Studies on the Antigenic Complexity of Human 7S γ -Globulin

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Tryptic hydrolysates of human 7S γ -globulin were fractionated by means of gel filtration on Sephadex gels with different degrees of cross-linking. The gel filtration fractions obtained were analyzed by means of agar gel and starch gel electrophoresis, showing electrophoretic heterogeneities especially in starch gels. By means of immunological analyses (immunoelectrophoresis and double diffusion in gel) using a number of various immune sera the presence of several antigenic determinants on different enzymatic fragments of 7S γ -globulin could be assumed. A few pathological γ -globulins investigated showed to be antigenically deficient in comparison with two of the gel filtration fractions of normal γ -globulin.

Since the work of Porter¹ on the proteolytic degradation of rabbit γ -globulin and subsequent chromatographic separation of the hydrolysate into three fractions, several authors have pursued similar studies of human 7S γ -globulin.²⁻⁹ The possibilities of obtaining a group-separation of proteolytic break-down products of human γ -globulin by gel filtration on Sephadex G-75 have been studied in previous communications.¹⁰⁻¹² It was thought that the new types of Sephadex gels with lower degrees of cross-linking than Sephadex G-75 might give further possibilities for the separation of rather high molecular break-down products and such gels have been applied in the present study.

The cited investigations of human γ -globulin demonstrate the antigenic complexity of this protein. The present investigation further illustrates this complexity by means of immuno-diffusion analysis of the antigenic determinants present in the fragments obtained by the employed separation techniques.

MATERIALS

Normal human 7S γ -globulin prepared from Cohn fraction II by an ion exchange method using DEAE-Sephadex was kindly supplied by AB Kabi, Stockholm. It was pure 7S γ -globulin when tested with an anti-human blood plasma serum in immunoelectrophoresis.

Paraproteins from a few patients with multiple myeloma were purified by chromatography of blood serum on DEAE-cellulose. Serum (30–50 ml) was dialysed overnight

at $+4^{\circ}\text{C}$ against 0.005 M tris-phosphate buffer, pH 8.0 and applied to a 3×40 cm column. Elution was performed with stepwise increases in ionic strength of tris-phosphate buffer pH 8.0. Fractions were collected and treated in the same way as in the gel filtration procedure described. All paraproteins used were homogeneous in agar electrophoresis and situated in the γ -globulin region. Immuno-electrophoretically the paraproteins were of 7S γ -globulin nature.

Trypsin ($2 \times$ cryst. containing 50 % MgSO_4) was a commercial preparation obtained from Worthington Biochem. Corp., New Jersey.

Antisera. Rabbit antisera against human 7S γ -globulin were prepared by weekly subcutaneous injections of an emulsion of γ -globulin in sterile paraffin oil. The animals were bled once a month. A commercial rabbit anti- γ -globulin serum from Behringwerke AG, Marburg/a. Lahn, Germany, was also employed.

Anti-blood plasma sera were prepared in sheep and goats by injection of fresh blood plasma with an immunization schedule similar to that used for production of anti- γ -globulin sera.

METHODS

Tryptic hydrolysis was performed in a pH-stat as described earlier.¹¹ Enzyme:substrate ratios of 1:25 were used.

Gel filtration was performed with gels of different cross-linkings (Sephadex G-75, G-100 and G-200). Three types of Sephadex G-75 with different particle size (*coarse*, *medium* and *fine*) were also used. Filtrations were usually performed in a buffer medium, containing 0.02 M tris-HCl, pH 8.0 and 0.2 M sodium chloride. In experiments where the quantitative composition of the hydrolysate was analysed, tris-HCl was substituted by phosphate-buffer. The light absorption at $254\text{ m}\mu$ of the effluent was continuously recorded by a "Uvicord" ultra violet absorptiometer (LKB-produkter AB, Stockholm). The effluent was then collected in fractions of suitable size and the optical density at $280\text{ m}\mu$ determined with a Zeiss PMQ II spectrophotometer. Constant flow rates, when necessary, were obtained by a peristaltic pump. Pooled fractions from the gel filtration experiments were dialysed against ice-cold de-ionised water for 48 h and lyophilised.

Protein determinations were performed by a micro Kjeldahl technique.

Agar gel electrophoresis was performed on microscope slides in an apparatus from LKB-Produkter AB, Stockholm. Agar gels containing 1 % Difco Noble agar were used. Electrophoretic runs were performed for 60 min with a potential gradient of 8 V/cm in sodium barbital buffer (pH = 8.4; $I = 0.05$).

Starch gel electrophoresis was carried out as described by Poulik.¹³

Immunological techniques. Immuno-electrophoresis and comparative immuno-electrophoresis were performed as described by Wadsworth and Hanson.¹⁴ The double diffusion-n-gel analyses were made as the micromodification described by Wadsworth.¹⁵

RESULTS

The separation of tryptic hydrolysates of normal 7S γ -globulin on Sephadex G-75 was studied using three commercially available types of Sephadex G-75 with different particle sizes (G-75, *coarse*, *medium* and *fine*). The separations of γ -globulin with these different gels were performed under comparable conditions.

It was found that Sephadex G-75 *coarse* gave a very poor separation (Fig. 1 A), while Sephadex G-75 *medium* gave a satisfactory separation (Fig. 1 B). With Sephadex G-75 *fine* an excellent separation was obtained (Fig. 1 C). The use of the last mentioned gel, however, seems to be greatly restricted by the very low flow-rates obtainable.

Fig. 1. Gel filtration of tryptic hydrolysates of normal human γ -globulin on Sephadex G-75. Column dimensions 3×50 cm. Application of about 100 mg material.

- A. Filtration on Sephadex G-75 *coarse*. Flow rate 8 ml/h.
 B. Filtration on Sephadex G-75 *medium*. Flow rate 8 ml/h.
 C. Filtration on Sephadex G-75 *fine*. Flow rate 3 ml/h.

The yield of material calculated from nitrogen determinations on fractions from Sephadex G-75 medium were found to be 100 % and 101 % of the applied amount in two experiments. The distribution of nitrogen in per cent of the applied nitrogen in the different gel filtration fractions is seen in Table 1.

Table 1. Nitrogen analyses of gel filtration fractions obtained from Sephadex gels with different cross linkings. The gel filtrations were performed on 3×60 cm columns in 0.02 M sodium phosphate buffer containing 0.2 M sodium chloride. Samples from one γ -globulin hydrolysate containing 24.7 mg nitrogen were applied to each column. The values are expressed in per cent of total nitrogen applied.

	Fraction				% Recovery of applied nitrogen
	I	II	III	IV	
Sephadex G-75 <i>medium</i>	32.8	38.1	4.2	25.9	101.0
» » »	31.1	37.4	3.8	27.8	100.1
Sephadex G-100	33.4	35.3	4.4	29.2	102.3
Sephadex G-200	29.2	40.5	31.7	—	101.4

Sephadex G-100 and Sephadex G-200 were also tested for separating ability. With Sephadex G-100 an enhanced resolution compared to Sephadex G-75 *medium* was obtained (Fig. 2 A). The peaks were somewhat asymmetric. This gel also had very good flow-rate properties. Using Sephadex G-200 the gel filtration diagram seen in Fig. 2 B was obtained. The maximal flowrates obtained with this gel were low compared to Sephadex G-100.

Fig. 2. Gel filtration of tryptic hydrolysates of normal human γ -globulin.

- A. Filtration on Sephadex G-100, 3×60 cm. 100 mg of material applied. Flow rate 8 ml/h.
- B. Filtration on Sephadex G-200, 3×100 cm. 200 mg of material applied. Flow rate 12 ml/h.

The agar electrophoretic patterns obtained by the fractions I—III from Sephadex G-75 *medium* and G-100 were quite similar (Fig. 3). Agar electrophoretic analyses of the fractions from Sephadex G-200 showed that fractions I and II were electrophoretically comparable to the corresponding fractions obtained with Sephadex G-75 and G-100 while fraction III probably contained low-molecular material only. The recovery of applied material on Sephadex

Fig. 3. Agar electrophoresis of the fractions obtained by gel filtration of normal 7S γ -globulin and of a myeloma protein fraction (M.P.) from three patients after degradation by trypsin. Gel filtration performed on Sephadex G-75 medium. The same patterns were obtained for normal γ -globulin hydrolysates gel filtered through Sephadex G-100. (Agar electrophoresis of the gel filtration fraction III from the myeloma protein fractions designated V.K. and S.S. could not be performed due to denaturation during the protein concentration procedure.)

Fig. 4. Starch gel electrophoresis of fractions from a tryptic γ -globulin hydrolysate after gel filtration on Sephadex G-75 medium.

G-100 and G-200 was found to be quantitative as determined by Kjeldahl analyses (Table 1).

The fractions from gel filtration were also investigated by means of starch gel electrophoresis. Fractions I—III from Sephadex G-75 and G-100 gave patterns which are seen in Fig. 4. In fraction I only a broad zone around the starting line was observed. Fraction II was very heterogeneous with at least seven bands moving toward the anode and several poorly separated bands moving cathodically. Fraction III was also heterogeneous with five to seven bands moving cathodically and a few diffuse zones moving toward the anode.

A few samples of isolated paraproteins were also subjected to tryptic hydrolysis and gel filtration. No significant differences in the gel filtration diagrams compared to the diagrams obtained with hydrolysates of normal

γ -globulin were found. The agar gel electrophoretic patterns of the gel filtration fractions, however, differed obviously from the patterns of normal γ -globulin fractions (Fig. 3).

Immuno-electrophoretic analyses were performed on the gel filtration fractions of the tryptic hydrolysate of normal γ -globulin. Identical patterns

Fig. 5. Immuno-electrophoretic patterns obtained with gel filtration fractions I—III on Sephadex G-75 and G-100 using various immune sera:
 (A) (D) and (E) different *rabbit* anti- γ -globulin sera, (B) and (C) two *goat* anti- γ -globulin sera and (F) a *sheep* anti-blood plasma serum. Hypothetical determinants are indicated. (G): Schematic representation summarizing the various γ -globulin determinants found in each of the three fractions.

The antigenic determinants forming the precipitates are indicated by letters a to h.

were obtained with gel filtration fractions I and II from gel filtrations on Sephadex G-75, G-100, and G-200. The patterns of fraction III from G-75 and G-100 were also identical while fraction III from G-200 contained no immunoprecipitating material.

The antigenic determinants of the three fractions obtained after separation of the tryptic hydrolysate on Sephadex G-75 *medium* were studied using a large number of antisera. A pronounced variability was observed in the precipitation patterns formed by several of these sera (Fig. 5). These precipitation patterns were compared and interpreted according to the type patterns suggested by Ouchterlony.¹⁶

A few of the immune sera formed the patterns illustrated in Fig. 5 A. Comparative immunoelectrophoretic studies of the three fractions showed antigenic determinants common to the components of these fractions (an example of such experiments is shown in Fig. 6). The smallest number of

Fig. 6. Exemplification of a comparative immunoelectrophoretic analysis. Gel filtration fractions I and II were run electrophoretically and compared immunologically with fraction II using a rabbit anti- γ -globulin serum.

different determinants (*a*, *b*, *c*) that might explain these patterns on the basis of the comparative experiments is indicated in Fig. 5 A.

A goat antiserum to human γ -globulin formed the patterns shown in Fig. 5 B with the three gel filtration fractions. This antiserum gave no precipitate with fraction III. This may be due to a lack of antibodies to the presumed determinants *a*, *b* and *c* of the components of this fraction. The formation of a precipitate with the fast-moving component of fraction II might be due to the presence in the antiserum of antibodies against another determinant *d* of the fast component. Antibodies against the determinant *d* also form the major precipitate of fraction I as indicated by comparative experiments. The small cathodic precipitate of fraction I, however, should be formed by antibodies to another determinant *e*, as the antiserum probably did not contain antibodies against the determinants *a*, *b*, and *c*. The reaction of partial interference or of inhibition between the two precipitates of fraction I should indicate that the determinant *e* is present on both of the components forming these precipitation lines.

Another goat antiserum formed the patterns shown in Fig. 5 C with the three gel filtration fractions. The determinant *d* was found in these precipitates too as demonstrated by comparative studies with the goat serum employed in the experiment of Fig. 5 B. In addition, however, the second goat antiserum (Fig. 5 C) should also contain antibodies against another determinant *f* to form the small anodal precipitate with fraction I. Due to the reaction of partial interference or of inhibition between the two precipitates of fraction I the determinant *f* should be present on both of the components forming these precipitates.

A rabbit anti- γ -globulin immune serum gave the precipitation patterns illustrated in Fig. 5 D. Comparative studies of this rabbit serum with the antisera described above indicated that the rabbit antiserum contained antibodies against the determinant *a*, but not against the *b*, *c*, and *d*. To explain the pattern obtained with this antiserum the presence of a further determinant called *g* on the fast moving component of fraction II has to be suggested.

One of the rabbit antisera obviously contained antibodies to a determinant (*h*) common to the fast and slow components of fraction II (Fig. 5 E).

The patterns shown in Fig. 5 F were formed by a sheep anti-blood plasma serum. This antiserum was found to have antibodies against the determinant *a*, giving the precipitate of the third fraction. Comparative studies gave evidence that the precipitate of the fast-moving component of the second fraction contained the determinant *d*. The slow-moving fragment of fraction II formed two parallel precipitates, one of which might have been formed with antibodies to the determinant *a*. The other parallel precipitate gave a reaction of partial interference or of inhibition with the line formed by the fast component. This might be due to the presence in the antiserum of antibodies against the determinant *h* which was found to be common to the fast and the slow fragments of fraction II (*cf.* Fig. 5 E). With fraction I this sheep antiserum formed two main parallel precipitates and in addition the two minor ones also observed with other antisera (*cf.* Figs. 5 A—F). The minor anodical line might have been formed by antibodies to the determinants *f* or *g*. It was not possible to obtain decisive experimental evidence to support this supposition. The minor cathodically situated precipitate could be shown to be formed by antibodies to the determinant *a*. The two parallel precipitates may be due to the presence of several different determinants on the components forming these precipitates, possibly occurring on different molecules. This sheep antiserum regularly forms two and often three parallel lines with native γ -globulin.

All of these various antigenic determinants of normal γ -globulin are summarized in Fig. 5 G. The smallest number of determinants that might possibly explain the precipitation patterns in Figs. 5 A—F are illustrated. Each determinant and its probable distribution on the different components of the three gel filtration fractions is represented by a line in the diagram.

Immunoelectrophoretic analyses of the gel filtration fractions of the myeloma proteins showed principally the same patterns as the corresponding fractions from normal γ -globulin, with the exception of some changes of the shape of the precipitates due to quantitative differences.

Comparative double diffusion studies of the fractions of the normal and pathologic γ -globulins showed that fractions I and II of the myeloma proteins were antigenically deficient compared to the corresponding normal material. No such deficiency could be demonstrated for the components of the third gel filtration fractions of the myeloma protein.

DISCUSSION

Gel filtration gives a separation of proteolytic breakdown products of γ -globulin mainly correlated to differences in molecular sizes.¹¹ As shown by earlier studies as well as by the present investigation a dominant part of the separated material is of relatively high molecular weight, comprising about 70 % of the total hydrolysate. Sephadex G-100 was found to be the most suitable gel for the separation compared to Sephadex G-75, medium, and Sephadex G-200. The latter gel gave a considerable retardation of native γ -globulin, and seems to be more suitable for separation of undegraded immune globulins of different molecular sizes.¹⁷ A resolution into several fractions of the slightly asymmetric peak I obtained by Sephadex G-100 might be obtained by "recycling chromatography".¹⁸ Such studies are now in progress.

The tryptic hydrolysis of γ -globulin has certain similarities to the degradation of γ -globulin with papain. Gel filtration of papain hydrolysates of human γ -globulin yields two peaks with similar immunoelectrophoretic patterns as the two corresponding fractions obtained by gel filtration of tryptic hydrolysates of γ -globulin. The third peak, however, obtained by gel filtration of tryptic hydrolysates is not obtained after the action of papain. This fraction has proteolytic activity and contains material precipitating with anti- γ -globulin immune sera.¹¹ It is probable that papain (and other sulfhydryl-activated proteases) gives a more limited (specific) cleavage of the γ -globulin molecules than trypsin.¹⁹

Important information about the structure of 7S γ -globulin has been obtained by studies of subunits or fragments of γ -globulin molecules. These studies have been performed by the cleavage of disulfide bonds cross-linking the polypeptide chains,^{20,21} or by the fragmentation of the molecules by proteolytic enzymes.²⁻⁹ On the basis of these findings as well as from studies of multiple myeloma proteins Edelman and Benacerraf²² have recently presented a hypothetical pattern of the structure of the antibody molecule. The γ -globulin should consist of two high molecular weight polypeptide chains ("heavy chains") probably making up the fast moving (F) fragment obtained by papain degradation.³ The slow moving (S) fragment which has antibody activity should consist of two chains of lower molecular weight ("light chains") possibly with a portion of "heavy chains" as well. The heterogeneity of γ -globulin seems to be confined mainly to the "light chains" which in starch gel electrophoresis give somewhat different patterns for antibodies of different specificities.^{23,24}

The heterogeneity of the S fragment has been further illustrated by Mannik and Kunkel and others. They demonstrated two antigenically different groups of normal human γ -globulin. This antigenic difference was located to the S-fragment. The genetic variation of human γ -globulin found in the InV-system²⁶ has also been shown to be confined to the S-fragment, whereas the characteristics of the Gm-groups are confined to the F-fragment.^{27,28}

It is conceivable that all these cited antigenic characteristics might be reflected in the complex mosaic of antigenic determinants of the 7S γ -globulin. Therefore it could be of interest to try to simplify this antigen mosaic by relating the demonstrable antigenic determinants to the smaller pieces of the γ -globulin molecules obtainable by different means. The present note indicates

the possibility of illustrating the localisation of some of these determinants to the fragments produced by tryptic degradation of the γ -globulin. For further studies of the smaller structural parts of the γ -globulin molecules it might be possible to use differently localised determinants as "markers" for the identification or recognition of the various fragments.

Preliminary experiments with a few cases of multiple myeloma of γ -globulin type showed that the antigenic deficiency found in the M-proteins of such cases compared to normal γ -globulin could be confined to the fragments of the first and second gel filtration fractions. The small fragments found in the third gel filtration fractions of the degraded myeloma proteins could not be shown to be antigenically deficient compared to the corresponding fragments of normal γ -globulin. This might indicate a possibility for further localisation of some of the characteristics of these proteins.

Acknowledgements. We are greatly indebted to Mrs. Anita Larsson and to the Misses Anita Wallin and Marianne Wikström for skilful technical assistance. The investigation was supported by a grant from the *Swedish Medical Research Council*.

REFERENCES

1. Porter, R. R. *Biochem. J.* **73** (1959) 119.
2. Edelman, G. M., Heremans, J.-F., Heremans, M.-Th. and Kunkel, H. G. *J. Exptl. Med.* **112** (1960) 203.
3. Heremans, J.-F. *Med. Diss.* Masson et Arscia, Paris/Bruxelles 1960.
4. Franklin, E. C. *J. Clin. Invest.* **39** (1960) 1933.
5. Augustin, R. and Hayward, B. J. *Nature* **187** (1960) 129.
6. Skvaril, F. *Nature* **185** (1960) 475.
7. Shu-Hsi Hsiao and Putnam, F. W. *J. Biol. Chem.* **236** (1961) 122.
8. Schultze, H. E. and Schwick, G. *Behringswerke-Mitteil.* **33** (1957) 11.
9. Burtin, P. in *Protides of the Biological Fluids, Proceeding of the 8th Colloquium, Bruges 1960, Elsevier Amsterdam 1961*, p. 119.
10. Hanson, L. Å. and Johansson, B. G. *Nature* **187** (1960) 599.
11. Hanson, L. Å. and Johansson, B. G. *Clin. Chim. Acta* **8** (1963) 66.
12. Cederblad, G., Hanson, L. Å. and Johansson, B. G. *Clin. Chim. Acta* **8** (1963) 78.
13. Poulik, M. D. *Nature* **180** (1957) 1477.
14. Wadsworth, C. and Hanson, L. Å. *Intern. Arch. Allergy* **17** (1960) 165.
15. Wadsworth, C. *Intern. Arch. Allergy* **10** (1957) 355.
16. Ouchterlony, Ö. *Progress in Allergy, Vol. 6.* Karger, Basel/New York 1962.
17. Killander, J. and Flodin, P. *Biochim. Biophys. Acta* **63** (1962) 403.
18. Porath, J. and Bennich, H. *Arch. Biochem. Biophys. Suppl.* **1** (1962) 152.
19. Putnam, F. W., Easley, C. W. and Lynn, L. T. *Biochim. Biophys. Acta* **58** (1962) 279.
20. Edelman, G. M. *J. Am. Chem. Soc.* **81** (1959) 3155.
21. Edelman, G. M. and Poulik, M. D. *J. Exptl. Med.* **113** (1961) 861.
22. Edelman, G. M. and Benacerraf, B. *Proc. Natl. Acad. Sci. U.S.* **48** (1962) 1035.
23. Benacerraf, B., Ovary, Z. and Poulik, M. D. *Proc. Natl. Acad. Sci. U.S.* **47** (1961) 1751.
24. Grossberg, A. L., Stelos, P. and Pressman, D. *Proc. Natl. Acad. Sci. U.S.* **48** (1962) 1203.
25. Mannik, M. and Kunkel, H. G. *J. Exptl. Med.* **117** (1963) 213.
26. Steinberg, A. G., Wilson, J. and Lanset, S. *Vox. Sang.* **7** (1962) 151.
27. Harboe, M., Osterland, C. K. and Kunkel, H. G. *Science* **136** (1962) 979.
28. Franklin, E. C., Fudenberg, H., Meltzer, M. and Stanworth, D. R. *Proc. Natl. Acad. Sci. U.S.* **48** (1962) 914.

Received July 12, 1963.