TWO MAJOR TYPES OF NORMAL 7S γ-GLOBULIN*

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The biological and physical heterogeneity of normal human 7S γ-globulin is well established as a result of numerous investigations (1–3). However, since most antisera to highly purified specimens of 7S γ-globulin give a single precipitin line in agar diffusion analysis and one long precipitin line in immunoelectrophoretic experiments, the impression has been gained that this serum fraction is homogeneous antigenically. Certain observations have appeared recently suggesting that this is not entirely true. Evidence for the antigenic heterogeneity of 7S γ-globulin has been obtained through the use of antisera made against serum proteins that cross-react with 7S γ-globulin (4). Double precipitin lines are characteristically observed between such antisera and various preparations of 7S γ-globulin. Additional evidence of antigenic heterogeneity of this immune protein has been furnished by use of *rhesus* monkey antisera to human γ-globulin (5).

Recent studies have demonstrated that two mutually exclusive antigenic determinants occur on γ type multiple myeloma proteins, Bence Jones proteins, β_{2A} type myeloma proteins, and Waldenström type macroglobulins. On the basis of these antigenic determinants all the above mentioned proteins can be categorized into two antigenic groups, termed group 1 and group 2 (6). The ubiquitous distribution of these antigenic determinants among the pathological immune proteins suggested that these determinants might also exist on the molecules of normal 7S γ-globulin. The present study demonstrates that approximately 60 per cent of normal 7S γ-globulin molecules carry the antigenic determinants of group 1 myeloma proteins, and approximately 30 per cent of molecules carry the antigenic determinants of group 2 myeloma proteins. The presence of these two populations of γ-globulin is related to the double precipitin line phenomenon in agar diffusion, described previously (4).

Materials and Methods

1. Gamma Globulin.—A single lot of pooled human Fr II (Lederle Laboratories, Pearl River, New York, lot C-715) was used throughout these studies. At times the Fr II was further purified by zone electrophoresis. Normal γ-globulin from individuals was prepared from freshly

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collected sera by zone electrophoresis on starch medium (7). The isolated γ-globulin was stored in solution in phosphate-buffered normal saline at pH 7.4 at 4°C.

2. Antisera.—Antisera to 7S normal γ-globulin were produced by immunizing rabbits with aggregate-free human Fr II and complete Freund's adjuvant by subcutaneous weekly injections of 2 to 3 mg of Fr II over a 4 month period (rabbits 5-28, 5-29). Antisera to Bence Jones proteins of group 1 and group 2 were produced by immunizing rabbits in a similar manner with the electrophoretically isolated Bence Jones proteins and complete Freund's adjuvant over a 3 month period. The antisera to group 1 Bence Jones proteins (rabbits 5-80, 6-00, 6-45, 6-46) reacted with other Bence Jones proteins from group 1 and not with Bence Jones proteins from group 2. Similarly, antisera to group 2 Bence Jones proteins (rabbits 6-01, 6-18, 6-19, 6-48) reacted with other group 2 proteins and not with group 1 proteins (6). Antisera to Waldenström type macroglobulins were produced by immunizing rabbits (rabbits 3-40, 4-38, 4-39, 4-45) with the isolated macroglobulins and complete Freund's adjuvant. Antisera to β_{2A} type myeloma proteins were produced by immunizing rabbits (rabbit 6-42) with the isolated myeloma proteins and complete Freund's adjuvant.

3. Multiple Myeloma Proteins and Bence Jones Proteins.—Sera were obtained from patients with the diagnosis of multiple myeloma, established by the presence of myeloma protein peaks in the serum along with plasmacytosis of the bone marrow. The multiple myeloma proteins were isolated from other serum components by zone electrophoresis on starch medium (7). Urine was collected from patients with confirmed diagnosis of multiple myeloma, and Bence Jones proteins were isolated as described previously (6).

4. Enzymatic Digestion of γ-globulin.—Human Fr II or the slowly migrating portion of human Fr II, separated by zone electrophoresis on starch, was subjected to digestion under the conditions recommended by Porter (8), using twice recrystallized papain (Worthington Biochemical Corporation, Freehold, New Jersey). The electrophoretically slowly migrating digested fragments (subsequently termed S fragments) and the fast migrating digested fragments (subsequently termed F fragments) were separated by zone electrophoresis on starch medium (7).

5. Gel Diffusion Methods.—(*a*) Ouchterlony plates: Ouchterlony agar diffusion studies (9) were performed in 2.0 per cent agar (agar agar from Baltimore Biological Laboratory, Baltimore) in barbital buffer, pH 8.6, ionic strength 0.05. Each antigen well received 0.25 mg of protein, and the antibody wells were filled with the antisera as indicated. At least 72 hours of incubation at 37°C was allowed prior to the final interpretation of the precipitin lines. (*b*) Immunoelectrophoresis: micromethod of immunoelectrophoresis was carried out as previously described (4).

6. I-131 Labeling of Proteins.—Labeling of γ-globulin preparations with radioactive iodine (carrier-free I-131 was obtained from the Oak Ridge National Laboratory, Oak Ridge) was carried out by the method of Masouredis (10). Radioactive iodine was slowly added to the protein solution with constant mixing in cold room; the mixture was allowed to incubate at 4°C for 30 minutes. The unbound free iodine was removed from the iodinated protein by gel filtration. After incubation the mixture was passed over a sephedex G-25 column (obtained from Pharmacia, Upsala, Sweden; column 50 cm long and 1.2 cm in diameter) and eluted with a phosphate buffer, pH 7.5, ionic strength 0.2. The unbound radioactivity in γ-globulin preparations did not exceed 2 per cent as determined by precipitation with 5 per cent trichloracetic acid. The labeled protein preparations were stored at 4°C for further use.

7. Precipitin Curves.—For construction of precipitin curves the desired aliquot of I-131–labeled antigen was brought to 0.2 ml volume with normal saline, and 0.2 ml of the desired antiserum was added. The mixture was left at room temperature for 1 hour and then stored at 4°C overnight. On the following morning the precipitate was separated by centrifugation at 3000 RPM at 4°C for 15 minutes. The supernatant and the first washing were saved for counting.

In some experiments the first washing was not added to the supernatant for counting since further tests were performed on the supernatants. The precipitates were washed three times with saline and then dissolved in 0.1 N NaOH. The protein determinations were performed by Folin-Ciocalteu method (11). The entire dissolved precipitate was used for counting. The supernatants and dissolved precipitates were counted simultaneously. All counting was performed in the automatic well type scintillation counter (Nuclear of Chicago, Des Plaines, Illinois, model C-120). Precipitin curves were plotted with the increasing amounts of antigen on the abscissa and the amount of precipitated protein on one ordinate and the per cent of precipitated radioactivity on the other ordinate. Prior to use in precipitin studies all antisera were heated at 56°C for 30 minutes to inactivate complement.

RESULTS

A. Double Diffusion Studies in Agar.—In studies published separately (6) it was shown that all pathological immune proteins fall into two categories on the basis of two ubiquitous, mutually exclusive antigenic determinants.

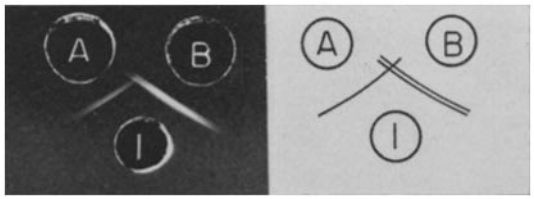

FIG. 1. Ouchterlony plate and line drawing of the same plate, showing reaction of Fr II with antisera to both antigenic groups of Bence Jones proteins. Well *A* contains an antiserum to a group 2 Bence Jones protein, and well *B* contains an antiserum to a group 1 Bence Jones protein. Well 1 contains Fr II. The lines are faint at the crossing but definite spurring occurs in both directions, as shown on the diagram.

Rabbit antisera to Bence Jones proteins of the two groups proved most useful for the identification of these antigenic determinants and for classification of the pathological immune proteins. It was thought that these antisera would be useful to test the possibility of existence of two antigenic groups of molecules in normal 7S γ-globulin. Ouchterlony double diffusion in agar was used to test this hypothesis.

Pooled normal human Fr II and isolated 7S γ-globulin from five individuals were used as antigens against antisera to Bence Jones proteins of group 1 and group 2. Fig. 1 illustrates typical results of these double diffusion experiments in agar. The crossing of precipitin lines formed by the two antisera with Fr II in Ouchterlony plates shows the presence of two non-identical antigenic determinants in Fr II and normal 7S γ-globulin. Furthermore, the complete crossing of lines suggests that the antigenic determinants occur on separate molecules (9). The double precipitin line formed between the Fr II and the antiserum to a group 1 Bence Jones protein is further discussed below.

B. Precipitin Curves with I-131-labeled γ-Globulins and Antisera to Bence

Jones Proteins.—Since the double diffusion studies in agar suggested the existence of two groups of molecules of normal γ-globulin on the basis of antigenic dissimilarity, precipitin studies with I-131–labeled γ-globulin were undertaken.

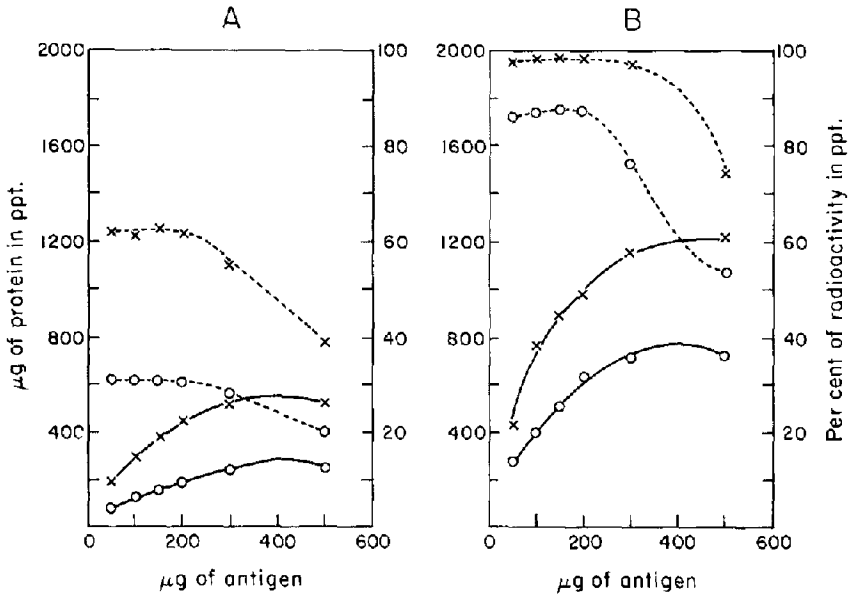

FIG. 2. Precipitin curves with I-131–labeled Fr II and various antisera. In this and all subsequent precipitin curves the broken line indicates per cent of precipitated radioactivity, and the solid line indicates amount of precipitated protein.

A. ×—×—×—× indicates protein and ×---× indicates per cent of radioactivity precipitated by an antiserum to a group 1 Bence Jones protein. ○——○ indicates protein and ○---○ indicates per cent of radioactivity precipitated by an antiserum to a group 2 Bence Jones protein.

B. ×——× indicates protein and × - - × indicates per cent of radioactivity precipitated by an antiserum to normal 7S γ-globulin. ○—— ○ indicates protein and ○---○ indicates per cent of radioactivity precipitated by a mixture of equal aliquots of antisera used in *A* of this figure.

Freshly prepared I-131 labeled human Fr II was used as antigen in constructing precipitin curves. The total amount of precipitated protein was plotted against the increasing amounts of antigen. The amount of radioactivity in the precipitate and in the supernatant was determined for each point of the precipitin curve, and the per cent of radioactivity in the precipitate was calculated and plotted against the increasing amounts of antigen. Typical experiments of this nature are illustrated in Fig. 2. As seen on the right side of Fig. 2, an antiserum to normal human 7S γ-globulin precipitated 98 per cent of the

I-131–labeled Fr II at antibody excess. As expected, in antigen excess the per cent of the precipitated antigen declined sharply. Similar experiments were performed with an antiserum to a group 1 Bence Jones protein. At antibody excess only approximately 60 per cent of the labeled Fr II was precipitated, as indicated on the left half of Fig. 2. Addition of an antiserum to normal human γ-globulin precipitated additional protein, and the total per cent of precipitated radioactivity exceeded 98 per cent. Under the same conditions an antiserum to a group 2 Bence Jones protein precipitated approximately 30 per cent of the labeled Fr II at antibody excess, as shown on the left half of Fig. 2. Addition of an antiserum to normal human γ-globulin precipitated additional protein and the total per cent of precipitated radioactivity exceeded 98 per cent.

Further experiments were performed to determine whether the two antigenic determinants occur on different or the same molecules. If the two antigenic determinants in question occur on different molecules, then the per cent of antigen precipitated simultaneously by a mixture of the two antisera should equal the sum of the percentages of the antigen precipitated by the two antisera individually. On the other hand, if the same molecules carry both antigenic determinants, then the per cent of antigen precipitated by the mixture of the two antisera should be less than the sum of the percentages of antigen precipitated by the two antisera individually. For this reason equal aliquots of antisera to group 1 and group 2 Bence Jones proteins were mixed, and 0.4 ml of the mixture was added to the aliquots of the labeled human Fr II to construct precipitin curves. As seen from the right half of Fig. 2, the mixture of the two antisera precipitated approximatey 85 per cent of the labeled Fr II at antibody excess. On the other hand, the sum of the percentages of Fr II precipitated by the antisera individually exceeded 90 per cent.

Gamma globulin was isolated from five individuals, labeled with I-131, and subjected to similar studies as described above for I-131–labeled Fr II. In all instances approximately 60 per cent of the molecules were precipitated by antisera to group 1 Bence Jones proteins and approximately 30 per cent by antisera to group 2 Bence Jones proteins in antibody excess. Furthermore, approximately 85 per cent of the I-131–labeled γ-globulin preparations were precipitated by the mixture of the two antisera, whereas the sum of the percentages of antigens precipitated by the two antisera individually exceeded 90 per cent. An antiserum to normal 7S γ-globulin precipitated in excess of 98 per cent of the isolated individual γ-globulins.

These observations indicated that pooled human Fr II and individual normal 7S γ-globulins contain approximately 60 per cent of group 1 molecules (molecules with antigenic determinants present in group 1 myeloma proteins) and approximately 30 per cent of group 2 molecules (molecules with antigenic determinants present in group 2 myeloma proteins).

C. Precipitin Curves with I-131–Labeled γ-Globulins and Antisera to Normal 7S γ-Globulin Absorbed with Myeloma Proteins.—In the previous section it was shown that approximately 60 per cent of normal γ-globulin molecules carry the antigenic determinants of group 1 Bence Jones proteins and approximately 30 per cent the determinants of group 2 Bence Jones proteins. Potent antisera to normal human γ-globulin distinguish between these two groups of Bence Jones proteins as well as between the two groups of myeloma proteins (6). If a potent antiserum to normal 7S γ-globulin was absorbed with a group 1 γ type myeloma protein, it reacted only with the group 2 antigenic determinants and other antigenic determinants not present in the group 1 γ type myeloma protein. Similarly, if the same antiserum was absorbed with a group 2 γ type myeloma protein, the remaining antibodies reacted only with group 1 antigenic determinants and other antigenic determinants not present in group 2 γ type myeloma proteins. Antisera absorbed in this manner should then also distinguish the two antigenic groups of normal human γ-globulin molecules.

A potent antiserum to normal human 7S γ-globulin was absorbed precisely at equivalence with a group 2 γ type myeloma protein so that the absorbed antiserum contained no excess antigen or antibodies to this protein. The absorbed antiserum was used to construct precipitin curves with I-131–labeled Fr II and isolated γ-globulin from individuals. Typical experiments are illustrated in Fig. 3. Prior to absorption the antiserum precipitated 98 per cent of the labeled Fr II at antibody excess. However, the same antiserum after absorption with a group 2 γ type myeloma protein precipitated only 72 per cent of the I-131–labeled Fr II. Further addition of the unabsorbed antiserum to normal 7S γ-globulin precipitated additional protein, and the total precipitated radioactivity exceeded 98 per cent. These observations again indicate that approximately 30 per cent of normal γ-globulin molecules carry the determinants of group 2 myeloma proteins. In these experiments the antibodies to group 2 determinants were removed by absorption and therefore the molecules carrying group 2 determinants were not precipitated. Similar results were also obtained with antisera directed to group 1 Waldenström type macroglobulins and with antisera directed to group 1 β_{2A} type multiple myeloma proteins. Contaminating antibodies to 7S γ-globulin in these antisera were absorbed with a group 2 γ type myeloma protein. These absorbed antisera precipitated 70 per cent of the labeled γ-globulin preparations and left 30 per cent of the labeled proteins in the supernatant at antibody excess (12).

In the experiments with absorbed antisera to normal 7S γ-globulin the possibility was considered that the non-precipitated I-131–labeled γ-globulin was due to formation of soluble complexes between the rabbit antibodies and γ-globulin. If soluble complexes are present, radioactivity should be precipitated by addition of a sheep antiserum to rabbit γ-globulin. Five ml of sheep anti–rabbit γ-globulin was added to each supernatant in the precipitin curve

shown in Fig. 3. At antibody excess, where 30 per cent of the I-131–labeled γ-globulin was not precipitated, the addition of 5 ml of sheep anti–rabbit γ-globulin failed to bring down any additional radioactivity. However, at antigen excess, where a large percentage of I-131–labeled Fr II had not precipitated, the addition of 5 ml of sheep anti–rabbit γ-globulin to the supernatants precipitated high percentages of additional radioactivity, indicating the presence of soluble complexes formed between the rabbit anti–human

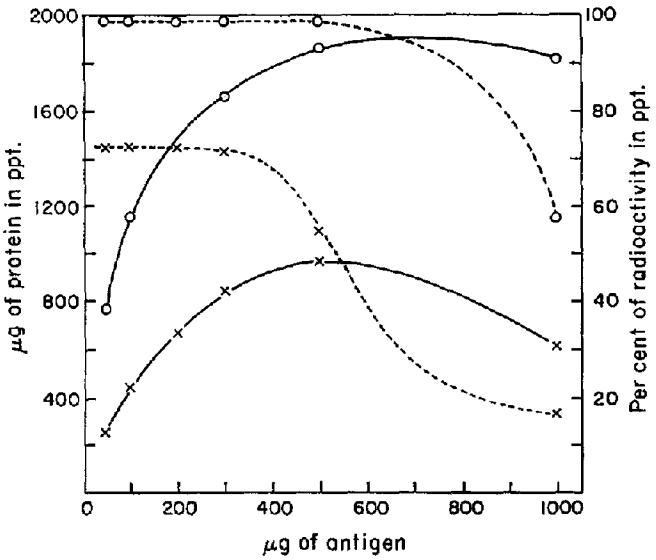

FIG. 3. Precipitin curves with I-131–labeled Fr II and an absorbed antiserum to 7S γ-globulin. O——O indicates protein and O---O indicates per cent of radioactivity precipitated by an unabsorbed antiserum to normal 7S γ-globulin. X——X indicates protein and X---X indicates per cent of radioactivity precipitated by the same antiserum absorbed with a group 2 myeloma protein.

γ-globulin and the I-131–labeled human γ-globulin. From these experiments it is clear that soluble complexes of rabbit antibodies and human Fr II did not account for the unprecipitated I-131–labeled Fr II in antibody excess.

Subsequently antisera to normal 7S γ-globulin were absorbed with group 1 γ type myeloma proteins at equivalence. Such an antiserum should precipitate approximately 30 per cent of the normal I-131–labeled γ-globulin, particularly in view that the antisera to group 2 Bence Jones proteins precipitate 30 per cent of the I-131–labeled γ-globulin. However, in many attempts antisera to 7S γ-globulin absorbed with group 1 γ type myeloma proteins failed to give a 30 per cent plateau of precipitated normal γ-globulin at antibody excess. The amount of protein precipitated in these experiments was small and good

quantitative precipitin curves were not obtained. Nevertheless, qualitative experiments with double diffusion in agar indicated that the antisera to normal γ-globulin absorbed with group 1 γ type myeloma proteins reacted with group 2 γ type myeloma proteins (6), with normal human Fr II and with isolated γ-globulins from individuals. The implications of these observations are considered in the discussion of the results below.

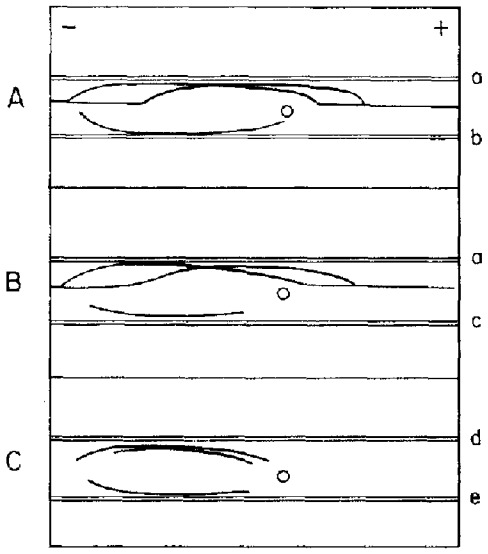

FIG. 4. Semidiagrammatic tracings of immunoelectrophoretic experiments localizing the group-specific antigenic determinants on the S fragments of 7S γ-globulin. All wells contain papain-digested human Fr II. Troughs *a* contain unabsorbed antiserum to normal 7S γ-globulin; trough *b* contains the same antiserum absorbed with a group 1 γ type myeloma protein; trough *c* contains the same antiserum absorbed with a group 2 γ type myeloma protein; trough *d* contains an antiserum to a group 1 Bence Jones protein; and trough *e* contains an antiserum to a group 2 Bence Jones protein. The S fragments migrate towards the cathode.

An antiserum to normal 7S γ-globulin was absorbed at equivalence with a group 1 and with a group 2 γ type myeloma protein. This absorbed antiserum still reacted with the S fragments of normal Fr II, but very faintly.

D. Localization of the Group-Specific Antigenic Determinants on the S Fragments of Normal γ-Globulin.—In a previous publication evidence was provided that the group-specific antigenic determinants of γ type myeloma proteins are localized on the S fragments of the papain-digested myeloma proteins (6). These observations suggest that the group-specific antigenic determinants of the normal 7S γ-globulin molecules also occur on the S fragments of this protein.

Antisera to normal 7S γ-globulin were absorbed individually with a group 1 γ type myeloma protein and with a group 2 γ type myeloma protein. These

absorbed antisera were reacted with papain-digested human Fr II in immunoelectrophoresis, as shown by a typical experiment in Fig. 4. It is evident that the absorbed antisera react only with the S fragments of γ-globulin. Similarly, as seen in Fig. 4, the antisera to group 1 and group 2 Bence Jones proteins react only with the S fragments of normal human γ-globulin.

In connection with localizing the group-specific antigenic determinants on the S fragments of normal γ-globulin, the question was raised, do all molecules

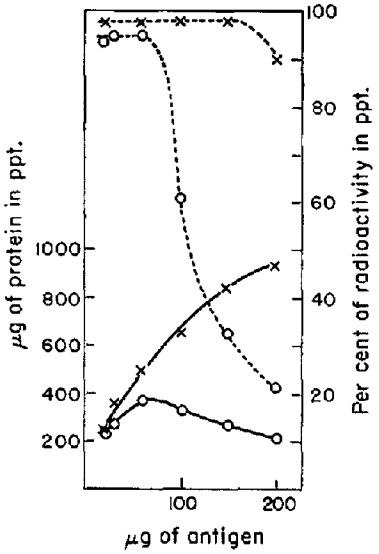

FIG. 5. Precipitin curves with I-131–labeled Fr II and antisera specific to S fragments and F fragments of normal Fr II showing complete precipitation of Fr II at antibody excess. ×——× indicates protein and ×---× indicates per cent of radioactivity precipitated by an antiserum to normal 7S γ-globulin absorbed at antigen excess with F fragments of human Fr II (in effect an antiserum to S fragments). O——O indicates protein and O---O indicates per cent of radioactivity precipitated by an antiserum to normal 7S γ-globulin absorbed at antigen excess with S fragments of human Fr II (in effect an antiserum to F fragments).

of normal γ-globulin possess S fragments and F fragments? If all molecules of γ-globulin possess S fragments, then an antiserum to normal 7S γ-globulin absorbed with F fragments will precipitate 100 per cent of the whole molecules. Similarly, if all normal γ-globulin molecules possess the F fragments, an antiserum to 7S γ-globulin absorbed with S fragments will still precipitate 100 per cent of whole γ-globulin molecules. Such experiments were performed and are illustrated in Fig. 5. An antiserum to normal 7S γ-globulin was absorbed at antigen excess with isolated F fragments of human Fr II, thus leaving an antiserum specific to S fragments. This antiserum was used to construct precipitin curves with I-131–labeled Fr II. As seen from Fig. 5, at antibody excess

the antiserum to S fragments precipitated 98 per cent of the I-131–labeled Fr II. Similarly, the same antiserum was absorbed at antigen excess with isolated S fragments of human Fr II, leaving an antiserum specific to the F fragments. This absorbed antiserum was used to construct precipitin curves with I-131–labeled Fr II. As seen from Fig. 5, at antibody excess, the antiserum to F fragments precipitated 95 per cent of the I-131–labeled Fr II.

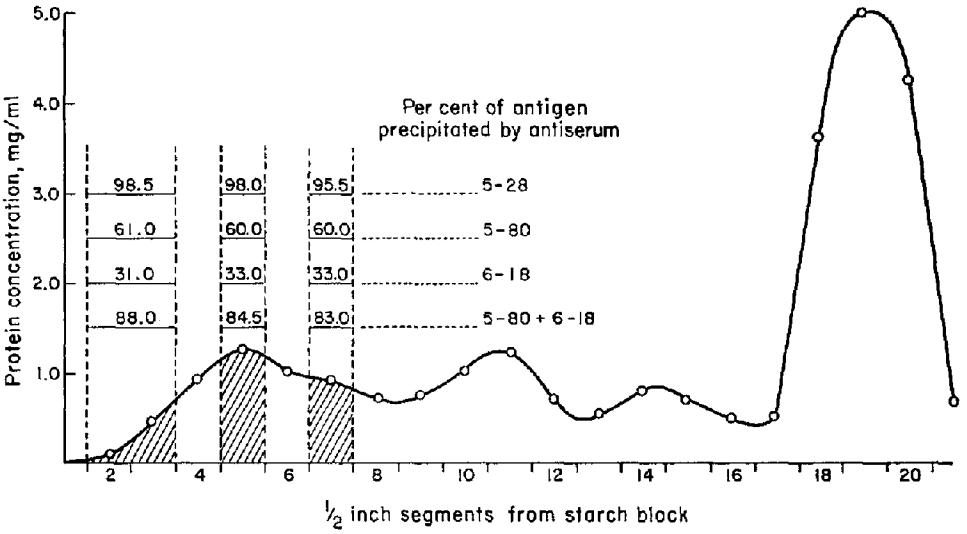

FIG. 6. Percentages of group 1 and group 2 molecules in different electrophoretic fractions of normal γ-globulin. Serum of a normal individual was subjected to zone electrophoresis on starch medium, the solid line indicates the concentration of protein eluted from ½ inch segments of the starch block. Segments 7, 5, and combined segments 1 and 2 were labeled with I-131. The labeled proteins were used as antigens against the various antisera indicated. All percentages of the precipitated γ-globulin are from the antibody excess region of the respective precipitin curves. Rabbit antiserum 5-28 is directed to normal 7S γ-globulin, rabbit antiserum 5-80 to a group 1 Bence Jones protein, and rabbit antiserum 6-18 to a group 2 Bence Jones protein.

E. Distribution of Antigenic Determinants over the Electrophoretic Range of Normal 7S γ-Globulin.—Immunoelectrophoretic studies of the antigenic heterogeneity of 7S γ-globulin show that the double precipitin line extends over the entire electrophoretic distribution of 7S γ-globulin (4), suggesting that the two antigenic groups of molecules exist throughout the entire electrophoretic distribution of 7S γ-globulin. To confirm this impression, individual fractions of γ-globulin with differing electrophoretic mobility were isolated, labeled with I-131, and studied for the presence of the two group-specific antigenic determinants.

Serum from two individuals was subjected to zone electrophoresis on starch

medium. Fractions of γ-globulin with slow, medium, and fast electrophoretic mobility were individually labeled with I-131 as illustrated in Fig. 6. Each labeled protein fraction was used as antigen in constructing precipitin curves with the antisera to group 1 and to group 2 Bence Jones proteins and a mixture of the two antisera. In all fractions the antiserum to a group 1 Bence Jones protein precipitated approximately 60 per cent of the I-131–labeled γ-globulin, and an antiserum to a group 2 Bence Jones protein precipitated approximately 30 per cent of the I-131–labeled γ-globulin, whereas the mixture of the two antisera precipitated approximately 85 per cent of the γ-globulin fractions.

F. Relationship of the Group 1 and Group 2 Molecules of Normal γ-Globulin to the Double Precipitin Line Phenomenon on Immunoelectrophoresis.—In a previous study it was shown that certain antisera to multiple myeloma proteins and to Waldenström type macroglobulins gave distinct double precipitin lines with normal 7S γ-globulin (4). In the same study the precipitin line closer to the antibody trough was related to antibodies to the F fragments of normal γ-globulin, and the precipitin line closer to the antigen well was related to antibodies to the S fragments of normal γ-globulin.

The experiments described by Edelman and collaborators were repeated with an antiserum to a group 1 Waldenström type macroglobulin. On modified immunoelectrophoresis, as seen in Fig. 7, double precipitin line formation was demonstrated with pooled human Fr II. The precipitin line closer to the antibody trough fused with the precipitin line of the F fragments, and the line closer to the antigen well fused with the precipitin line of the S fragments of papain-digested human γ-globulin (see Fig. 7 *A*). In additional experiments the papain-digested γ-globulin was replaced by a mixture of a group 1 and a group 2 γ type myeloma proteins. Under these conditions the precipitin line closer to the antibody trough fused with the line of the group 2 myeloma protein, and the line closer to the antigen well fused with the line of the group 1 myeloma protein (see Fig. 7 *B*). Subsequently, the same antiserum was exhaustively absorbed with the F fragments of human Fr II. This absorbed antiserum gave only a single precipitin line with human Fr II and reacted only with the S fragments of papain-digested γ-globulin (see Fig. 7 *C*), and did not react with group 2 γ type myeloma proteins.

The experiments described under section *D* above showed that all γ-globulin molecules possess F fragments and S fragments. The double precipitin line formation with the above described antiserum to a group 1 Waldenström type macroglobulin can be explained by the presence of antibodies to the F fragments and to the group 1–specific antigenic determinants and the presence of two distinct antigenic groups of γ-globulin molecules, 60 per cent group 1 molecules and 30 per cent group 2 molecules. After electrophoresis the γ-globulin molecules and antibody molecules diffuse; group 1 molecules diffuse faster due to their higher concentration, and they meet the antibodies to group

1 antigenic determinants, the antibodies present in high concentration in the antiserum under study, resulting in the formation of the first precipitin line. The group 2 molecules of γ-globulin continue to diffuse and penetrate the first precipitin line since this was formed by group 1–specific antibodies. Eventually

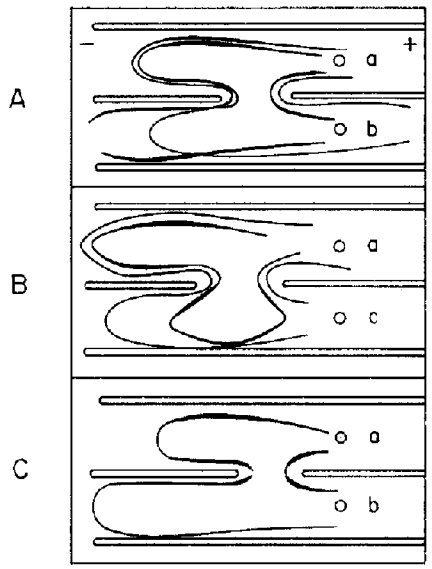

FIG. 7. Semidiagrammatic tracings of immunoelectrophoretic experiments, illustrating the relationship of double precipitin lines to the two groups of molecules of 7S γ-globulin.

A. Well *a* contains Fr II and well *b* papain-digested Fr II. The antibody troughs contain a typical antiserum giving double precipitin lines. The outer precipitin line fuses with the precipitin line of F fragments, and the inner precipitin line fuses with the precipitin line of S fragments.

B. The same system is used as in *A* except that well *c* contains a mixture of a group 1 and a group 2 γ type myeloma proteins. The outer precipitin line fuses with the precipitin line of group 2 myeloma protein, and the inner precipitin line fuses with the precipitin line of group 1 myeloma protein.

C. The same system is used as in *A*, except the antiserum was absorbed with F fragments of Fr II. Only the inner precipitin line is present, and it fuses with the precipitin line of S fragments.

the group 2 molecules meet with the antibodies to F fragments and the second precipitin line forms. The removal of the second precipitin line by absorption of the antiserum with F fragments, the fusion of the second precipitin line with the precipitin line of the F fragments, and the fusion of the same line with a group 2 myeloma protein precipitin line support the above explanation of the formation of double precipitin lines and relates this to the presence of the two antigenic groups of γ-globulin molecules. The location of the lines greatly

depends on the relative concentration of antibodies to each antigenic determinant. If an overabundance of antibodies to F fragments is present, double precipitin line formation will not occur. The molecules with the group-specific antigenic determinants will not penetrate the precipitin line formed by the antibodies to the F fragments and the first molecules of γ-globulin to reach them, since all 7S γ-globulin molecules carry the F fragments.

The double precipitin line formation described above could also be produced by mixing specific antisera to group 1 and group 2 antigenic determinants. If an antiserum to a group 1 Bence Jones protein was mixed with an antiserum to a group 2 Bence Jones protein, a double precipitin line was formed with normal γ-globulin. At the same time single precipitin lines were formed by the antisera individually. Of particular interest for further studies was the observation that some antisera to group 1 Bence Jones proteins, obtained after 6 months of immunization, produced double precipitin lines with 7S γ-globulin, but still failed to react with group 2 myeloma proteins and with the F fragments of human Fr II. This suggests that further antigenic and molecular heterogeneity exists among the group 1 molecules of normal γ-globulin.

DISCUSSION

Previous studies with antisera to multiple myeloma proteins and Waldenström type macroglobulins have suggested that two or even perhaps three populations of normal 7S γ-globulin (at times termed γ_2-globulin) molecules exist (4, 12–14). The present study shows with quantitative precipitin curves that indeed at least two, and possibly three, such populations can be identified and quantitated. The grouping of normal 7S γ-globulin into two populations of molecules is based on the group-specific antigenic determinants found in γ type myeloma proteins, β_{2A} type myeloma proteins, Bence Jones proteins, and Waldenström type macroglobulins described in a previous publication (6). On the basis of these ubiquitous antigenic determinants the pathological immune proteins fall into two groups, termed group 1 and group 2. In this study evidence is given that approximately 60 per cent of 7S γ-globulin from pooled specimens as well as from normal individuals possess the specific group 1 antigenic determinants of the above mentioned pathological immune proteins. For the sake of simplicity it is suggested that this group of molecules be termed group 1 molecules of normal 7S γ-globulin. Further, evidence is provided that approximately 30 per cent of 7S γ-globulin molecules from pooled specimens as well as from normal individuals possess the specific group 2 antigenic determinants of the above mentioned pathological immune proteins. For the sake of simplicity it is suggested that this group of molecules be termed group 2 molecules of normal 7S γ-globulin.

Antisera to group 1 and group 2 Bence Jones proteins proved to be most useful in this investigation for precipitation of group 1 and group 2 normal

γ-globulin molecules. Precipitation of the two groups of molecules individually resulted in total precipitation of above 90 per cent of normal γ-globulin. On the other hand, if equal aliquots of both antisera were mixed, only 85 per cent of normal γ-globulin was precipitated at antibody excess. This discrepancy in the percentages of precipitated γ-globulin could in part be due to experimental error, but an alternative explanation might be that a few molecules of γ-globulin possess both group 1 and group 2 antigenic determinants. The observation that not 100 per cent of normal γ-globulin was precipitated by both antisera individually or in mixture, indicates that a third antigenic group of 7S γ-globulin molecules exists, not related to group 1 or group 2 molecules. This group would at best constitute 10 to 15 per cent of normal γ-globulin. The existence of a third group of 7S γ-globulin molecules is further supported by the observation that an antiserum to 7S γ-globulin absorbed with group 1 and group 2 γ-type myeloma proteins still reacts faintly with normal γ-globulin.

The presence of the two antigenic groups of normal γ-globulin also was demonstrated by absorption of antisera to normal human γ-globulin with group 1 and group 2 γ type myeloma proteins. An antiserum to normal γ-globulin after absorption with a group 2 γ type myeloma protein precipitated 70 per cent of the I-131–labeled Fr II. The precipitated 70 per cent consists of the 60 per cent of group 1 molecules precipitated by antisera to group 1 Bence Jones proteins and the 10 per cent belonging to the possible third antigenic group. By similar reasoning an antiserum to normal γ-globulin absorbed with a group 1 γ type myeloma protein should precipitate 40 per cent of I-131–labeled Fr II, the 40 per cent consisting of the 30 per cent of group 2 molecules precipitated by antisera to group 2 Bence Jones proteins and the 10 per cent belonging to the possible third antigenic group of γ-globulin molecules. Such quantitative results, however, could not be obtained in spite of repeated absorption of several antisera to normal γ-globulin with several group 1 myeloma proteins. These experiments are technically difficult because the antisera to normal γ-globulin contain a relatively small number of antibodies to group 2 antigenic determinants. On the other hand, it is significant that qualitatively immunoelectrophoretic experiments and Ouchterlony double diffusion experiments invariably showed that antisera to normal γ-globulin absorbed with group 1 γ type myeloma proteins react with group 2 myeloma proteins and with the S fragments of normal 7S γ-globulin.

Studies of the electrophoretic mobility of a large number of γ type myeloma proteins has resulted in a frequency distribution histogram of these proteins, plotted according to their electrophoretic mobility, that resembles the normal electrophoretic distribution of γ-globulin (15, 16); *i.e.*, relatively small proportion of γ-globulin with extremely fast and extremely slow mobility and a relatively large proportion of γ-globulin with intermediate mobility. These observations have been construed as support to the hypothesis that only one clone of γ-globulin producing plasma cells has undergone malignant alterations

in multiple myeloma, producing then the myeloma protein with mobility specific for the globulin from that clone of plasma cells. If this hypothesis is true, then statistically about 30 per cent of γ type myeloma proteins should be of group 2 and 60 per cent of group 1, since these are the percentages of group 1 and group 2 molecules in normal 7S γ-globulin. The grouping of 134 γ type myeloma proteins disclosed 27.6 per cent group 2 myeloma proteins and 72.4 per cent group 1 myeloma proteins (17). The latter group may also contain the possible third group of myeloma proteins (6). Thus the ratio of the two antigenic types of molecules in normal γ-globulin closely resembles the ratio of the incidence of the two antigenic groups of γ type multiple myeloma proteins.

Immunoelectrophoretic experiments with antisera to group 1 and group 2 Bence Jones proteins, and the studies with antisera absorbed with myeloma proteins demonstrated that the antigenic determinants, permitting the classification of normal 7S γ-globulin into two antigenic groups, are located on the S fragments of papain-digested γ-globulin. This is in good agreement with the previous observations that the group-specific antigenic determinants of γ type myeloma proteins occur on the S fragments (6).

The present investigations demonstrate that electrophoretic fractions of normal γ-globulin with slow, medium, and fast electrophoretic mobility contain the same percentages of group 1 and group 2 molecules as the electrophoretically unseparated γ-globulin. This was suggested already by the observation that the double precipitin lines occur in similar electrophoretic fractions of γ-globulin (4, 13). The double precipitin lines were noted with euglobulin, pseudoglobulin, 7S γ-globulin prepared by density gradient ultracentrifugation, and zinc precipitation of γ-globulin (4), suggesting that these methods will not yield separation of the two antigenic groups of normal γ-globulin.

The double precipitin line of normal γ-globulin on immunoelectrophoresis is related to the two antigenic groups of γ-globulin molecules. The occasional observation of three parallel precipitin lines (14) could be explained by the presence of a third antigenic group of γ-globulin. The fundamental factors in the formation of double precipitin lines are the antigenically different molecules of γ-globulin diffusing independently and the presence of antibodies to the different antigenic determinants. In the presence of overabundance of antibodies to the F fragments, the fragments common to all molecules, γ-globulin molecules with different antigenic determinants are precipitated as a single line. The two precipitin lines may form under the following conditions: (*a*) antibodies exist only to group 1 and group 2 molecules of γ-globulin; (*b*) the majority of antibodies are directed to group 1 molecules and only very few antibodies are directed to F fragments; (*c*) the majority of antibodies are directed to group 2 molecules and only few to the F fragments. Additional possibilities exist for double precipitin line formation since certain antisera to group 1 Bence Jones proteins give double precipitin lines.

Antisera to normal human γ-globulin, produced in *rhesus* monkeys, have been

reported to show three antigenic determinants of normal γ-globulin with somewhat different electrophoretic mobility (5). These observations are difficult to correlate with the present findings since the group 1 and group 2 molecules both had the same broad distribution electrophoretically.

The present investigations have been limited to the normal 7S γ-globulin. It is speculated that the same two antigenic groups also exist among the normal β_{2A} proteins and the normal macroglobulins. Experiments are in progress to determine the percentage of the two antigenic groups among the latter two normal proteins. Previous studies have shown that two antigenic groups exist among the low molecular weight γ-globulin molecules in urine of normal individuals (18). No experiments have been conducted to establish antigenic groups of γ-globulin in other species. However, the observation of double precipitin line with mouse γ-globulin on immunoelectrophoresis (19) suggests that at least two antigenic groups of γ-globulin are present in mice.

During this investigation the possible relationship of the group-specific antigenic determinants to the Gm and Inv genetic factors of γ-globulin were considered. The Gm characters occur only on the F fragments of normal 7S γ-globulin and γ type multiple myeloma proteins (20–24); therefore, these factors appear not to be related to the group-specific antigenic determinants of γ-globulin. The Inv factors on the other hand, occur on the S fragments of 7S γ-globulin and also on some of the β_{2A} myeloma proteins, Bence Jones proteins, and Waldenström type macroglobulins (20–24), and might well be related to the group-specific antigens. Observations on the group 1 and group 2 myeloma proteins indicated that both types possessed the Inv factors. However, the rather surprising observation was made that only group 1 Bence Jones proteins carried the Inv trait (24).

At present the reasons for the existence of these two fundamental antigenic groups of normal 7S γ-globulin and other immune proteins are not apparent. Preliminary work has disclosed specific human antibodies in both group 1 and group 2 molecules of 7S γ-globulin. Work is in progress to group a variety of human antibodies and to attempt to elucidate the significance of this grouping of immune proteins in relation to problems of antibody formation.

SUMMARY

Normal 7S human γ-globulin was found to contain two fundamental antigenic groups of molecules. The group 1 molecules of normal γ-globulin correspond antigenically to group 1 multiple myeloma proteins and Bence Jones proteins; and group 2 molecules of normal γ-globulin correspond antigenically to group 2 multiple myeloma proteins and Bence-Jones proteins. Among pooled human Fr II and several individual γ-globulin preparations, approximately 60 per cent of molecules belong to group 1 and approximately 30 per cent of molecules to group 2 in this classification. The possible existence of a third

minor antigenic group, constituting about 10 per cent, is discussed. Antisera to Bence Jones proteins of antigenic group 1 and group 2, in conjunction with I-131–labeled 7S γ-globulin proved to be the most useful system for defining the antigenic groups of normal γ-globulin. The group-specific antigenic determinants of normal 7S γ-globulin molecules were located on the S fragments of these proteins.

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