Human Monoclonal γG-Cryoglobulins with Anti-γ-Globulin Activity

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ABSTRACT Seven human γG-myeloma proteins which were also cryoglobulins were studied with respect to their reactivity with γG-globulins as well as with regard to their antigenic classification within the γG–heavy chain subclasses. Five of the seven cryoglobulins studied were positive in at least two of the three tests used to assay for anti-γ-globulin activity. One protein was only weakly positive in one test system and another was negative in all test systems. The structures which were recognized by the cryoglobulins were localized to the Fc-fragment. Only primate γG-globulins contained these antigenic determinants and in some cases the cryoglobulin appeared to show specificity for one human heavy chain subclass over the others. Antigenic analysis revealed that four of the five cryoglobulins with definite antibody activity belonged to the γG3-subclass, the fifth belonged to the γG1-subclass. The two cryoglobulins which reacted only weakly or failed to combine with γG-globulins were both of the γG1-subclass. These findings taken together with the localization of the combining site to the Fab-fragment suggests that many of these cryoglobulins may represent antibodies to γG-globulin, and that the cryoprecipitate in these cases represents antigen-antibody complexes of such a nature that they precipitate only in the cold.

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Received for publication 11 March 1968.

INTRODUCTION

Immunoglobulins are a physically and chemically heterogeneous group of proteins. In some instances, they precipitate or gelify on cooling and such proteins are known as cryoglobulins. While cryoglobulinemia is occasionally seen as a primary or idiopathic disease, it is most often associated with multiple myeloma or Waldenström's macroglobulinemia. In these subjects a major or minor fraction of the abnormal homogeneous protein behaves as a cryoglobulin. Recently it has become apparent that small amounts of cryoglobulins can also occur in other diseases, most often in those included in the group of collagen diseases such as systemic lupus erythematosus or related symptom complexes resembling this disorder (1–3). The frequency of these cryoglobulins is born out by a recent study of 29 unselected cases of cryoglobulinemia which indicated that about 40% of the proteins studied possessed rheumatoid factor activity, in which 11 of 12 instances was shown to be a γM–rheumatoid factor (2, 3). In one case the anti-γ-globulin activity was associated with a γG-protein. The biochemical basis for the phenomenon of cold precipitation of these proteins is largely unknown.

The present study was performed first, to study a group of γG–myeloma proteins which were also cryoglobulins for the purpose of determining whether these γG-cryoglobulins possessed antibody activity to normal γ-globulin. Second, since previous studies on the γG–heavy chain subclasses indi-

cated that they differ considerably in certain biological and biochemical properties (4–6 and footnote 1), it was of considerable interest to classify the γG-cryoglobulins with respect to heavy chain subclasses.

METHODS

Source and preparation of proteins. γG–myeloma cryoglobulins as well as other γG– and γA–myeloma proteins were obtained from sera of patients with a diagnosis of multiple myeloma or benign monoclonal gammopathy. The homogeneous nature of the cryoglobulins was established by starch-gel electrophoresis of the pepsin-digested proteins using a modification of Smithie's vertical gel method (7); γM-globulin was obtained from the serum of a patient with Waldenström's macroglobulinemia. Human serum albumin (HSA) was obtained from Cutter Laboratories, Berkeley, Calif. Heterologous γ-globulins were a gift from Dr. William Weigle. These were prepared by *O*-diethylaminoethyl (DEAE) cellulose chromatography of whole serum or commercially obtained fraction II.

Myeloma proteins were isolated by starch-block electrophoresis (8) and in the case of the γA- and γM-proteins, further purified by gel filtration on Sephadex G-200 (9).

Myeloma cryoglobulins were prepared by cold precipitation of the cryoglobulins followed by five washes in large volumes of phosphate-buffered saline, pH 7.2. Immunoelectrophoresis (10) of the washed cryoglobulins revealed only γG-globulin when tested with an antiserum prepared against whole human serum.

Antisera. Antisera to heavy chain subclasses of γG were obtained from rhesus monkeys immunized with γG1, γG2, or γG4–myeloma proteins and rendered subclass specific by appropriate absorption. Antiserum to γG3 was prepared by immunizing rhesus monkeys with pooled human γG and absorbing the antiserum with γG1– and γG2–myeloma proteins.

Anti-K and λ antisera were obtained from rabbits immunized with K or λ Bence Jones proteins.

Diffusion in gel. Proteins were studied by double diffusion in 1% agarose gel. Several of the cryoglobulin preparations precipitated in the gel at room temperature, and it was therefore necessary to modify the usual procedure. Antiserum was added to appropriate wells and allowed to completely diffuse into the agar while the plates were heated to 37°C in a humid chamber. All antigen solutions were heated at 50°C, the temperature required to totally solubilize the least soluble cryoprotein. Antigens were rapidly added to wells, and the plate incubated at 37°C until all solutions had completely diffused into the agar. The plate was then submerged in heated mineral oil and incubated at 50°C. Under these conditions proteins did not spontaneously precipitate in the gel. Plates were evaluated at 6, 24, and 48 hr.

Estimates of the quantity of each subclass present in the washed cryoprecipitates were obtained by finding the highest dilution of each preparation which would give a precipitin line with the subclass specific antisera. Dilutions of known concentrations of myeloma proteins representing each of the subclasses were similarly tested with the same antisera on the same plate. It was assumed that the highest dilution of the cryoglobulin that gave a precipitin line contained the same quantity of a subclass as that present in the highest dilution of the myeloma protein that gave a precipitin line.

Rheumatoid factor test. The test for rheumatoid factor was performed by the latex agglutination test using a commercial source (Hyland Laboratory, Los Angeles, Calif.) of gamma globulin coated latex particles. Serial dilutions of purified cryoglobulins were tested for their capacity to agglutinate the latex particles. Results are expressed as the lowest concentration of cryoglobulin that gave definite agglutination.

Coprecipitation of radioiodinated proteins with cryoglobulins. Normal γ-globulin from a variety of species, human serum albumin, as well as human γA- and γM-paraproteins, were labeled with ^{131}I (I*) by means of the chloramine T method (11). Radioisotope detection was done with a gamma well-type scintillation counter. To test for binding of the labeled proteins to the cryoglobulin precipitate, 1-ml aliquots containing 10 mg of the purified cryoglobulin in phosphate buffered saline, pH 7.2, were mixed with 0.1 ml of the buffer containing 0.1–1 μg of the labeled protein to be tested. The mixture was incubated at 37°C for 1 hr and then for 16 hr at 4°C. The cryoglobulin-I* myeloma protein mixture was centrifuged at 2000 rpm for 30 min in the cold; the supernatant was decanted and the precipitate washed and resuspended in 1 ml of cold, phosphate-buffered saline. The centrifugation was repeated, the supernatant discarded, and the precipitates assayed for radioactivity. It was found that the percentage of radioactivity precipitated did not vary over the range of concentrations of I* protein used.

Enzymatic digestion of γ-globulin. Fragments of cryoglobulins and normal human γ-globulin were produced by papain digestion (12) using a protein: enzyme ratio of 100:1. Digestion was carried out in the presence of 0.01 M cysteine and 0.002 M ethylenediaminetetraacetate (EDTA) at pH 7.5. Digestion was allowed to proceed for 45 min at 37°C and was stopped by the addition of a five-fold molar excess of iodoacetamide. After 1 hr at 4°C, the digestion products were dialized against 0.1 M phosphate buffer, pH 7.5. Pepsin digestion (13) was performed using 100:1, protein: enzyme ratio at pH 4.0. Digestion proceeded for 16 hr at 37°C and was stopped by dialysis against a large volume of 0.1 M phosphate buffer, pH 7.5.

Ultracentrifugation. Analytic ultracentrifugation was carried out in a Spinco model E ultracentrifuge equipped with schlieren optics. Centrifugation was performed at 20°C, at a speed of 52,640 rpm. Sedimentation coefficients and areas were calculated with the aid of a Gaertner

[1] Müller-Eberhard, H. J., M. A. Calcott, and H. M. Grey. Interaction between C'1q and γG-globulins of different heavy chain subtypes. In preparation.

comparator (Gaertner Scientific Corp., Chicago, Ill.). The sedimentation coefficients were corrected to the $s_{20,w}$ values by the accepted procedures (14). The partial specific volumes were assumed to be 0.73.

RESULTS

Electrophoretic and antigenic analysis. Seven γG-cryoglobulins were available in sufficient quantity to perform detailed studies. It was difficult to perform agar or starch-gel electrophoresis on these proteins to establish their monoclonal nature because of their tendency to aggregate, even at room temperature. In order to circumvent this problem we treated the proteins with pepsin which destroyed the property of cryoprecipitation and thereby made it possible to evaluate the electrophoretic homogeneity of these proteins. The results of starch-gel electrophoresis of the pepsin-treated cryoglobulins is shown in Fig. 1 and illustrates the discrete bands that are the typical patterns obtained with the pepsin-treated myeloma proteins compared with the diffuse smear obtained with normal γG-globulin (*HGG*, Fig. 1, slot 7).

In contrast to the starch-gel electrophoresis data which indicated the cryoglobulins were essentially homogeneous, immunodiffusion analysis revealed that in several instances the cryoprecipitates were antigenically heterogeneous, in that more than one light chain type or heavy chain subclass was present in the washed cryoprecipitate. However, a predominant light and heavy chain type was always found. Since the electrophoretic analysis indicated that the major protein moiety in each of the cryoprecipitates was "monoclonal" with regard to electrophoretic mobility, it was assumed that the major antigenic type present in the cryoprecipitate represented the antigenic structure of the "monoclonal" protein. Table I gives the results of this antigenic analysis. In those cases where significant amounts of more than one heavy chain subclass or light chain type were present the minor antigenic type is placed in parenthesis next to the major type. In some instances the minor "contaminating" antigenic type made up as much as one-fourth to one-third of the total cryoprecipitate. Five of seven proteins had type K light chains as the sole or major class present. Four proteins were of the γG3–heavy chain subclass and three were of the γG1-subclass.

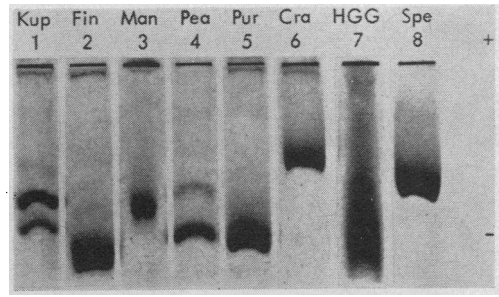

FIGURE 1 Starch-gel electrophoresis (glycine buffer, pH 8.8) of the $F(ab')_2$ fragments of cryoglobulins and normal γ-globulin, obtained by pepsin digestion. The $F(ab')_2$ fragments of the cryoglobulins yielded discrete bands whereas the $F(ab')_2$ fragment of normal γ-globulin gave a diffuse smear.

Determination of anti–γ-globulin activity. The finding that in some instances the cryoprecipitate contained the myeloma protein plus other γ-globulin suggested the possibility that the myeloma protein cryoglobulin might be interacting with the

TABLE I

Antigenic Classification and Anti–γ-Globulin Activity of γG–Monoclonal Cryoglobulins

Cryoglobulin	γG–heavy chain subclass	Light chain type	Latex agglutination of cryoprecipitate	% Radioactivity in cryoprecipitate		
				γA	HSA	Normal γG
Man	γG3(γG1,γG2)*	κ	0.1‡	3.0	2.2	33.0
Kup	γG3	κ(λ)	0.06	4.7	5.9	33.8
Pea	γG1	λ	0.06	3.2	2.1	13.7
Cra	γG3(γG1)	κ	0.06	5.7	5.7	35.3
Spe	γG1	λ	>10	3.4	2.3	15.1
Pur	γG3(γG1)	κ(λ)	>10	5.1	3.1	26.1
Fin	γG1	κ	>10	2.5	3.5	6.8

* Figures in parentheses indicate the presence and classification of significant amounts (>5%) of other immunoglobulin antigens.

‡ Lowest concentration of protein (mg/ml) to give positive reaction.

normal γ-globulin present in the patient's serum. The first system used to test this possibility was the latex agglutination method for measuring rheumatoid factor activity. The results of this test are shown in Table I. Four of the seven cryoglobulins tested possessed a high titer of rheumatoid factor activity and three proteins were negative. Tests done on the serum supernatants obtained after cryoprecipitation demonstrated only weakly positive or negative reactions.

Another indication of interaction between the cryoglobulins and human γG-globulin was obtained by determining the extent of coprecipitation of several radioiodinated proteins. The degree of retention of I*γG, I*γA, and I*HSA in the cryoglobulin precipitate is given in Table I. Only 2–6% of the added γA–myeloma protein or HSA was present in the cryoprecipitate whereas there was 13–35% of the added normal γG in six of seven cryoprecipitates. When the data obtained with the iodinated human γ-globulin is compared with the rheumatoid factor activity as measured by the latex fixation method (Table I), good but imperfect correlation is observed. Three proteins, Man, Kup, Cra, that had high I*γG-binding had a high rheumatoid factor activity; the one protein, Fin, which did not bind I*γG had a negative rheumatoid factor test and one protein, Spe, was weakly positive in one and negative in the other test. However, two proteins showed discordant results. One protein, Pea, only weakly bound the I*γG but gave a strongly positive rheumatoid factor test, whereas another protein, Pur, that showed good binding of the I*γG was negative by the latex agglutination technique.

Ultracentrifuge studies. There are obvious difficulties in evaluating the interaction of a γG-immunoglobulin with another protein of the same immunoglobulin class. Since several of the cryoglobulins behaved as rheumatoid factors, i.e. antibodies to γG-globulin, it was considered of particular interest to digest the cryoglobulins with papain and pepsin in order to separate the Fab- and Fc-fragments thus allowing each to be studied independently. If specific interaction between the Fab or $F(ab')_2$ of the cryoglobulin and γG-globulin could be demonstrated, this would represent further evidence that the cryoglobulins may act as classical antibodies with regard to the localization of the combining sites to the Fab-fragment. When

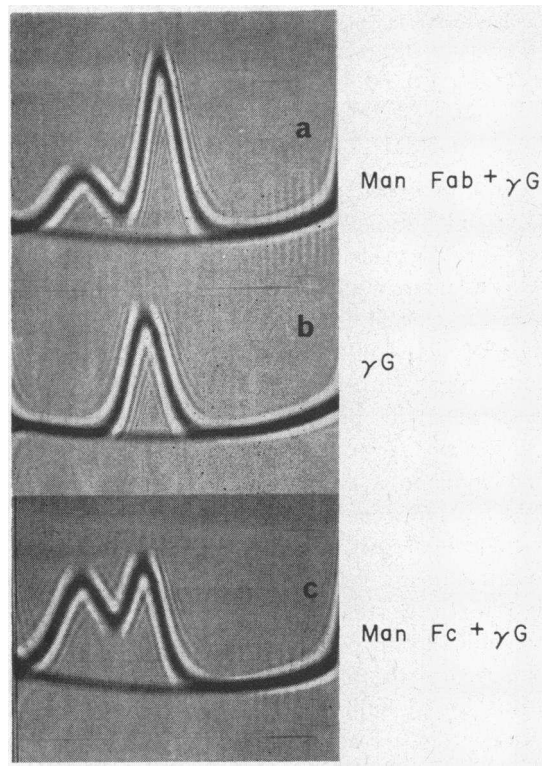

FIGURE 2 Analytic ultracentrifuge analysis of the interaction between papain fragments of cryoglobulin Man and normal γG-globulin. Pictures taken 64 min after reaching speed of 52,640 rpm. Man Fab formed complex (*a*) which sedimented more rapidly than γG by itself (*b*) whereas, the Fc-fragment did not complex with γG (*c*).

the seven cryoglobulins were digested with pepsin or papain, all of them lost the property of cryoprecipitation. When analyzed in the analytic ultracentrifuge the usual 3.5S and 5S peaks were observed with the papain and pepsin digested proteins, respectively, and no suggestion of higher polymers were seen when analyzed at 10 or 20°C.

In order to determine whether the Fab-fragment of the cryoglobulins was responsible for the interaction observed with normal γ-globulin in the previous test systems, Fab- and Fc-fragments of Man cryoglobulin were isolated, mixed with normal γ-globulin, and studied in the analytic ultracentrifuge (Fig. 2). The papain Fab-fragment of the Man protein interacted with normal γG to form a complex which sedimented with an *s* rate of 6.9, 0.6 U greater than the γG-protein by itself (Fig. 2*a* and *b*); whereas when the Man Fc-fragment was mixed with γG-globulin no evidence of interaction was obtained (Fig. 2*c*). The area

under the faster sedimenting peak in Fig. 2 *a* was greater than that observed when γG was centrifuged separately (Fig. 2 *b*) and the area under the 3.5S peak was less than that observed when the Fab-fragment was centrifuged separately. This also indicates that complexing had occurred between the Man Fab-fragment and normal γG-globulin.

For further ultracentrifugal analysis of the interaction between the cryoglobulins and γG-globulin, pepsin digested $F(ab')_2$ fragments were used. Representative myeloma proteins of the four heavy chain subclasses were used as antigens rather than normal γ-globulin so that it could be determined whether the cryoglobulins recognized structures specific for the heavy chain subclasses or whether the reactive regions were common to the four subclasses. Complexing between the cryoglobulin $F(ab')_2$ and the γG–myeloma proteins is illustrated in Figs. 3 and 4. The complexing was characterized, as was the case for the papain Fab-fragments, by a diminution of the 5S $F(ab')_2$ peak and an increase in the area and *s* rate of the faster sedimenting peak. Fig. 3 illustrates the interaction between $F(ab')_2$ fragment of the myeloma protein Man with a γG1–myeloma protein. The 5S peak was almost absent in the cell which contained both $F(ab')_2$ and a γG1–myeloma protein (Fig. 3 *b*). A single, slightly assymetrical peak was observed which was larger and sedimented slightly ahead of the peak observed when the γG1-protein was centrifuged by itself (Fig. 3 *c*) at the same protein concentration. The $s_{20,w}$ of the γG-myeloma protein was 6.6 and that of the 5S–7S complex 8.2. The lower two frames show a mixture of Man $F(ab')_2$ with a γA–myeloma protein. The γA–protein used in this case was a polymer. No diminution in the 5S peak was observed nor was there a noticeable change in the sedimentation pattern of the γA-polymer. Fig. 4 illustrates the reactions between the $F(ab')_2$ of the myeloma protein Pur and two γG– and a γA–myeloma protein. Fig. 3 *b* and *g* show the interaction between the $F(ab')_2$ of the cryoglobulin and the γG-proteins and Fig. 2 *d* demonstrates the lack of interaction with a γA-protein. This figure also demonstrates that Pur $F(ab')_2$ reacted to a greater degree with the protein of the γG2-subclass than with that of the γG4-subclass. Although the 5S peaks were depleted to almost the same extent when the schlieren pattern

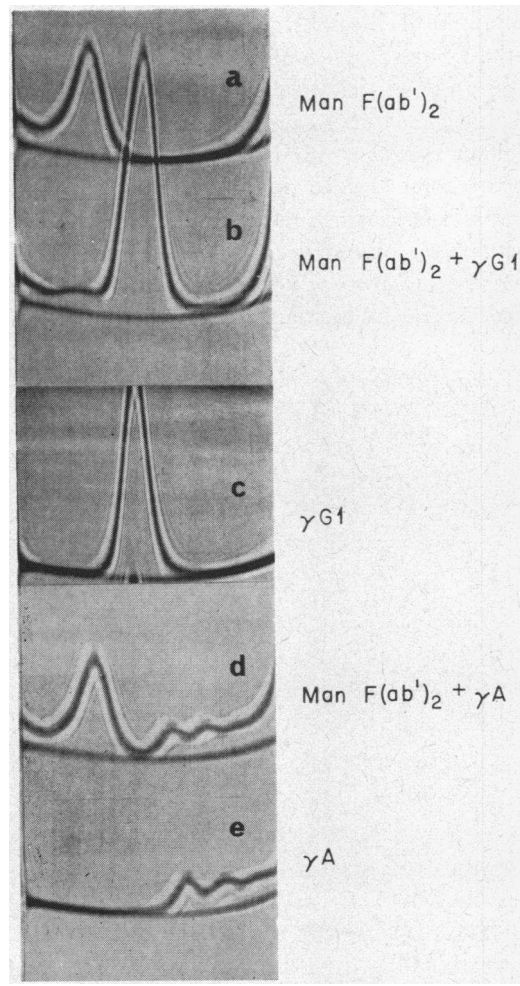

FIGURE 3 Analytic ultracentrifuge analysis of the interaction between the $F(ab')_2$ fragment of cryoglobulin Man and myeloma proteins. Pictures taken 64 min after reaching speed of 52,640 rpm. Man $F(ab')_2$ complexed with γG1-protein (*b*) but not with γA-protein (*d*). Complexes caused diminution of 5S peak and increase in *s* rate and area of faster sedimenting peak when compared to unmixed proteins (*a* and *c*).

of Fig. 3 *b* and *g* were compared, the complexes formed between Pur $F(ab')_2$ and γG2 were larger, having a sedimentation rate of 8.4, whereas the complexes formed between Pur $F(ab')_2$ and γG4 had an *s* rate of 6.7 only 0.2 Svedberg units greater than the *s* rate of the uncomplexed γG4-protein shown in Fig. 3 *f*.

Table II summarizes the ultracentrifuge results obtained with the $F(ab')_2$ of the seven cryoglobulins studied and indicates the maximum degree of interaction observed and gives a comparison of the relative intensity of the reaction obtained with each

of the four γG–heavy chain subclasses as measured by the *s* rates of the complexes formed. Five of the seven proteins showed definite interaction between their pepsin $F(ab')_2$ and one or more of the γG–myeloma proteins used. Three of the reactions showed some degree of specificity for one heavy chain subclass over another; although wherever a reaction was observed, all subclasses interacted to some extent. Two proteins, Fin and Spe, showed no detectable interaction.

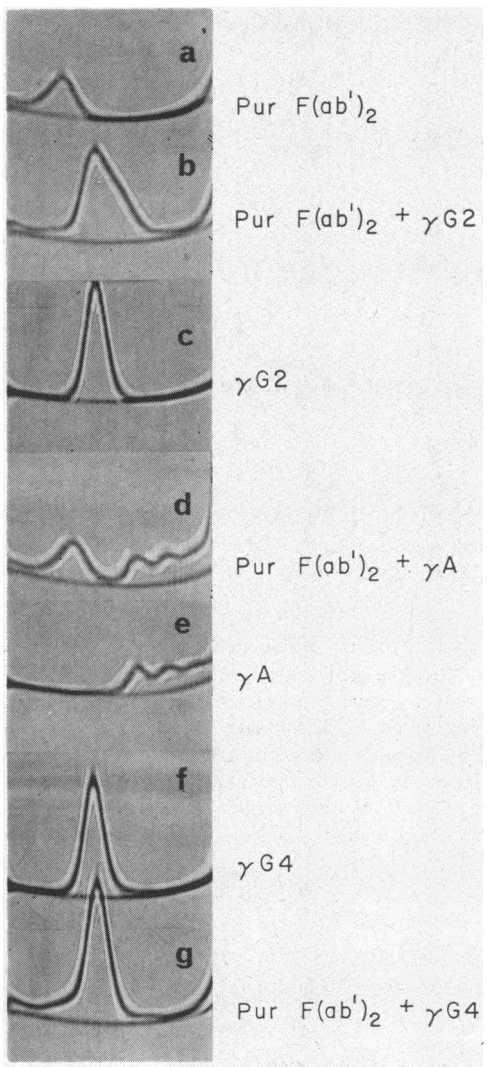

FIGURE 4 Analytic ultracentrifuge analysis of the interaction between $F(ab')_2$ fragments of cryoglobulin Pur and myeloma proteins. Pictures taken 64 min after reaching speed of 52,640 rpm. Pur $F(ab')_2$ complexed with γG2- and γG4-proteins (*b* and *g*) but not with γA (*d*). The complexes formed with γG2 had a faster sedimentation rate than those formed with γG4.

TABLE II

Ultracentrifuge Analysis of Complex Formation between $F(ab')_2$ of Cryoglobulins and γG–Myeloma Proteins of Different Heavy Chain Subclasses

Cryoglobulin	Degree of interaction: Diminution of 5S peak*	Degree of interaction: Increase in *s* rate of "7S" peak due to 5S-γG-complex	Heavy chain subclass specificity of interaction
Man	4+	1.6	$1>4\approx3\approx2$
Kup	1+	0.2	$1\approx2\approx3\approx4$
Pea	3+	0.3	$1\approx2\approx3\approx4$
Cra	3+	1.0	$3>1>2>4$
Spe	0	0	—
Pur	4+	1.9	$2>1\approx3\approx4$
Fin	0	0	—

* 1+10–25%; 2+25–50%; 3+50–75%; 4+75–100%.

When the three tests (rheumatoid factor activity, I*γ-globulin precipitation, and $F(ab')_2$–γ-globulin complexing) used for evaluating anti–γ-globulin activity of the cryoglobulins were compared, the correlation was again good but not complete. Four proteins showed positive reactions in all three tests (Man, Kup, Pea, and Cra); one protein, Fin, was negative in all three tests; one protein, Spe, was weakly positive in one test and negative in the other two; and one protein, Pur, was strongly positive in two tests but negative in the rheumatoid factor assay. It is of interest that this last protein which showed the most discordant results was the only protein which demonstrated specificity for the γG2-subclass.

It has previously been shown that most γM–rheumatoid factors react with the Fc-fragment of γG-globulin. The finding that some of the cryoglobulins reacted preferentially with certain of the γG–heavy chain subclasses suggested that the determinants that the cryoglobulins reacted with were also located on the Fc-fragment. This was directly demonstrated by studying the interaction between the $F(ab')_2$ of a cryoglobulin with the Fc- and Fab-fragments of normal γ-globulin. The ultracentrifuge analysis of this interaction is shown in Fig. 5 and demonstrates the complexing of normal γG–Fc-fragment with the $F(ab')_2$ from Man cryoglobulin to produce a complex which had a sedimentation rate of 6.5S (Fig. 5 *a*). There was no evidence of complex formation between γG–Fab-fragment with the Man $F(ab')_2$ (Fig. 5 *b*).

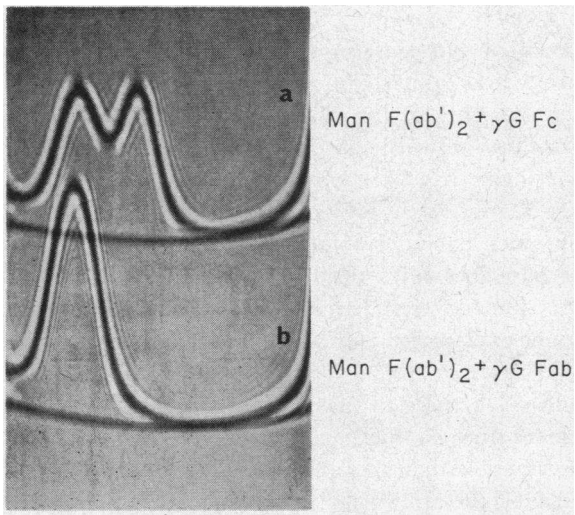

FIGURE 5 Analytic ultracentrifuge analysis of the interaction between $F(ab')_2$ fragment of cryoglobulin Man and papain fragments of normal γ-globulin. Picture taken 64 min after reaching speed of 52,640 rpm. Man $F(ab')_2$ interacted with the Fc-fragment (*a*) to form a 6.5S complex, whereas no complex formed with the Fab-fragment of normal γ-globulin (*b*).

Reaction of cryoglobulins with heterologous γ-globulins. In order to further investigate the specificity of the reaction between the cryoglobulins and γ-globulin, we made a study of the reaction of two cryoglobulins, Man and Kup, with radioiodinated γ-globulins of several infra-human species. The γ-globulins available for study were obtained from: gorilla, monkey, horse, cow, dog, guinea pig, and turkey. Both the gorilla and monkey γ-globulin coprecipitated with the two cryoglobulins studied to the same extent as human γ-globulin (35–40%); whereas, none of the five nonprimate γ-globulins reacted to a significant extent. A labeled preparation of human γM from a case of Waldenström's macroglobulinemia was also studied by the coprecipitation procedure. None of the seven cryoglobulins reacted with the γM-preparation.

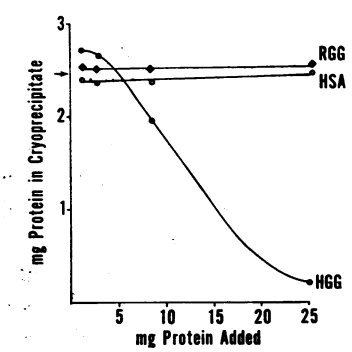

FIGURE 6 Inhibition of cryoprecipitate by normal human γ-globulin. Increasing amounts of human γ-globulin (*HGG*), rabbit γ-globulin (*RGG*), or human serum albumin (*HSA*) were added to 3 mg of Man cryoglobulin, incubated at 37° for 1 hr, and 16 hr at 4°C before centrifugation. Inhibition of cryoprecipitate formation occurred only after the addition of HGG.

Inhibition of cryoprecipitation by normal γG-globulin. In a further attempt to characterize some cryoglobulins as a special form of precipitating antibody to γ-globulin, the effect of the addition of increasing amounts of γ-globulin on the formation of the cryoprecipitate was studied. Fig. 6 shows these results and indicates the manner in which increasing amounts of normal human γ-globulin added to the cryoglobulin led to a decrease in the total cryoprecipitate formed, whereas rabbit γ-globulin and human serum albumin had no such effect. This inhibition of precipitation is similar to that observed in classical immunsystems studied by quantitative precipitin analysis where addition of antigen in excess of that needed to maximally precipitate the antibody results in the formation of soluble antigen-antibody complexes.

DISCUSSION

The present study of seven monoclonal γG-cryoglobulins has demonstrated that these proteins differ from noncryoglobulin monoclonal γG-proteins with respect to two important features. First, there is an extremely high incidence (five of seven) of proteins that interact with γG-globulins and may be considered to possess antibody activity to γG-globulin; second, there is a similarly high incidence of the γG3–heavy chain subclass in this group of myeloma proteins compared to that found in noncryoglobulin γG–myeloma proteins where the incidence ranges from 5–10% (15, 16). It is of interest in this regard that one cryoglobulin which was negative by all three tests for anti–γ-globulin activity and another protein which was only weakly positive to one test were both of the γG1-subclass, whereas, of the other five proteins with anti-γG–activity, four were of the γG3-subclass. That the anti–γ-globulin activity was associated with the myeloma protein which was the major component of the cryoprecipitate and not

due to small amounts of nonmyeloma 19S or 7S rheumatoid factor is indicated by the ultracentrifuge analysis of the interaction between the pepsin treated cryoglobulins and γG–myeloma proteins. As shown in Figs. 3 and 4 most, if not all, of the $F(ab')_2$ was capable of complexing with the γG–myeloma proteins as evidenced by the almost complete disappearance of the 5S peak. If the anti–γ-globulin activity was associated with a minor fraction of nonmyeloma rheumatoid factor only minor alterations in the size of the 5S peak should have been observed.

The significance of why complexes between the $F(ab')_2$ of cryoglobulins and certain γG-myeloma proteins sedimented at *s* rates only 0.1–0.3 units greater than the uncomplexed γG-myeloma proteins is unclear but it may reflect a highly dissociating complex, whereas those combinations which yielded complexes which sedimented with *s* rates 1–2 units greater than the uncomplexed γG-proteins may have formed complexes of higher affinity that sedimented with an *s* rate compatible with stable 5S–7S complexes. An alternative explanation involving differences in the frictional coefficients of the complexes formed is also possible, but appears less likely.

It would seem likely that both of the distinctive features of these cryoglobulins; viz. antibody activity toward γ-globulin and the γG3–heavy chain structure, are important in the formation of the cryoprecipitate. A possible explanation for the formation of the cryoprecipitate in those proteins that possess anti–γ-globulin activity would be that it represents antigen-antibody complexes of such a nature that precipitating complexes are formed only in the cold. The temperature dependence of the precipitation could be explained by postulating the antigen-antibody bond formed is one of very low affinity, since with complexes of low affinity, the antigen-antibody equilibrium tends to be shifted toward dissociation at higher temperatures.[2]

Why cryoglobulins with anti–γ-globulin activity tend to be of the γG3-subclass is not immediately apparent; however, there are at least two possible reasons for this. First, it is possible that there is a close relationship between the structure of an antibody site and the light or heavy chain class or subclass to which the γ-globulin molecule belongs. Or, put in more precise terms, the type of variation that can exist in the variable regions of the heavy and light chain is limited by and related to the structure of the constant region. Such a relationship between antigenic class and antibody specificity has been observed in the case of cold agglutinins with I blood group specificity: most of which have only type K light chains (17), and certain antibodies produced in the guinea pig to the dinitrophenyl hapten (18). The second possible explanation for the preponderence of the γG3-subclass in this group of cryoglobulins with anti-γG–activity is that the antigen-antibody complexes formed with γG3–antibody molecules are less soluble than those formed with antibody molecules of the other subclasses. The complexes made up of γG3-molecules would therefore have the observed property of cryoprecipitability, whereas complexes made up of the other subclasses would be more soluble. Observations on γG3–myeloma proteins without antibody activity give some indirect evidence for this hypothesis in that many γG3-proteins have a tendency to form aggregates.[3]

Another indication that the over-all structure of the protein is important with regard to the phenomenon of cryoprecipitability came from observations made with the pepsin digested cryoglobulins. Pepsin treated cryoglobulins lost their ability to precipitate in the cold. Combination with γG still occurred however, since addition of normal γG-globulin to the $F(ab')_2$ fragments of the cryoglobulins resulted in the formation of soluble complexes demonstrable in the ultracentrifuge. However, on the addition of normal γG to the $F(ab')_2$ fragment of the cryoglobulin, over a wide range of γG-concentrations, no cryoprecipitate formed. This would indicate that the phenomenon of cold precipitation is dependent on the integrity of the whole molecule and suggests that the structure of the Fc-fragment is important in determining the solubility of the complexes that form.

The finding in the present study, that there appears to be a high incidence of antibody activity to γ-globulin among γG–cryoglobulin myeloma proteins, confirms and extends previous reports in which studies of unselected cases of cryoglobulinemia were studied (2, 3, 19). In a previous series (2, 3), 12 of the 29 cases of cryoglobu-

[2] Grey, H. M. Unpublished observations.

[3] Kunkel, H. G., and H. M. Grey. Unpublished observations.

linemia studied had a positive test for rheumatoid factor. In one case the rheumatoid factor was associated with a γG–myeloma protein, whereas, in the remaining 11 cases the cryoglobulin was of the mixed type (19) in which the γM-component acted as rheumatoid factor and the γG-globulin served as antigen for the rheumatoid factor.

Most anti–γ-globulin antibodies studied come from the sera of patients with connective tissue disorders and have been γM-immunoglobulins. However, γG-antibodies directed against γG–globulin antigen are also quite common but less amenable to study because of the difficulty in isolating the antibody from the antigen since both are 7S γG-globulins. The technique used in the present study was one employed by Schrohenloher in studying γG–rheumatoid factor; that is, pepsin digestion of the antigen-antibody complex, thereby destroying the antigenic determinants which reside in the Fc-fragment (20). Using this technique, Schrohenloher was able to demonstrate complexing between the $F(ab')_2$ of the γG–rheumatoid factor with normal γG-globulin by ultracentrifuge analysis. The complexing observed was similar in degree and size of complex formed to those observed in the present study using myeloma proteins.

The current study adds to the recently expanding list of paraproteins which may be considered to have antibody activity. Several cases of monoclonal γM-globulins with anti–γ-globulin activity have been described (21–23). Recently, one such case which also was a "mixed" cryoglobulin in a patient with Waldenström's macroglobulinemia was studied in detail and was shown to combine specifically with γG-globulin from human or other primate sera but, as in the present study, not with other mammalian γ-globulins (23). It was also possible to demonstrate that the binding site was located in the Fab-fragment of the γM-molecule. Other antibody specificities that have been ascribed in the past to monoclonal immunoglobulins are: cold agglutinins with specificity for the I red cell antigen (24); an antigen present on aged erythrocytes, but not on fresh ones (25); streptolysin O and staphlysin T (26); α- and β-lipoproteins (27); and dinitrophenyl hapten (28). It is of considerable interest that most of the monoclonal proteins studied with antibody activity have specificity for autoantigens. The significance of this at present is unclear but it may be an important factor in the pathogenesis of certain dysproteinemic states.

The question is often raised whether all or indeed any of the myeloma proteins mentioned above which exhibit affinity for a variety of substances are truly antibodies. In the present study two of the most important criteria for antibodies have been met by the cryoglobulins. First, the degree of specificity with which the cryoglobulins reacted with primate γG-globulin and not with other primate immunoglobulins or mammalian γG-globulins is in keeping with, and is considered strong evidence for the antibody nature of the cryoglobulins. Second, the reactive region on the cryoglobulin molecule has been shown to be the Fab-fragment which also points to the antibody nature of these proteins. A third property which classical antibodies share but which is not shared by these paraproteins because of the very nature of the disease from which they arise, is the inducibility of antibody formation. It is perhaps this last point which has led to much scepticism and caution in designating some paraproteins as antibodies. However, the great similarities these proteins share with antibodies with respect to the nature of their reactivity with a specific ligand and the localization of the reactive site to the same portion of the molecule in which it occurs in induced antibodies, make it appear likely that they are indeed antibodies. As more information becomes available on the nature and further localization of the antibody combining site within the immunoglobulin molecule, it will be possible to test the myeloma "antibodies" by these more refined criteria as well.

ACKNOWLEDGMENTS

We wish to acknowledge the generous gift of serum (Man) we received from Dr. Stanley J. Altman. It is also a pleasure to acknowledge the fine technical assistance of Miss Sharon Ford.

This is Publication No. 264 of the Department of Experimental Pathology, Scripps Clinic and Research Foundation. This investigation was supported by Research Grant AI-07007 and AI-06894 of the U. S. Public Health Service, and in part, by Atomic Energy Commission Contract AT(04-3)-410, and the American Heart Association grant No. 67-795. This study was done during the tenure of an Established Investigatorship of the American Heart Association to one of the authors (H.M.G.).

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