MACROGLOBULINEMIA

I. The Antigenic Relationship of Pathological Macroglobulins to Normal γ-Globulins*, ‡

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Plate 39

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In 1944 Waldenström (1) described a clinical syndrome characterized by a marked increase of macroglobulins in the blood, with sedimentation constants of 19S and greater. Since this first description an increasing number of patients with macroglobulinemia has been reported, and extensive reviews dealing with the literature of the clinical and physico-chemical aspects of this disease have appeared during the last few years (2–4). Because the antigenic nature of the pathological macroglobulins has not been studied too extensively, we decided to investigate ten of these proteins with the Ouchterlony gel diffusion technique and to compare them with each other and with the normal γ-globulins.

Materials and Methods[1]

Macroglobulins (MG) and Macrocryoglobulins (MCG)[2].—

MCG I.—Serum I (patient A.P.) was placed in the cold room (5°C.) and the resulting precipitate, which contained the macrocryoglobulin, was separated by centrifugation, redissolved in saline (37°C.) and precipitated as before. After a third precipitation, it remained soluble in cold saline but came out of solution on dialysis against distilled water. This euglobulin was redissolved in saline and found to be insoluble after the addition of a few drops of veronal buffer, pH 8.6. It was further purified by two such precipitations and resolution in

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[1] The following samples were kindly supplied by: Serum I (A.P.) Dr. E. L. Crumpacker; urine (A.P.) Dr. E. L. Crumpacker; Serum II (I.R.) Dr. S. Ginsburg; Serums III (B) and V (S.W.) Dr. Ralph Engle; Serums VIII (C.S.) and X (F.S.) Dr. J. H. Neiman.

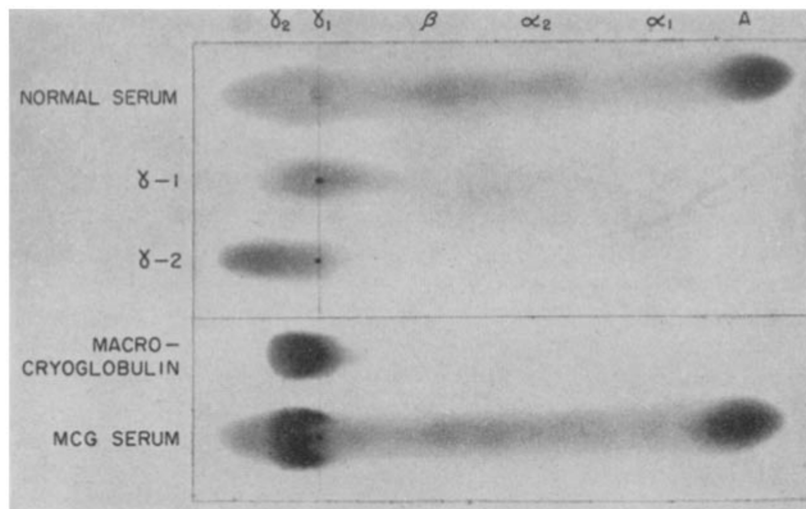
[2] The following abbreviation will be used: MG, macroglobulin; MCG, macrocryoglobulin. The designation γ_1-globulin is used in preference to β_2-globulin because the 7S component of γ_1-globulin is antigenically identical with the 7S component of γ_2-globulin, and the 19S globulin is related to it. (See following.)

saline. The final product was lyophilized. Its electrophoretic mobility was that of a γ_1-globulin (Text-fig. 1).

Macrocryoglobulins II(IR), III(B), and VIII(CS) were purified by precipitation in the cold: MCG II was precipitated four times; MCG III and MCG VIII, three times. These proteins remained insoluble in cold saline and were preserved in the deep freeze.

MCG IV was given to us by Dr. V. N. Tompkins.

MG V (S.W.) was purified by zone electrophoresis on starch followed by dialysis against phosphate buffer, pH 6.0, $\Gamma/_2 = 0.025$. The jelly-like precipitate was dissolved in saline and frozen. It had the same mobility as MCG I. Lyophilized preparations of MG VI and VII were kindly supplied by Dr. F. W. Putnam; (see reference 5 for the electrophoretic mobility as determined by moving boundary electrophoresis and clinical data). MG IX(C) and the



TEXT-FIG. 1. Paper electrophoresis of normal serum, γ_2-globulin, γ_1-1 globulin, Serum I and MCG I. (Phosphate buffer; pH 7.6 $\Gamma/2 = 0.1$)

serum from which it was isolated by zone electrophoresis on starch were kindly supplied by Dr. H. G. Kunkel.

MG X(F.S.) was not isolated.

The physico-chemical properties of most of these proteins are listed in Table I.

Bence Jones Protein.—A sample of urine from patient A.P. gave a positive heat test for Bence Jones proteins. The urine was dialyzed against distilled water, lyophilized, and the Bence Jones protein was isolated by zone electrophoresis on starch (6). It had the same mobility as the macroglobulin of A.P. (MCG I), but ultracentrifugal analysis proved it to be a Bence Jones protein with sedimentation constant (s_{20w}) of 3.66S.

Multiple Myeloma Cryoglobulins.—The cryoglobulins MM XVI and MM XVII, from patients with multiple myeloma have been described (7, 8).

Normal Human γ-Globulins.—Pooled human serum was fractionated by zone electrophoresis on starch and the γ_2- and γ_1-globulins were checked by paper electrophoresis and in the ultracentrifuge. The γ_2-globulin contained the 7S component primarily and only traces of the 19S globulin; γ_1-globulin contained considerable amounts of the 19S globulin as well as

the 7S component. Similar preparations have recently been described in detail by others (9, 10).

A sample of normal γ_1-macroglobulin prepared by repeated ultracentrifugation of the γ-globulin fraction (10) was kindly supplied by Dr. H. G. Kunkel. This preparation contained approximately 90 per cent macroglobulin and 10 per cent of the 7S component.

Antisera.—The immunization procedure has been described previously (8). Rabbits were injected with human γ-globulin (Armour) or with γ_2-globulin prepared by fractionating fraction II (Squibb)[3] by zone electrophoresis. The immunizing antigen was incorporated in Freund adjuvant; each rabbit received 50 to 100 mg. protein per injection. Anti-γ-globulin serum C has been described (11); anti-γ-globulin serum D was prepared in the same manner as anti-γ-globulin B (6).

TABLE I

Ultracentrifugal and Electrophoretic Data for the Pathological Macroglobulins

Macroglobulin	Relative per cent*		Mobility
	19S	26S	(Paper electrophoresis, phosphate buffer; pH 7.6 $\Gamma/2$ =0.1)
MCG I (A.P.)‡	54	27	γ_1
MCG II (I.R.)	82	12	γ_1
MCG III (B)§			γ_1
MCG IV§			γ_2
MG V (S.W.)	80	10	γ_1
MG VI (K)‖	70	15	slow γ_2
MG VII			γ_1
MCG VIII	89	9	γ_2
MG IX¶	76	13	slow α-2
MG X§			γ_1

* Most preparations also contained small amounts of components with sedimentation constants of 7 and > 30S.

‡ Ultracentrifugal data and sedimentation records supplied by Dr. Mary L. Petermann and Mrs. Mary G. Hamilton.

§ Ultracentrifugal data not available, but these preparations are macroglobulins as determined by immunological analysis.

‖ See reference 5 for additional data.

¶ Ultracentrifugal data supplied by Dr. H. G. Kunkel.

Anti-γ_2-globulin E was the antiserum from a single rabbit injected twice at weekly intervals with γ_2-globulin.

Antisera C and D were absorbed with human fraction V (Squibb)[3] and a β-globulin fraction prepared by zone electrophoresis. Anti-serum E was absorbed with the β-globulin fraction only, because it did not react with fraction V. In a few experiments the antisera were also absorbed with lyophilized γ_2-globulin or macroglobulin I.

Immunological Analysis.—The gel diffusion technique of Ouchterlony (12) was used as described (13).

[3] Human fractions II and V were obtained through the courtesy of J. N. Ashworth of the American National Red Cross and E. R. Squibb & Sons.

RESULTS

From Table I it can be seen that three of the ten pathological macroglobulins have electrophoretic mobilities of γ_2-globulin; one moved as a slow α_2-globulin[4] and the other have the same mobility as MCG I (Text-fig. 1). The purified proteins were heterogeneous in the ultracentrifuge; the major components had sedimentation constants of 19S and 26S; the minor components were 7S (usually present only in minute amounts) and 35S. The sedimentation patterns of these proteins are similar to those that have appeared in the literature (3, 14); that of MG VII has been reproduced in reference 5. (The concentration of the 19S component in the patient's serum was as low as 0.8 gm. per cent (for serum III) and as high as 3.9 gm. per cent (for serum V). The 19S component of normal serum is 0.15 gm. per cent or less than 2 per cent (3, 14) of the total protein and this includes the antigenically unrelated 19S component of α_2-globulins.

The macroglobulin of normal human gamma globulin is a potent immunizing antigen, even when present in such small amounts as in fraction II (15). Many of the rabbits immunized with either fraction II or γ_2-fraction obtained from it by zone electrophoresis responded with the production of high titered anti-macroglobulin antibodies (Figs. 1, 3, 5). Similar findings have been obtained by others (15). That these antibodies were indeed against the macroglobulin is apparent from the following considerations:—

1. The convex curvature of the precipitin line with respect to the antibody reservoir is characteristic for an antigen that is considerably larger, and diffuses slower, than antibody (16),
2. Highly purified macroglobulins form a single line, which coalesces with the line formed by the macroglobulin of normal serum (Figs. 1, 7),
3. A preparation containing 90 per cent normal macroglobulin reacted in a similar manner (Fig. 4).

The 19S component of normal serum γ_1-globulin is not an artifact because the same antigenic unit is present in unfractionated serum (Fig. 1).

All antisera against normal γ-globulin preparations contained antibody against the 7S component of both γ_2- and γ_1-globulin. It is of some interest that these antisera do not differentiate between these two 7S globulins (Fig. 1) which differ so markedly in electrophoretic mobility (Text-fig. 1). The lines formed by these proteins as well as the 7S globulin of unfractionated serum coalesce, indicating that our antisera lack antibody that is unique for any single member of the 7S family of globulins. The failure of the antisera to distinguish between 7S components of normal serum is in contrast to their capacity to distinguish between normal γ_2-globulin (7S) and the abnormal globulins found

[4] This slow α_2-globulin should not be confused with the normal α_2-globulin (19S), which is antigenically unrelated to the macroglobulins encountered in macroglobulinemia.

in the blood of patients with multiple myeloma (6, 8, 11). For example, the cryoglobulins obtained from such patients form precipitin lines that coalesce partially with the line formed by γ_2-globulin (Fig. 2) resulting in the formation of spurs, which are characteristic for cross-reacting systems (11).

The purified macroglobulins contain relatively little of the 7S component and at concentrations of 3 mg./ml. they produce one line only (Figs. 1, 3, 4, 5). It was therefore of some interest to determine whether the macroglobulins have any antigenic groupings in common with the 7S globulin. From Fig. 3 it can be seen that the lines formed by the macroglobulins are very dense in the center, and that this density diminishes suddenly beyond the point of intersection with the 7S line. This suggests that some antibodies capable of combining with the macroglobulins are prevented from diffusing through the precipitate formed by the γ_2-globulin. From previous experience with cross-reacting systems (11) it can be assumed that these antibodies react with groupings both proteins have in common.

When normal and pathological macroglobulins were compared, it became apparent that the latter are antigenically deficient; *i.e.*, spurs are formed between the lines produced by the normal 19S globulin and the patient's proteins (Fig. 4). Furthermore, marked differences were found between the macroglobulins from different patients; single spurs and double spurs, previously observed with multiple myeloma proteins (8, 11), were encountered regularly (Figs. 5, 6, 8). That these differences cannot be attributed to the methods of isolation is apparent from Fig. 6. Both the isolated MG IX (cup B) and this macroglobulin in the serum (cup F) have the same antigenic properties; neither is distinguishable from MCG I (cup A). However, these proteins are antigenically more deficient than MCG VIII (cup C) or MG X which had not been isolated (cup E) (The second line, which is nearest to the antigen reservoir, is formed by the small amounts of 7S globulin of this serum.)

When antisera were absorbed with MCG I they still retained their reactivity with the other macroglobuins and the 7S globulin.

It should be noticed that cryoglobulins (7S) obtained from patients with multiple myeloma are antigenically distinct from the macrocryoglobulins (Fig. 2).

The data presented so far were obtained with antisera that contained antibodies against both the 7S and 19S components of γ-globulin. When such antisera are absorbed with γ_2-globulin they form one line only, namely that with macroglobulin of the γ_1-fraction or normal plasma (Figs. 7 and 8). After this absorption, however, the lines of all pathological and normal macroglobulins coalesce completely indicating that the 7S globulin also removed antibody against groupings on the macroglobulin molecule. Consequently, the groupings responsible for the antigenic differences among the macroglobulins must be groupings they have in common with γ_2-globulins (7S) and this is ad-

ditional evidence that γ_2-globulins (7S) and macroglobulins are immunologically related.

The cross-reactivity between γ_2-globulin and macroglobulin is basically different from that of γ_2-globulin with multiple myeloma proteins. In the latter instance, antibodies against γ_2-globulin react with groupings the multiple myeloma proteins and the normal γ_2-globulin have in common; spurs are formed because some of the antibodies cannot react with the deficient myeloma proteins (11). The cross-reactivity between 7S and 19S globulins, however, depends on antibodies formed in response to the injection of both proteins; some of these antibodies can combine with groupings that these proteins have in common, and the other antibodies are specific for the 7S or 19S components. Immunization with 100 per cent pure 7S globulin may result in antisera capable of precipitating 100 per cent pure macroglobulin, because antibody can be formed against those groupings that the two antigens have in common; absorption with γ_2-globulin should remove all antibodies. In the second paper of this series (17) it will be shown that antisera against highly purified macroglobulin cross-react with the 7S globulin in this manner.

The Bence Jones protein isolated from the urine of A.P. was immunologically identical with the Bence Jones proteins of group A described previously (6). When it was compared with both γ_2-globulin and MCG I the precipitin pattern showed that it had antigenic groupings in common with both globulins (Fig. 9). It may be of some interest to compare this shadowgraph with a similar picture (Fig. 2, reference 16) in which human serum albumin was substituted for the Bence Jones protein and the antiserum had not been absorbed with fraction V. Whereas the lines formed by the albumin intersect with those of the globulins, the lines formed by Bence Jones protein coalesce partially. Since the curvature of the lines formed by the albumin (16) is similar to that of the light Bence Jones proteins this partial coalescence must be a function of the cross-reactivity between Bence Jones protein and the globulins rather than an artifact caused by the curvature of the line towards the antibody reservoir. When the anti-γ-globulin serum was absorbed with γ_2-globulin it no longer reacted with the Bence Jones protein, which indicates that γ_2-globulin, Bence Jones protein and macroglobulin have a few groupings in common.

DISCUSSION

The study of the antigenic nature of γ-globulin has been complicated by its heterogeneity. The presence of two antigenically distinct components, which cannot be completely separated from each other, has been a source of confusion, especially since either protein, if present in trace amounts as a contaminant of the immunizing material, may induce the formation of considerable amounts of anti-body.

Kabat and Murray (18)—who used a very specific anti-7S serum—found that various preparations of γ_2-globulin produced identical precipitin curves. γ_1-globulin, however, precipitated very much less nitrogen per unit weight, but all antibody was removed in the region of antigen excess. It was concluded that γ_1-globulin contained at least two substances; one similar to γ_2-globulin and one that was immunologically inert.

Cohn *et al.*, (19) using an antiserum against highly purified γ_2-globulin found that γ_1 precipitated more nitrogen than did the homologous antigen. The amount of nitrogen precipitated by γ_1-globulin could be reduced if the 19S component were removed. It was concluded that the antiserum contained antibody against the heavy component and that, since the antiserum had been produced against highly purified γ_2-globulin (7S), the heavy component had to be antigenically related to a component in γ_2-globulin. Their data also led them to believe that γ_2-globulin is antigenically heterogeneous.

The Ouchterlony gel diffusion technique permits the determination of the minimal number of antigens that participate in an immunological reaction. Recent studies with this technique (15, 20, 21) have shown that human gamma globulin contains two distinct antigens, one with a molecular weight of 160,000, the other with a molecular weight of over a million. Since the relative proportions of these two components differ in various γ-globulin preparations (9), it may be expected that the quantitative precipitin curves for them will vary considerably, expecially if the antiserum contains antibody against both proteins or groupings that the two proteins have in common. (Ultracentrifuge data indicating the "absence" of the heavy component from a preparation are of little value in the more sensitive immunological studies, especially in the antigen excess region.)

Franklin and Kunkel (15) have recently presented quantitative precipitin data from which they conclude that the 7S and 19S globulins have antigenic groupings in common.

Our studies confirm these conclusions in three independent ways:—

1. Highly purified macrocryoglobulins combine with antibody capable of reacting with the 7S globulin (Fig. 3).

2. The absorption of anti–γ-globulin serum with γ_2-globulin removes antibody capable of reacting with macroglobulins.

3. Bence Jones proteins of group A (6) cross-react with both the macroglobulin and the 7S component because of groupings these 3 proteins have in common.

The literature on macroglobulinemia contains but a few references on the antigenic nature of the pathological macroglobulins.

Luey *et al.* (22) and McFarlane, Dovey and Slack (23) found that pathological macroglobulins reacted very weakly or not at all with antiserum to normal γ-globulin. Franklin and Kunkel (15) found that these macroglobulins are antigenically related to normal γ-globulins. Other workers prepared antisera against the pathological

sera (24, 25) or purified macroglobulin (26) and their results will be discussed in our second paper (17).

The experiments reported here clearly show that pathological macroglobulins are antigenically related to the normal 19S globulin. Furthermore, these proteins differ among each other in many respects: solubility, electrophoretic mobility, and antigenic structure. Most macroglobulins have the mobility of γ_1-globulin, but mobilities of γ_2-globulin and β-globulin are not uncommon (3). There is no correlation between antigenic structure and electrophoretic mobility. For example, greater antigenic differences, as indicated by the size of the spur, (Figs. 5 and 6) were observed between some of the proteins of the same mobility than between some of the macroglobulins with widely divergent mobilities (compare MCG I with MCG III and MG VII or MG IX with MCG I). Nor was there any correlation between antigenic structure and the protein's solubility in the cold. Similar findings have been reported for the multiple myeloma serum proteins (8).

The ease with which macroglobulins can be distinguished from the 7S globulins by our antisera against normal γ-globulin has resulted in a relatively simple immunological test for macroglobulinemia. The practical diagnostic application of this study will be discussed elsewhere (27).

SUMMARY

Highly purified pathological macroglobulins, which had been characterized electrophoretically and in the ultracentrifuge, were studied by the Ouchterlony gel diffusion technique.

These macroglobulins were shown to be antigenically related to normal γ_1-macroglobulin (19S) as well as the 7S γ-globulins.

The pathological macroglobulins differ among each other and they are antigenically deficient when compared with the normal macroglobulin.

There is no correlation between the macroglobulin's antigenic structure and its physico-chemical properties.

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EXPLANATION OF PLATE 39

FIG. 1. Center cup: anti–γ_2-globulin E absorbed with β-globulin.
A. γ_2-globulin, 2 mg./ml.
B. γ_1-globulin, 4 mg./ml.
C. human plasma (1/4)
D. MCG I, 3 mg./ml.

FIG. 2. Center cup: anti–γ_2-globulin E absorbed with β-globulin.
A. γ_2-globulin, 2 mg./ml.
B. MM XVI, 2 mg./ml.
C. MM XVII, 1 mg./ml.
D. MCG I, 3 mg./ml.

FIG. 3. Center cup: anti-γ-globulin C absorbed with β-globulin and fraction V.
A. γ_2-globulin
B. MCG I
C. γ_2-globulin
D. MCG II
E. γ_2-globulin
F. MCG IV

FIG. 4. Center cup: anti-globulin C absorbed with β-globulin and fraction V.
A. MCG IV
B. Normal macroglobulin
C. MCG I
D. MCG III
E. MCG II
F. MG V

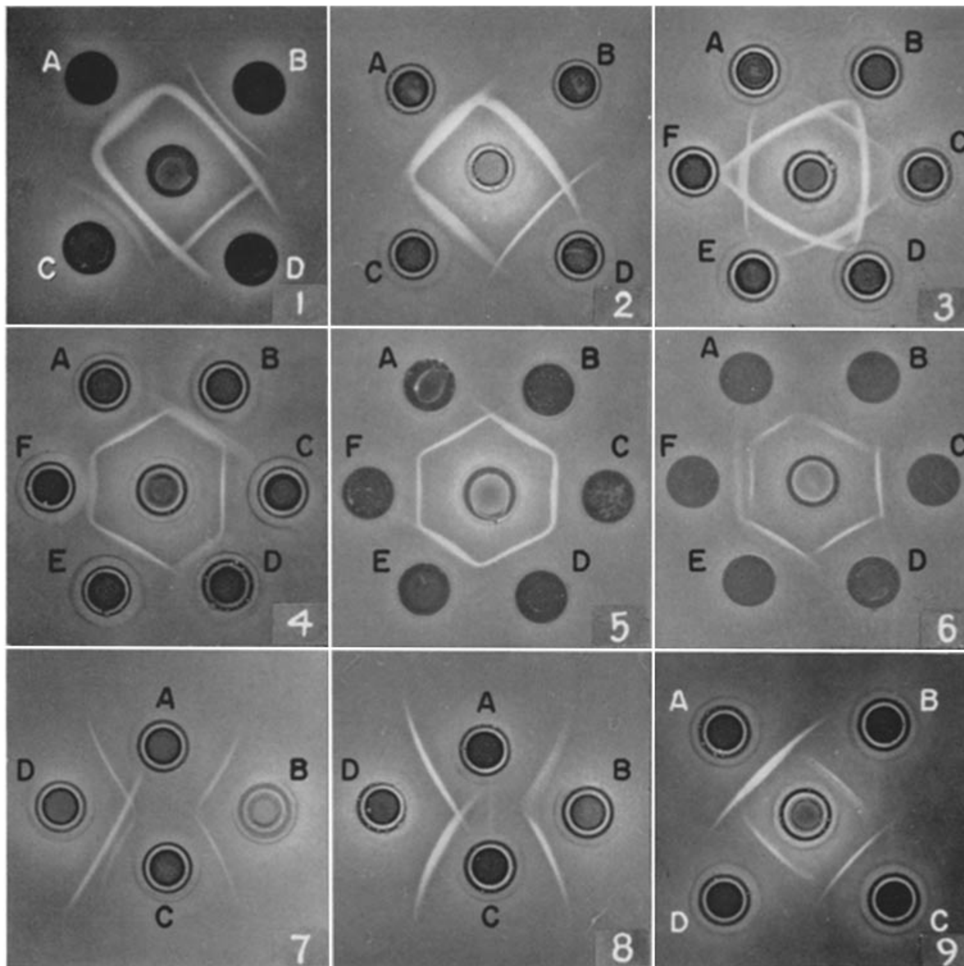
FIG. 5. Center cup: anti–γ_2-globulin E absorbed with β-globulin.
A. MCG I
B. MCG III
C. MG V
D. MG VI
E. MCG IV
F. MG VII

FIG. 6. Center cup: anti–γ-globulin C absorbed with β-globulin and fraction V.
A. MCG I
B. MG IX
C. MCG VIII
D. MCG I
E. Serum X (1/200)
F. Serum IX (1/50)

FIG. 7.
A. MCG I, 3 mg./ml.
B. anti-globulin D absorbed with fraction V, γ-2 and β-globulin.
C. γ_1-globulin, 4 mg./ml.
D. anti-globulin D absorbed with fraction V and β-globulin.

FIG. 8.
A. MCG I
B. anti–γ-globulin D absorbed with fraction V, γ-2 and, β-globulin.
C. MCG II
D. anti–γ-globulin D absorbed with fraction V and β-globulin.

FIG. 9. Center cup: anti–γ-globulin C absorbed with fraction V and β-globulin.
A. γ_2-globulin
B. Bence Jones protein (A.P.)
C. MCG I.
D. Bence Jones protein (A.P.)



(Korngold and Van Leeuwen: Macroglobulinemia. I)