MACROGLOBULINEMIA

II. Antisera Specific for Pathological Macroglobulins*, ‡

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In previous reports we have shown that pathological macroglobulins are antigenically deficient when compared with normal 19S γ_1-globulin (1), and that multiple myeloma proteins are deficient when compared with normal 7S γ_2-globulin (2). Furthermore, these multiple myeloma proteins contain antigenic determinants that are patient-specific (2); that is, these proteins are specific for the patient from whom they were obtained.

It was therefore of interest to determine whether pathological macroglobulins, when injected into rabbits, might induce antisera specific for pathological macroglobulins or the homologous macroglobulins. Moreover, the heterogeneity of the macroglobulins, which consist of two major components with sedimentation constants (s_{20w}) of 19S and 26S raised the question whether this heterogeneity would be reflected immunologically.

The resulting experiments have shown that the pathological macroglobulins contain two patient-specific proteins.

Materials and Methods

Antigens.—The macroglobulins have been described previously (1); they contained at least two components, 19S and 26S, as determined by ultracentrifugal analysis.

Antisera.—Antisera against normal macroglobulin have been described (1). Antisera against MCG I,[1] MCG III and MG V were prepared by injecting rabbits twice with 10 to 50 mg. of the antigens in Freund adjuvant. These injections were given at weekly intervals. Three weeks after the last injection the animals were bled. If the antiserum was sufficiently strong the rabbits were exsanguinated, otherwise a third injection was administered and the animals were exsanguinated 3 weeks later. The gel diffusion technique has been described (3).

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[1] The abbreviations MCG and MG stand for macrocryoglobulin and macroglobulin (1).

RESULTS

All antisera obtained at the first bleeding were weak; they either produced very faint lines or otherwise the spurs between the homologous and cross-reacting lines were too short for comparative studies. Anti-MCG IB, the antiserum obtained at the second bleeding, was slightly better (Fig. 1), but was bypassed for the more potent antiserum obtained at the third bleeding, anti-MCG IC serum (Fig. 3). The other antisera were from the second bleeding. All antisera usually contained antibodies against a β-globulin which were removed by absorption with a β-fraction obtained by zone electrophoresis on starch.

Since the immunizing antigens contained small amounts of a component with a sedimentation constant of 7S it was of interest to determine whether the antisera would react with normal γ_2-globulin (7S). In our first paper (1) it was shown that anti-γ_2-globulin sera contain antibody against antigenic groupings common to the 19S and 7S γ-globulins as well as antibody specific for each protein. Consequently, when these antigens were compared with such an antiserum, the lines intersected (Fig. 2). The anti-MG sera, however, did cross-react with the 7S component in the conventional manner; *i.e.*, the lines coalesced partially because these antisera contain antibody against groupings that the macroglobulin and 7S globulin have in common as well as antibody against groupings specific for the macroglobulins. No antibody specific for the 7S globulin could be demonstrated and it may be concluded that the rabbit's response was primarily to the injected macroglobulins rather than the trace amounts of the lighter component.

When anti-MCG IC, absorbed with γ_2-globulin, was used for comparative studies of several macroglobulins, two spurs extended beyond the point of fusion of the lines formed by the homologous and heterologous antigens (Fig. 3). Consequently, the precipitate formed by the homologous MCG I should consist of two different immunological systems. Partial absorption of the antiserum with normal γ_1-globulin showed this to be the case (Fig. 4). (The normal serum actually formed a line which was too faint to be photographed.) Further absorption of this antiserum resulted in a reagent which was specific for the homologous antigen (Fig. 5). Both lines still show up very clearly; from their curvature (1) it can be concluded that the antigens are large, slowly diffusing proteins. They will be referred to as the 19S and 26S components.

The other anti-macroglobulin sera produced two lines with their homologous antigens even prior to absorption (Figs. 6, 7). Absorption of these antisera with normal γ_1-globulin made them patient-specific (Figs. 8 to 11), and again both components of the macroglobulin preparation contain the patient-specific determinants. The precipitin pattern with the unabsorbed antisera (Figs. 6, 7) shows that one of the macroglobulins has its counterpart in the heterologous antigens, including normal macroglobulin. This macroglobulin is the 19S

component, since it only is present in normal serum in sufficiently high concentration to be detected. The lines partially coalesce, and spurs are formed indicating that the homologous proteins contain determinants that are unique. The second component, 26S, is detectable only in the homologous macroglobulin, and this is true for all three macroglobulins against which antisera were produced. In the discussion arguments will be presented suggesting that the 26S component is not completely patient-specific, but that it shares a few groupings with normal 19S globulin.

The fact that spurs are formed between the lines of the homologous and the lines of the heterologous 19S globulins, including the normal, is already sufficient evidence that the pathological macroglobulins are antigenically abnormal. These spurs are the product of an exhaustive antibody absorption *in situ*. The limited experience with this technique, however, made it desirable to test the antisera in the classical manner by absorbing them with cross-reacting antigen. That our antisera were exhaustively absorbed can be seen from Fig. 12. Reservoir A contains absorbed anti-MCG IC serum, which no longer reacts with the macroglobulin of normal γ_1-globulin (cup B) or normal undiluted serum. It still reacts with MCG I in cup D. Cup C contains anti-γ-globulin serum C absorbed with γ_2-globulin. This antiserum reacts with both normal and pathological macroglobulin as well as with the normal macroglobulin used for the absorption of anti-MCG IC serum, proving that the absorbing antigen is in excess. (This proof is an adaptation of the supernatant test used to determine the region of antigen excess in the quantitative precipitin reaction (4)). The fact that both lines formed by the anti-MCG IC serum with MCG I coalesce with the line of the anti-γ-globulin serum suggests that the two pathological macroglobulins and the normal 19S globulin share the same antigenic grouping (See below).

DISCUSSION

The first immunological studies with antisera against sera from patients with macroglobulinemia were performed by Habich (5). Using several such antisera he found that they precipitated the homologous serum as well as sera from other macroglobulinemia patients, even after the antisera were absorbed with normal serum. He also absorbed one of his antisera with both normal serum and heterologous macroglobulinemia serum, and obtained a completely patient-specific antiserum. Most of his results were confirmed by Kanzow *et al.*, (6).

More recently Deutsch *et al.* immunized a rabbit with crystalline macroglobulin (7) isolated from a patient with macroglobulinemia (8). After absorption with a mixture of γ_2- and γ_1-globulins, the antiserum failed to react with the homologous antigen.

All these studies were performed in aqueous media, and consequently it is

difficult to determine which antigen(s) were involved in the immunological reactions. This difficulty is eliminated in the Ouchterlony technique because each immune system forms a separate line, the curvature of which is characteristic for the molecular weight of the antigen (9). It is therefore possible to differentiate precipitates formed by macroglobulins from those formed by the 7S globulin or other contaminating light proteins.

In order to study antigenic relationships among different proteins it is essential that the antisera contain antibody against those antigenic groupings by which these proteins may be distinguished. Failure to find differences by immunological techniques does not constitute evidence that a pathological protein is normal (7); it only indicates that the antiserum does not contain antibody against a possible abnormal determinant.

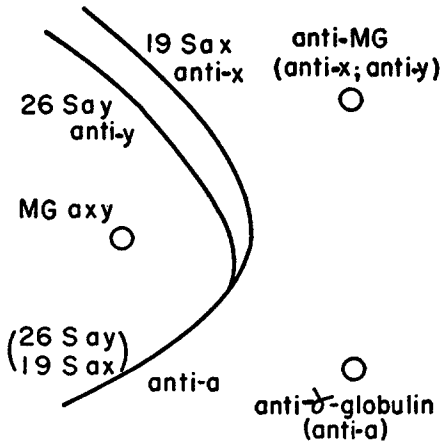
The use of hyperimmune anti-macroglobulin sera in this study shows that pathological macroglobulins contain at least two antigenic components, and that both are macroglobulins according to the curvature of their precipitin line. Whether these two macroglobulins are indeed the 19S and 26S components or whether they are two units of the 19S macroglobulin cannot be proven until a pure 26S component becomes available. However, since the antigens contain at least 10 per cent of the 26S component and because a molecule of that size would diffuse slower than the 19S component, we will assume that it is responsible for the line closest to the antigen cup. This 26S globulin is detectable only in the homologous antigen, suggesting that its antigenic structure is different for each patient. Whether such a protein is also present in normal serum is as yet undetermined. Wallenius *et al.*, (10) have shown that repeated ultracentrifugation of normal γ_1-globulin leads to the accumulation of a 26S component.

The 19S components of pathological macroglobulins cross-react with normal 19S globulin and the equivalent proteins from heterologous macroglobulinemic sera. The fact that spurs are formed indicates that the homologous 19S globulin possesses antigenic groupings that are absent from all other pathological and normal macroglobulins.

In previous papers (1, 11) it was shown that Bence Jones proteins (M. weight 35,000) γ_2-globulin (molecular weight 150,000) and 19S globulin (M. weight 1,000,000) have antigenic groupings in common. It was therefore of interest to determine whether 26S and 19S were also antigenically related. A direct proof is as yet not possible because the 26S globulin is not available as an isolated protein. Let us therefore turn to the indirect evidence from which it can be deduced that these two macroglobulins share the same groupings with normal 19S γ_1-globulin.

From Fig. 12 it can be seen that the two lines produced by the pathological macroglobulins and homologous antiserum fuse with the single line formed by the same antigens and the antiserum against normal 19S globulin. Since anti-

MCG IC does not react with normal macroglobulin it follows that the two antisera contain antibodies against different groupings of the pathological proteins; *i.e.*, anti-γ-globulin serum contains antibodies against groupings that the normal and pathological antigens have in common, whereas the absorbed anti-MCG IC serum contains antibody only against those groupings that are unique for MCG I. The formation of two lines with this preparation and its homologous antiserum proves that the antiserum contains some antibodies that cannot react with the antigen forming the line closest to the antiserum cup; these antibodies react with the slower diffusing component. All antibodies of anti-γ-globulin serum react with the faster diffusing antigen of MCG I; if the antiserum contained antibody against groupings of the 26S components



TEXT-FIG. 1. Evidence for the antigenic relationship of normal macroglobulin and the two pathological macroglobulins.

absent from the 19S protein two lines rather than one should have been formed. The fusion of the two lines formed by anti-MCG IC serum with the line formed by anti-γ-globulin serum can be best explained by Text-fig. 1.

We assume that the two pathological proteins have the following antigenic structure:—

19S: *ax*
26S: *ay*

and that anti-MCG IC serum contains anti-*x* and *y* antibodies. Anti-γ-globulin serum contains only anti-*a* antibody.

Anti-*x* reacts with 19S and is precipitated by it; anti-*y* diffuses through the resulting line and reacts with 26S. Anti-*a* reacts with 19S and forms a line in the region of optimal proportions; as soon as 26S diffuses beyond the leading

edge of this line it will encounter free anti-*a* antibody and be precipitated so that the two precipitates will be superimposed to form one line. The three lines formed by the two antisera coalesce because neither groupings *x* nor *y* can diffuse independent of grouping *a*.

Recently there has been some controversy in the literature concerning the nature of the elevated serum proteins encountered in multiple myeloma and macroglobulinemia (2, 5, 7). The data presented here and elsewhere are not easily reconciled with the theory that these proteins are elevated components of normal serum constituents; rather, they are best explained by the hypothesis that the elevated proteins encountered in these two diseases are abnormal. These proteins are abnormal because they are deficient in some of the antigenic groupings present in the normal proteins, and they contain groupings that as yet have not been demonstrated in normal serum.

SUMMARY

Rabbits were immunized with three highly purified macroglobulins, from patients with macroglobulinemia. The antisera reacted with two macroglobulins with sedimentation constants of 19S and 26S of homologous antigen, and cross-reacted with the 7S and 19S globulins of normal γ_1-globulin and the heterologous pathological 19S macroglobulins. Exhaustive absorption of these antisera with normal γ-globulins rendered them specific for the homologous macroglobulins. The antigenic properties of the pathological macroglobulins indicate that these proteins are abnormal.

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EXPLANATION OF PLATE 40

FIG. 1. Center cup: anti-MCG IB absorbed with β-globulin.

A. MCG I
B. MCG II
C. MG VII
D. MCG I
E. MG VI
F. Normal serum

FIG. 2.

A. MCG II
B. anti-γ-globulin serum C absorbed with fr. V and β-globulins.
C. γ_2-globulin
D. anti-MCG II B

FIG. 3. Center cup: anti-MCG IC absorbed with γ_2- and β-globulins.

A. MCG I
B. MG V
C. MCG II
D. MCG I
E. MG VI
F. MG VII

FIG. 4. Center cup: anti-MCG IC absorbed with β- and γ_1-globulins (incomplete).

A. MCG I
B. MCG II
C. MG V
D. MCG IV
E. MCG III
F. Normal serum

FIG. 5. Center cup: anti-MCG IC absorbed with β- and γ_1-globulins (complete).

A. MCG I
B. MG V
C. MG VI
D. MCG I
E. MCG II
F. MCG IV

FIG. 6. Center cup: anti-MCG IIB absorbed with γ_2- and β-globulins.

A. MCG II
B. MCG I
C. MG V
D. MCG II
E. MG VI
F. Normal plasma (½)

FIG. 7. Center cup: anti-MG VB absorbed with β-globulins.

A. MG V
B. MCG I
C. MCG II
D. MG V
E. MG VI
F. Normal serum (½)

FIG. 8. Center cup: anti MCG IIB absorbed with β and γ_1-globulins.

A. MCG II
B. MCG I
C. MG V
D. MCG II
E. MG VI
F. Normal plasma (½)

FIG. 9. Center cup: anti-MG VB absorbed with β- and γ_1-globulins.

A. MG V
B. MCG I
C. MCG II
D. MG B
E. MG VI
F. MG VII

FIG. 10.

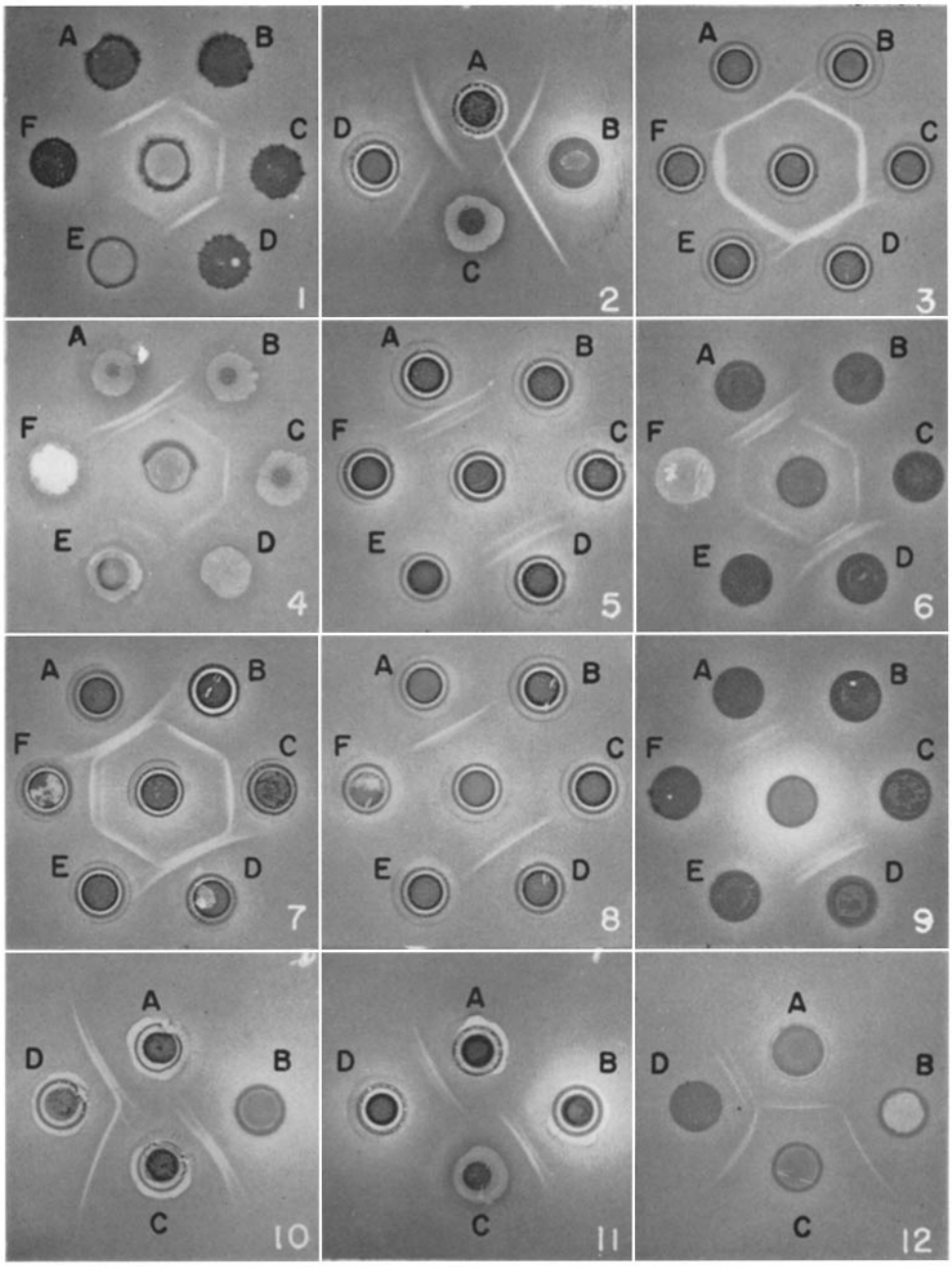
A. MCG I
B. anti-MG VB absorbed with β- and γ_1-globulins.
C. MG V
D. anti-MCG IC absorbed with β- and γ_1-globulins (incomplete).

FIG. 11.

A. MCG II
B. anti-MG VB absorbed with β- and γ_1-globulins.
C. MG V
D. anti-MCG II absorbed with β- and γ_1-globulins.

FIG. 12.

A. anti-MCG IC absorbed with β- and γ_1-globulins (complete).
B. γ_1-globulin, 40 mg./ml.
C. anti-γ_2-globulin E absorbed with fraction V, β- and γ_2-globulins.
D. MCG I, 3 mg./ml.



(Korngold and Van Leeuwen: Macroglobulinemia. II)