MACROGLOBULINEMIA

III. The Effect of Mercaptoethanol on the Antigenic Structure of Macroglobulins

By LEONHARD KORNGOLD, Ph.D., and GERDA van LEEUWEN*

(From the Division of Experimental Pathology, Sloan-Kettering Institute for Cancer Research and Sloan-Kettering Division of the Cornell University Medical College, New York)

Plate 1

(Received for publication, March 20, 1959)

Mercaptoethanol depolymerizes γ_1-macroglobulins to monomers with sedimentation constants of 7S (1, 2). These monomers are antigenically different from the 7S γ-globulins usually found in human serum (3). Recently Morton and Deutsch reported that they did not detect any qualitative antigenic differences between the native protein, the monomer, and the reaggregated monomer (4).

In previous paper (5, 6) we described the antigenic properties of normal γ-macroglobulin as well as those of the macroglobulins from patients with Waldenström's macroglobulinemia. These properties were determined with several potent antisera against the normal and pathological proteins. It was therefore of interest to investigate by means of these antisera the antigenic changes resulting from the depolymerization of the macroglobulins.

In the present communication it will be shown that the monomers lack several antigenic groupings characteristic of the native protein, and that some or all of these groupings can be reconstituted on reaggregation of the depolymerized protein.

Materials and Methods

Antisera.—The antisera used in this study have been described (5, 6). They included three antisera against pathological macroglobulins (anti-MCG IC, anti-MCG IIB, and anti-MG VB)[1] as well as two antisera against normal γ-globulins, anti-γ_2-globulin E, and anti-γ-globulin C.

Antigens.—Macroglobulin MCG I, MCG II, and MG V[1] have been described (5, 7). In addition we have used serum "Q" which was supplied by Dr. Putnam (3).

Depolymerization.—The depolymerization was accomplished according to Deutsch (1). After treatment with mercaptoethanol (0.1 M) some of the samples were dialyzed against

* This investigation was supported by research grant (C-2359 M & I) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

[1] The abbreviations MCG and MG stand for macrocryoglobulin and macroglobulin (5).

sodium iodoacetate in order to block the sulfhydryl groups and some were dialyzed against phosphate buffer, pH 7, to permit reaggregation. Most of the samples were kept in the cold room (5°C.), but some, for reasons that will become apparent later, were subjected to repeated freezing (−25°C.) and thawing. Controls included macroglobulins kept in sodium iodoacetate or in phosphate buffer. All three purified macroglobulin preparations, MCG I, MCG II, and MG V, were depolymerized as described above. Normal macroglobulin was studied in the same manner without attempting to purify the protein; in this instance the whole serum was treated with mercaptoethanol and iodoacetate. Included in these studies was a sample of serum "Q" treated with mercaptoethanol and iodoacetate by Dr. Putnam; the physicochemical properties of both the native protein and the monomer have been described (3).

Immunological Studies.—The antigenic analysis was performed by the double gel diffusion technic of Ouchterlony as described previously (5).

RESULTS

The preliminary experiments of this study were performed with the preparations of serum "Q" which have been characterized in the ultracentrifuge (3). From Fig. 1 it can be seen that the antiserum against normal γ-globulins reacts strongly with the macroglobulin of serum Q and its monomer (cups A and C; the line closest to cup A is formed by the 7S γ-globulin of this serum). From this figure it is also apparent that the monomer of macroglobulin Q (7S) is antigenically different from normal 7S γ-globulin (cup D). This difference was also observed when the depolymerized macroglobulin of normal serum was compared with 7S γ-globulin. Fig. 2 shows that the anti-γ-globulin C serum which was completely absorbed with 7S γ_2-globulin still reacts strongly with the monomer of normal macroglobulin.

Since the curvature of the precipitin line is a function of the diffusion coefficient of the antigen (8), it was to be expected that the curvatures of the lines formed by the native protein and the monomer would differ: the large and slowly diffusing macroglobulin should produce a line which curves towards the antigen cup, whereas the smaller monomer should produce a line without too much curvature since its sedimentation constant, and presumably its diffusion coefficient, are similar to that of antibody. The curvature of an immune precipitin line is best observed when there is no interference from an adjacent line; however, a marked difference between the curvatures of the lines formed by the monomer and by the native proteins is already apparent from Figs. 1 and 2. In Fig. 3 this difference is even more pronounced.

The experiments described above were primarily designed to check the reactivity of the monomer, and for this study the arrangement of the antigen cups in a square around the antiserum cup is suitable. However, for the study of cross-reactions of mcroglobulins, or of macroglobulins and their monomers, it is essential to place the antigen cups closer together. The reason for this is apparent from Figs. 1 and 2—the lines formed by the macroglobulins, or by the macroglobulins and monomers, barely meet. The failure of related or identical macroglobulins to form lines that coalesce when their sources of diffusion are

too far apart is due to the slow diffusion of these antigens. Towards the end of the line, which curves away from the center cup, the antibody concentration drops rapidly until it is no longer capable of forming a visible precipitate. Since the study of cross-reaction depends on the diffusion of enough specific antibody beyond the point of fusion in order to form a spur, it is obvious that studies in which the antigen cups are arranged in a square (4) will result in misleading data.

In a previous paper (6) we have pointed out that in the study of immunological cross-reactions it is essential to have antibody against those antigenic determinants that distinguish the homologous from the cross-reacting protein. Unfortunately, it is as yet not possible to produce at will antisera that fulfill this requirement, and some of the failures to differentiate between normal and pathological proteins (9, 10) or between a native macroglobulin and its monomer (4) can be traced to the lack of proper antisera. (It is of some interest that two coauthors of reference 10 later changed their conclusions regarding the identity of normal and pathological macroglobulins (11, 12).)

For example, if this study were limited to the reaction of macroglobulin Q and its monomer with anti-γ_2-globulin E serum, one would have concluded that these proteins are antigenically indistinguishable, since no spurs were formed (Fig. 4, antiserum in cup D). However, the use of anti-MCG IC-serum (Fig. 4, cup B) shows clearly that they are different. The lines formed by MG Q and its monomer coalesce partially and a distinct spur extends beyond the point of junction. This spur is the result of antibody against determinants of the native MG which no longer can combine with the depolymerized proteins, presumably because the chemical treatment destroyed these antigenic determinants. This antibody diffuses through the line formed by the monomer and then reacts with the native protein from the adjacent cup. Similar results were obtained with anti-MCG IC serum and its homologous native protein and monomer (Fig. 5). Note that the monomer gives rise to two lines as did the previously described monomer of protein Q. The loss of antigenic groupings after treatment with merceptoethanol was also apparent with MG V and MCG II, and in this latter instance both the homologous antiserum and the anti-gamma globulin serum were able to detect the difference (Fig. 6). The anti-macroglobulin sera produced two lines with the homologous native protein (6); however the lines formed by anti-MCG IC serum are superimposed and only one line appears. The action of mercaptoethanol resulted in the loss of antigenic determinants from both macroglobulins.

Since depolymerization resulted in the loss of antigenic groupings it was of interest to determine whether reaggregation of the depolymerized protein in phosphate buffer would lead to a reconstitution of these groupings and to a change in curvature of the precipitin line. When the depolymerized MCG I

was dialyzed against phosphate buffer all lost antigenic groupings reappeared. No spur could be detected between the lines formed by the reaggregated protein and the native antigen (Fig. 7), and at least one of the two lines of the reaggregated antigen had the curvature characteristic for a macroglobulin (cup D). The reaggregated MG V, on the other hand, never regained all its antigenic groupings, although most of the lost determinants of one of the macroglobulins of the native preparation were reformed (Fig. 8). The spurs between the native and reaggregated lines were so small that they cannot be seen on the reproduction; but the monomer, when compared with either the native or the reaggregated proteins, still gives rise to distinct spurs. MCG II, after depolymerization, never regained its lost antigen determinants (Fig. 6).

An unexpected finding of this study was the effect of sodium iodoacetate on the macroglobulins. If the native macroglobulins MCG I, MCG II, or MG V were dialyzed against iodoacetate and kept at 5 C., or in the deep freeze at −20 C., no antigenic changes were detected even after several months. However, if the same preparations were subjected to repeated freezing and thawing, all antigenic properties were ultimately lost, although freezing and thawing in themselves had no effect on the native protein when it was kept in phosphate buffer. The loss of immunological reactivity of MCG is shown in Fig. 9, (cup B) and similar results were obtained with the other two macroglobulins.

When MCG I was treated with mercaptoethanol and subsequently dialyzed against sodium iodoacetate, the monomer was stable if kept in the cold room or in the deep freeze. However, when a similar preparation was subjected to repeated freezing and thawing a marked antigenic change became apparent (Fig. 9, cup F). The monomer split into two components, one of which was antigenically indistinguishable from the Bence Jones protein (cup E) found in the urine of the patient (5) who had contributed MCG I. This change became apparent after freezing and thawing for approximately six times, and could be prevented by removing excess iodoacetate by dialysis against phosphate buffer. Fig. 9, cups C and D, clearly shows that the monomer which had been dialyzed against phosphate buffer to remove excess iodoacetate, is antigenically still intact as is the monomer that had been frozen and thawed only once in excess iodoacetate. The further degradation of the monomer of MCG I was unique and similar experiments with the monomers of the other two macroglobulins failed to show any antigenic changes.

DISCUSSION

The structure of the groupings responsible for the antigenic specificity of proteins is still unknown.

Recently it has been shown (13, 14) that differences in antigenic specificity of Bence Jones proteins can be correlated with the presence or absence of terminal *N*-aspartic acid. The finding of Deutsch and Morton (1) that mercaptoethanol de-

polymerizes γ_1-macroglobulins opened another line of approach, since the action of this chemical is directed against the disulfide bond of the protein. It was therefore of interest to determine whether the disruption of this bond would result in the loss of antigenic determinants.

The usefulness of the Ouchterlony gel diffusion technic for this study was suggested by the work of Lapresle and Durieux, (15) and of Porter (16) who applied it successfully to follow the enzymatic degradation of serum albumin.

The findings reported in this paper leave little doubt that both macroglobulins detectable by a homologous antiserum (6) are depolymerized by mercaptoethanol and that the monomers retain many, but not all, of the antigenic properties of the native proteins. Moreover, the monomers are antigenically different from the 7S γ-globulin of normal serum. The fact that the macroglobulin, which is antigenically related to 7S γ-globulin (5, 6, 17), can be degraded into antigenically distinct units with a sedimentation constant of 7S has been used in support of the hypothesis that the macroglobulins may be aggregates of the normally occurring 7S γ-globulin (1). The findings reported here, however, make this hypothesis rather untenable since the monomers of both normal and pathological macroglobulins are antigenically different from normal 7S γ-globulin.

It is still premature to speculate as to how the disulfide bond provides antigenic specificity; but the possibility that the bond supplies part of the molecular surface with a specific configuration must be considered. The finding that after reaggregation two of the depolymerized macroglobulins regained most or all of the antigenic groupings of the native proteins suggests that reaggregation is at least in part specific, since a random aggregation would not have resulted in the formation of these antigenic determinants. The data also reflect the physico-chemical differences among the pathological macroglobulins because one depolymerized preparation never showed signs of reaggregating, another regained most of the native protein's determinants, and the third regained all determinants that could be detected with the homologous antiserum.

In agreement with the findings of Deutsch and Morton (1) sodium iodoacetate had no effect on either the native protein or the monomer. The complete antigenic destruction of the three native macroglobulins by sodium iodoacetate after repeated freezing and thawing was therefore unexpected, as was the partial degradation of the monomer of MCG I to a "Bence Jones protein". The mechanism by which sodium iodoacetate destroys macroglobulins is not clear—however, this chemical can act as an alkylating agent capable of combining with several amino acids (18). The repeated freezing and thawing may make these amino acids more available to the chemical.

At present we cannot explain why only the monomer of MCG I was degraded by iodoacetate. The fact that the patient who supplied MCG I excreted Bence Jones protein, however, is of interest and it is planned to continue this study with macroglobulins of similar patients.

SUMMARY

The monomers obtained by treating γ_1-macroglobulins with mercaptoethanol have proved to be antigenically different from normal 7S γ-globulin.

The depolymerization of the macroglobulins resulted in the loss of several antigenic determinants, although the monomers still cross-reacted with antisera against macroglobulins.

Reaggregation of the monomers occasionally resulted in the reconstitution of some or all of the antigenic determinants that were lost during depolymerization.

Repeated freezing and thawing of pathological macroglobulins in iodoacetate resulted in their complete antigenic destruction.

Repeated freezing and thawing of one of the monomers in excess iodoacetate resulted in the degradation to a protein antigenically indistinguishable from Bence Jones protein. The other two monomers studied were stable under these conditions.

We wish to thank Mr. Rudolph Smith for his technical assistance.

BIBLIOGRAPHY

1. Deutsch, H. F., and Morton, J. I., Dissociation of human serum macroglobulins, *Science*, 1957, **125**, 600.
2. Glenchur, H., Zinneman, H. H., and Briggs, D. R., Macroglobulinemia: Report of two cases, *Ann. Int. Med.*, 1958, **48**, 1055.
3. Putnam, F. W., Abnormal human serum globulins I. The non-identity of macroglobulins, *Arch. Biochem. and Biophysics*, 1959, **79**, 67.
4. Morton, J. I., and Deutsch, H. F., Human serum macroglobulins and dissociation units II. Immunochemical properties, *J. Biol. Chem.*, 1958, **231**, 1119.
5. Korngold, L., and van Leeuwen, G., Macroglobulinemia I. The antigenic relationship of pathological macroglobulins to normal γ-globulins, *J. Exp. Med.*, 1957, **106**, 467.
6. Korngold, L., and van Leeuwen, G., Macroglobulinemia II. Antisera specific for pathological macroglobulins, *J. Exp. Med.*, 1957, **106**, 477.
7. Crumpacker, E. L., Peterman, M. L., and Irvine, W. D., A cryoglobulin with unusual characteristics associated with a rare hematologic disorder, *Virginia Med. Monthly*, 1957, **84**, 493.
8. Korngold, L., and van Leeuwen, G., The effect of the antigen's molecular weight on the curvature of the precipitin line in the Ouchterlony technic, *J. Immunol.*, 1957, **78**, 172.
9. Deutsch, H. F., and Morton, J. I., and Kratochvil, C. H., Antigenic identity of hyperglobulinemic serum components with proteins of normal serum, *J. Biol. Chem.*, 1956, **222**, 39.
10. Burtin, P., Hartmann, L., Heremans, J., Scheidegger, J. J., Westendorp-Boerma, F., Wieme, R., Wunderly, C., Fauvert, P., and Grabar, P., Études immunochimiques et immuno-électrophorétiques des macroglobulinemies, *Rev. franç. études clin. et. biol.*, 1957, **2**, 161.

11. Westendorp-Boerma, F., and Mandema, E., A serologic investigation of Waldenström's macroglobulinemia by means of the agar diffusion method, *J. Lab. and Clin. Med.*, 1957, **49,** 358.
12. Scheidegger, J. J., Weber, R., and Hässig, A. A., Zur Antigenstruktur der Makroglobuline beim Morbus Waldenström, *Helv. Med. Acta*, 1958, **25,** 25.
13. Korngold, L., and Lipari, R., Multiple-myeloma proteins. III. The antigenic relationship of Bence Jones proteins to normal gamma globulin and multiple myeloma serum proteins, *Cancer*, 1956, **9,** 262.
14. Putnam, F. W., and Miyake, A., Proteins in multiple myeloma. VII. The free amino acid groups of Bence-Jones proteins, *J. Biol. Chem.*, 1957, **227,** 1083.
15. Lapresle, C., and Durieux, J., Étude de la dégradation de la sérum albumine humaine par un extrait de rate de lapin V. Antigénicité de l'albumine dégradée, *Ann. Inst. Pasteur*, 1958, **94,** 38.
16. Porter, R. R., The isolation and properties of a fragment of bovine serum albumin which retains the ability to combine with rabbit antiserum, *Biochem. J.*, 1957, **66,** 677.
17. Franklin, E. C., and Kunkel, H. G., Immunologic differences between the 19S and 7S components of normal γ-globulin, *J. Immunol.*, 1957, **78,** 11.
18. Putnam, F. W., The chemical modification of proteins, *in* The Proteins, (H. Neurath and K. Bailey, editors), New York, Academic Press Inc., 1953, **1, B,** 893.

EXPLANATION OF PLATE 1

FIG. 1. Center cup: anti-γ-globulin C absorbed with β-globulin and fraction V.
A. "Q" serum ($\frac{1}{50}$)
B. MCG I, 3 mg./ml.
C. "Q" monomer
D. γ_2-globulin, 2 mg./ml.

FIG. 2. Center cup: anti-γ-globulin C absorbed with β-globulin, fraction V and γ_2-globulin.
A. Normal serum 1:2
B, C, and D. Normal serum treated with mercaptoethanol and iodoacetate

FIG. 3. Center cup: anti-γ-globulin C absorbed with β-globulin and fraction V.
A. MCG I, 3 mg./ml.
B and C. Empty
D. MCG I, monomer, 1 mg./ml.

FIG. 4.
A. "Q" serum 1:50
B. Anti-MCG I C absorbed with β- and γ_2-globulins.
C. "Q" monomer
D. Anti-γ_2-globulin E absorbed with β-globulin.

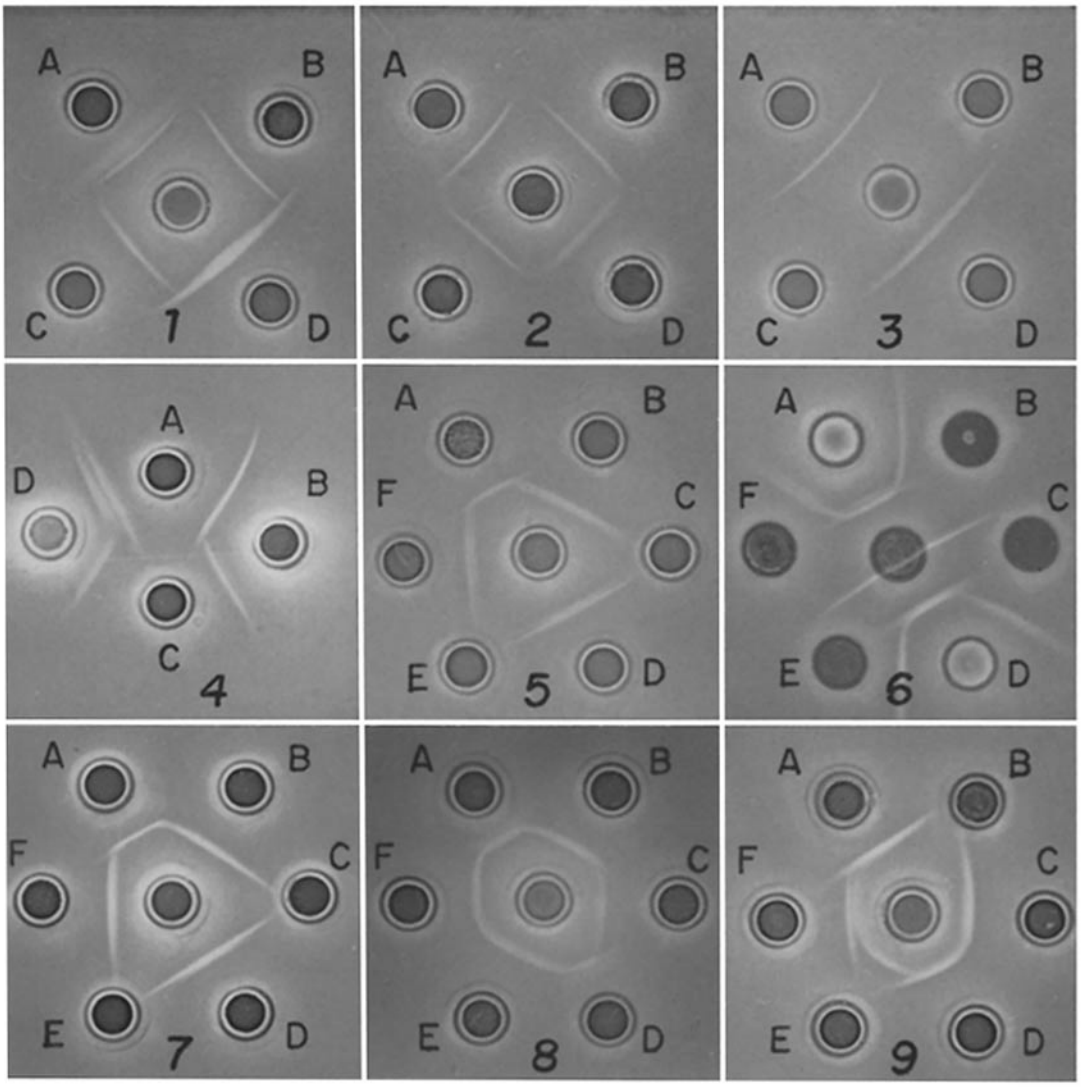
FIG. 5. Center cup: anti-MCG I C absorbed with β and γ_2-globulins.
A. MCG I, 3 mg./ml.
B. MCG I, monomer, 3 mg./ml.
C and E. Empty
D and F. MCG I, monomer, 1 mg./ml.

FIG. 6. Center cup: MCG II.
A. Anti-MCG II absorbed with β- and γ_2-globulins
B and C. MCG II, reaggregated
D. Anti-γ-globulin C absorbed with β-globulin and fraction V
E and F. MCG II, monomer
(The line in the center is formed by anti-γ-globulin C serum and the excess γ_2-globulin used for absorbing anti-MCG IIB).

FIG. 7. Center cup: anti-MCG I C absorbed with β- and γ_2-globulins.
A. MCG I, 3 mg./ml.
B and D. MCG I reaggregated, 1.5 mg./ml.
F. MCG I monomer, 1 mg./ml.
C and E. Empty

FIG. 8. Center cup: anti-MG-V absorbed with β- and γ_2-globulins.
A and D. MG V
B, E, F. MG V, reaggregated
C. MG V, monomer

FIG. 9. Center cup: anti-γ-globulin C absorbed with β-globulin and fraction V.
A. MCG I, 3 mg./ml.
B. MCG I, frozen and thawed in iodoacetate, 3 mg./ml.
C. MCG I, monomer (excess iodoacetate moved by dialysis), 1 mg./ml.
D. MCG I, monomer (in excess iodoacetate—one freezing and thawing), 1 mg./ml.
E. Bence Jones protein
F. MCG I, monomer (in excess iodoacetate—repeated freezing and thawing), 1 mg./ml.



(Korngold and van Leeuwen: Macroglobulinemia. III)