vivo* within cooler regions of the circulation. In some patients, such clinical events as Raynaud's phenomenon and the hyperviscosity syndrome could be based on such a mechanism. A better understanding of the nature of these interactions might provide insight into new therapeutic options for such problems.

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