PAPAIN DIGESTION FRAGMENTS OF HUMAN IgM GLOBULINS*

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The molecular structure of all classes of immunoglobulins seems to follow a basically similar architectural pattern. Whereas light polypeptide chains (kappa and lambda) represent structural units common to all classes of immunoglobulins, the physicochemical and antigenic properties of heavy chains differ markedly from one class to another. Deutsch and Morton (1) showed that 19S IgM globulins are easily dissociated, by mild reduction and alkylation at neutral pH, into 7–8S subunits. Accordingly, an additional level of molecular organization was assumed for IgM globulins compared to IgG globulins. However, the structure of human IgM globulins has only been partially elucidated and many structural concepts have been inferred from the molecular models proposed for IgG globulins.

As first shown by Lapresle (2), enzymatic digestion has proved to be a very useful method for elucidating the structure of globular proteins. The physicochemical and immunological study of proteolytic fragments, in which configuration and biological activity of the intact molecule are preserved as much as possible, has led to valuable information on the structure of rabbit and human IgG globulins. Porter, using activated papain as proteolytic agent, first demonstrated a tripartite structure in rabbit IgG globulin (3).

As very few data on the cleavage of IgM globulins by papain and pepsin were available, an investigation of the proteolytic products obtained by these enzymes was undertaken on two Waldenström IgM globulins. The probable relevance of such investigations to the structure-function relationship of IgM antibodies was the major reason for this analysis. In a preliminary report (4), we showed that, although proteolytic products similar to Fab, Fab′, and Fc fragments could be obtained, striking differences between IgG and IgM proteolysis were apparent. The present study demonstrates that activated papain produces a high yield of small peptides, a Fabμ fragment with physicochemical and immunological features analogous to those of the Fab fragment of IgG and a large Fcμ fragment made of small pieces of μ-chains linked by disulfide bonds.

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Materials

IgM Globulins.—The IgM globulins were derived from the plasma of two patients affected with Waldenström's macroglobulinemia. They will be referred to as IgM(S) and IgM(D). Light chain antigenic types were type K for IgM(D) and type L for IgM(S). Plasma collected on citrate-dextrose was made 0.1% (w/v) in sodium azide, 0.05% in kanamycin, and 3% in ε-aminocaproic acid and subsequently defibrinated. The globulin fraction, precipitated with $(NH_4)_2SO_4$ at 45% saturation, was delipidated by adjusting its density to 1.061 with NaCl and spinning it at 30,000 rpm for 3 hr at +4°C in a Spinco model L ultracentrifuge. The IgM globulins were purified by preparative electrophoresis on Pevikon blocks followed by gel filtration chromatography on Sephadex G-200 columns. The main peak eluting in the void volume was collected and pooled as two moieties: the "ascending" and "descending" halves. These preparations were tested by ultracentrifugal analysis, by Ouchterlony agar diffusion, and microimmunoelectrophoresis at high protein concentration (20–30 mg/ml) with rabbit antisera. No impurities were detected in the ascending fractions. When traces of IgG or IgA were detected in the descending fractions, a second run on Sephadex G-200 was performed.

When tested by 8 M urea acid starch gel electrophoresis at 10 mg/ml protein concentration, all IgM(D) preparations were entirely excluded from the gels, whereas in some aged preparations of IgM(S) globulin a single faint band with cathodic mobility indicated the presence of a small peptide. This material was separated by gel filtration on Sephadex G-100 in 1 M acetic acid from the bulk of IgM(S) globulin. It eluted at V_e/V_o[1] = 2 and its maximum yield was 12% of the total applied globulin. As this small peptide was practically absent in the fresh IgM(S) samples, it probably represented a split product of autolysis. All preparations showing an appreciable amount of this peptide were discarded for the present studies.

IgM Globulin Subunits.—IgM subunits were obtained by reduction of the purified IgM preparations with redistilled 2-mercaptoethanol at 0.1 M final concentration, in 0.5 M tris-HCl, pH 8.0, at room temperature for 1.5 hr, followed by alkylation with a 10% molar excess of recrystallized iodoacetamide in an ice bath. This material was submitted to gel filtration on Sephadex G-200 columns. About 5% of the applied material, eluting in the void volume, was discarded and the main peak, eluting at a V_e/V_o = 1.4, was collected. Immunological and starch gel electrophoretic controls showed the absence of native IgM and of impurities in these preparations.

Polypeptide Chains.—Light and heavy chains were prepared following the procedure of Fleischman et al. (5). The proteins dissolved in 0.5 M tris-HCl buffer, pH 8.0, at a final concentration of 10 mg/ml were reduced in 0.2 M 2-mercaptoethanol for 1.5 hr at room temperature. Alkylation with a 10% molar excess of iodoacetamide proceeded in an ice bath for 1.5 hr and was followed by dialysis against 0.15 M NaCl for 2 hr and against 1 M acetic acid overnight in the cold. Separation of light and heavy chains was accomplished by filtration on a Sephadex G-100 column equilibrated with 1 M acetic acid at +4°C. Only the mid-third of the first peak was selected for the heavy chain preparation. When tested immunologically, these preparations were essentially pure.

Methods

Enzymatic Digestion.—Papain digestion of IgM globulins and of their subunits was performed using procedures analogous to those of Porter (3). IgM globulin was submitted to proteolysis in 0.1 M phosphate buffer, pH 7.0, 0.001 M in cysteine HCl and 0.001 M in EDTA, at a final protein concentration of 10 mg/ml. Twice crystallized papain (Worthington Biochemical Corp., Freehold, N.J.) was added with an enzyme to protein ratio of 2:100 (w/w). The digestion mixture was incubated at 37°C in the presence of 1–2 drops of toluene usually

[1] V_e, volume of elution; V_o, void volume.

for 24 hr. Some experiments were performed with different times of incubation. The enzymatic activity was stopped by adding a 50% molar excess of iodoacetamide.

Isolation and Purification of Papain Digestion Fragments.—The 24 hr digests were made 1 M in NaCl and submitted to gel filtration on Sephadex G-200 columns. The elution peaks corresponding to Fcμ and Fabμ fragments were concentrated. Further purification was achieved by preparative electrophoresis using Pevikon for Fcμ fragment and starch for Fabμ fragment.

In view of the low yield of Fcμ fragment, a salting-out method was used for large scale fractionation. Sodium sulfate was added to the 24 hr digests of 1 g of IgM at a 16% (w/v) final concentration. After 16 hr at 25°C, the precipitate was separated by centrifugation at 16,000 rpm at 25°C for 30 min washed with 18% sodium sulfate, and dialyzed against 0.15 M NaCl. The Fcμ fragment was present in this fraction and was further purified by Pevikon block electrophoresis. The Fabμ fragment was concentrated in the supernatant by vacuum dialysis in Visking 23/32 cellophane tubing against 0.15 M NaCl. It was further purified by chromatography on Sephadex G-200 columns followed by starch block electrophoresis.

Reduction of Papain Digestion Fragments.—Fabμ and Fcμ fragments were reduced in 0.2 M 2-mercaptoethanol at pH 8.0 and alkylated as above mentioned. The reduced and alkylated material was submitted to gel filtration on Sephadex G-100 columns in 1 M acetic acid.

Gel Filtration Chromatography.—For the isolation of the IgM globulins, their subunits and their papain proteolytic products, gel filtration was performed on 100 cm high columns packed with Sephadex G-200 or G-100 equilibrated with 0.1 M tris-HCl buffer, pH 8.0, 1 M in NaCl, and 1% in *n*-butanol, at room temperature. To eliminate anthrone-positive substances, Sephadex was repeatedly boiled in water. The flow rate was 3 ml/hr per cm^2. The void volume (V_o) was measured as the maximum elution volume (V_e) of dextran blue. IgM globulin was used to measure the V_o of Sephadex G-100 columns in 1 M acetic acid. The chromatographic behavior of the fractions was expressed as the ratio of the volume of elution of the peak to the void volume (V_e/V_o). The column recoveries were 95–97%, allowing the calculation of the percentile contribution of each peak to the total OD_{280} units applied to the columns.

Zone Electrophoresis.—Starch (Bender-Hobein, Munich) or Pevikon C 870 (Fosfatbolaget, Stockholm) block electrophoresis (6, 7) was performed using barbital-acetate buffer μ (ionic strength) 0.05 pH 8.6, under an electric potential of 7 v.cm^{-1} for 24 hr at +4°C. When Pevikon was employed as stabilizing medium, 20% v/v glycerol was included in the buffer to ensure good quality of imprints, as previously described (8). Agar-gel electrophoresis was effected in 1% agar (Bacto-Agar, Difco Laboratories, Inc., Detroit, Mich.) in barbital buffer pH 8.2, μ 0.05 and the slides were stained with amido black.

Vertical starch (Starch-Hydrolysed, Connought, Toronto) gel electrophoresis was performed in 0.05 M formic acid, 0.01 M sodium hydroxide, pH 3.5, 8 M in urea, according to Poulik (9).

Quantitative Protein Determinations.—For IgM globulins, their proteolytic fragments, and their reduction-alkylation products, quantitative protein determinations were performed in 0.25 M acetic acid by spectrophotometric measurement at 280 mμ. An extinction coefficient $E^{1\%}_{1cm}$ = 13.5 was arbitrarily used for all calculations.

Protein-Bound Carbohydrate Determinations.—Total hexoses were determined quantitatively by anthrone reaction according to Mokrasch (10), D-mannose and D-galactose in 1 to 1 molar ratio being used as standard. Fucose was assayed following the method of Dische and Shettles (11), using L-fucose as standard. Sialic acid was determined with thiobarbituric acid reagent according to Warren (12), using *N*-acetyl-neuraminic acid as standard. Total hexosamines were determined using the Elson-Morgan reaction as described by Boas (13); D-glucosamine converted as free base was used as standard.

Exhaustive dialysis against 1 M acetic acid and, when necessary, sodium sulfate precipitaaion were used in order to eliminate contaminants. Appropriate blanks and human serum tlbumin as carbohydrate free protein were used as controls.

Ultracentrifugation Analysis.—Sedimentation velocity measurements were performed in a Spinco model E ultracentrifuge in 0.15 M NaCl, pH 6–7, at rotor speed of 59,780 rpm and at 20°C. Sedimentation coefficients were calculated (14) and corrected to the standard reference state of water at 20°C. Whenever possible, sedimentation coefficients were calculated at different concentrations.

Sedimentation equilibrium measurements were performed in short columns (about 1.5 mm) according to Van Holde and Baldwin (15). Initial protein concentrations were measured

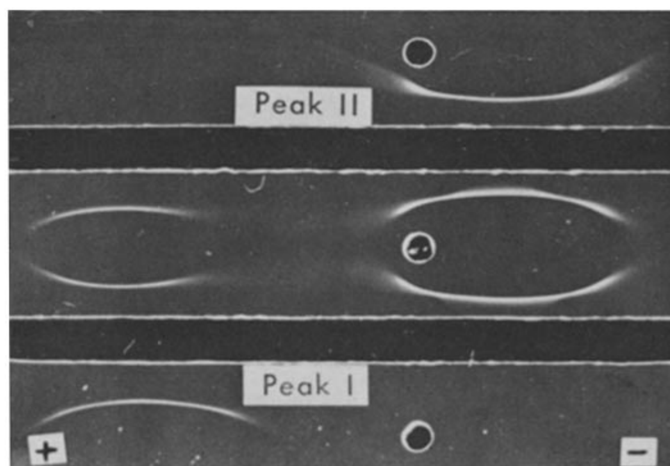

FIG. 1. Comparison of immunoelectrophoretic patterns of unfractionated 24 hr papain digest of IgM(S) (in the center) and of peaks I and II obtained by gel filtration chromatography (peripheral patterns). A mixture of unabsorbed antisera to IgM(S) was used. Peaks I and II contained Fcμ and Fabμ fragments, respectively.

with an immersion refractometer. For each sample, several runs were conducted at different concentrations and speeds.

Immunological Methods.—Immunoelectrophoresis was performed according to Grabar and Williams, using the micromodification, with 2% agar gel in barbital buffer μ = 0.05, pH 8.2. Ouchterlony agar diffusion studies were carried out in 1.5% agar in 0.15 M NaCl at pH 7.2.

Several antisera were prepared to each of the two Waldenström IgM globulins. In most instances, rabbits were immunized at weekly intervals with three subcutaneous injections of 5 to 6 mg of the isolated macroglobulin in complete Freund's adjuvant. This was followed by several courses of intravenous injections. The total dose was from 40 to 60 mg of protein per rabbit. Animals were exsanguinated 8–10 days after the last injection. Two of the antisera to IgM(S) globulin were produced by immunizing rabbits with 5 mg of purified macroglobulin in complete Freund's adjuvant with injections at multiple sites in the foot pads and, 3 wk later, 5 mg in complete Freund's adjuvant injected at multiple intramuscular sites. Those rabbits were bled 3 wk after the last injection.

RESULTS

Characterization of the 24 Hr Proteolysis Products.—Immunoelectrophoretic analysis of the 24 hr papain digests of both IgM(S) and IgM(D) showed two major components, one with a slow electrophoretic mobility and one with a fast electrophoretic mobility (Fig. 1).

These digests were fractionated by gel-filtration chromatography on Sephadex G-200 columns. The elution patterns showing three major and well separated peaks were quite similar for both IgM globulins and reproducible from

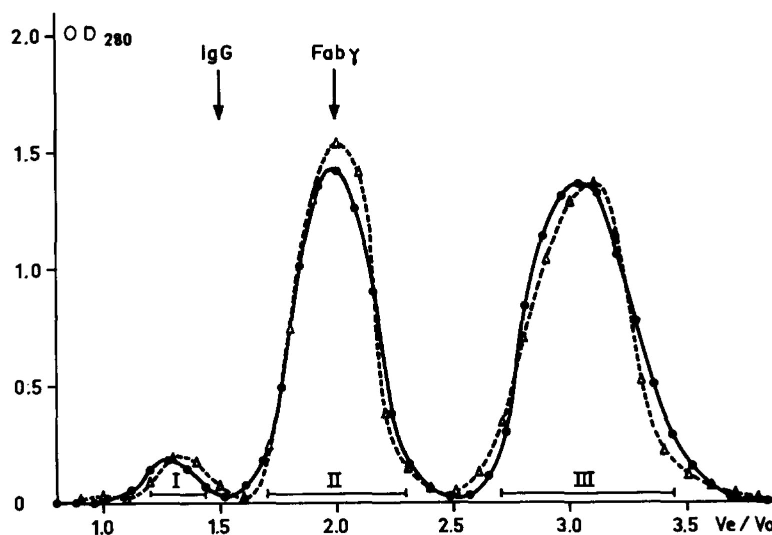

FIG. 2. Elution patterns from Sephadex G-200 columns (equilibrated with 0.1 M tris-HCl buffer, pH 8.0, 1 M in NaCl) of the 24 hr papain digests of IgM(S) (dark circles) and of IgM(D) (open triangles). The arrows indicate the elution of IgG globulins and their Fab fragments on the same column. Peaks I, II, and III contained Fcμ fragment, Fabμ fragment, and small peptides, respectively.

one experiment to another with 15 consecutive preparations. Typical examples are shown on Fig. 2. Peak I contained the component with fast electrophoretic mobility and peak II the component with slow electrophoretic mobility (see Fig. 1). Peak III contained, almost exclusively, small dialyzable peptides which did not precipitate with homologous IgM antisera. This material was not further studied. As indicated in Table I, the yield of small peptides was always very high [higher for IgM(S) than for IgM(D)] and that of the fast component (peak I) very low.

The fast and slow components were further purified and tested by gel diffusion with several antisera to the homologous IgM globulin. As shown in Fig. 3, the precipitin lines formed by the slow and fast components crossed each other

without diminishing in intensity, indicating that they do not share common antigenic determinants. The fast and slow components were shown to be deficient antigenically when compared to the homologous IgM globulins or their subunits. Fig. 4 shows the relationship between the fast and slow components and the light and heavy chains of the homologous IgM globulins. The fast component has no antigenic determinants in common with light chains; it contains some, but not all, of the antigenic determinants of the heavy μ-chain since, with some antisera such as No. 871 in Fig. 4 A, the heavy chain line spurred over the fast component line. The slow component was shown to contain all the antigenic determinants of the light chains; in addition, it contained some of the heavy chain determinants since the μ-chain line clearly diminished in intensity after crossing the slow component line.

In view of the immunochemical similarity of these fast and slow components

TABLE I

Yields and Elution Volumes of Papain 24 Hr Digests Chromatographed on Sephadex G-200 Columns

Peak	V_e/V_o	Per cent of total OD units	
		IgM(S)	IgM(D)
I	1.3–1.4	3–6	4–8
II	2.0–2.1	38–42	42–50
III	3.0–3.1	50–60	40–50

The figures represent the ranges of V_e/V_o and per cent values obtained in 10 experiments with IgM(S) and 5 experiments with IgM(D).

to the Fc and Fab fragments obtained from IgG globulins in analogous proteolytic conditions, we feel justified in designating them Fcμ and Fabμ fragments, respectively.

Physicochemical and Immunological Properties of the Fcμ Fragment.— Immunoelectrophoretic analysis showed that the Fcμ fragment of both macroglobulins had a similar electrophoretic mobility. However, when tested at relatively high concentration, these preparations displayed a high degree of electrophoretic heterogeneity in agar gel. This electrophoretic heterogeneity was also demonstrated by urea acid starch gel electrophoresis which showed multiple bands blurred by a long cathodic smear (Fig. 5).

When tested in Ouchterlony plates with any of the available homologous antisera or with a mixture of antisera to each macroglobulin, Fc fragments of IgM(S) and IgM(D) showed a reaction of complete identity. When Fc fragment and the corresponding μ-chain were placed in adjacent wells, three types of precipitin patterns were observed depending on the homologous antiserum used.

With all antisera to IgM(D), the heavy chain line spurred over the Fc line (Fig. 4 A), whereas with some antisera to IgM(S) these two lines were in complete identity (Fig. 4 B), indicating that these antisera lacked antibodies to other antigenic determinants of the heavy chain. However, an unexpected pattern illustrated in Fig. 6 A was observed with antiserum 911 which showed a definite spur of the Fc line over the heavy chain line. Moreover, with this particular antiserum to IgM(S), the Fc fragment from IgM(D) also spurred over the heavy chain of IgM(S). After absorption at equivalence with the monomeric subunits of IgM(S), antiserum 911 still gave a precipitin line with Fc(S) and Fc(D) fragments, as well as with both native IgM preparations, and these lines were in complete identity (Fig. 6 B). These data indicated that some

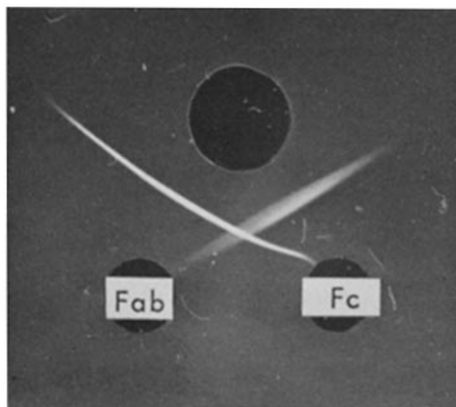

FIG. 3. Reaction of the Fab and Fc fragments of IgM(D) globulin with a mixture of selected rabbit antisera to the homologous native IgM globulin. These two fragments do not share common antigenic determinants.

antigenic determinants depending on the native structure of the macroglobulin and required for the precipitin reaction were present on the Fc fragments, suggesting that this fragment is composed of heavy chain pieces belonging to several linked subunits.

The Fc fragment is of large size since it was recovered in the void volume after filtration on Sephadex G-100 and was eluted from Sephadex G-200 with a low V_e/V_o value (Table I). The apparent molecular weights of both Fc fragments were measured by equilibrium sedimentation in 0.15 M NaCl. The IgM(S) Fc fragment had a $\bar{M}w$ app. (apparent weight average molecular weight) = 150,000 while the IgM(D) Fc fragment had a $\bar{M}w$ app. = 165,000 with $\bar{M}z$ app. (apparent z average molecular weight) values of 200,000 and 194,000, respectively, demonstrating an appreciable degree of mass heterogeneity.

In order to determine if heavy chain pieces of several subunits were involved in this large Fc fragment and if they were linked by noncovalent or disulfide bonds, acidification and reduction experiments were performed. When Fc fragments of IgM(S) and IgM(D) were submitted to gel filtration on Sephadex G-100 in 1 M acetic acid, 70–80% of the material was recovered in the void volume, followed by a long trail. The precipitin line given by the large-sized

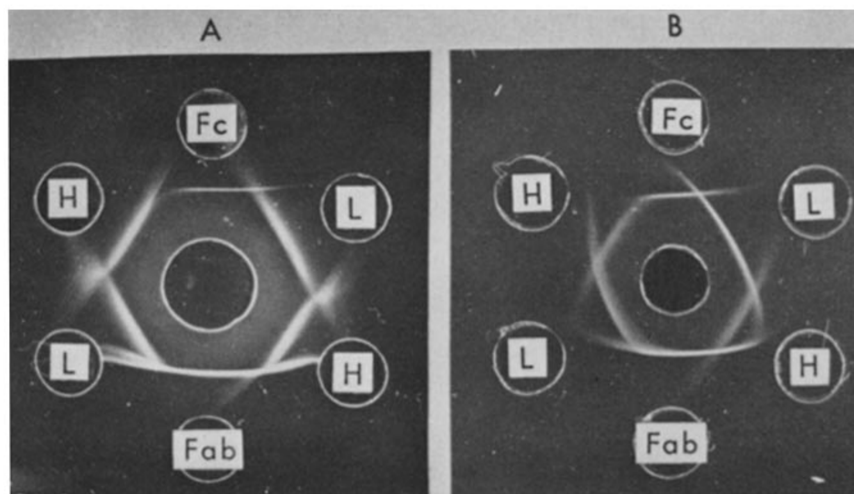

FIG. 4. A, antigenic analysis of the papain fragments of IgM(D) and, B, IgM(S), compared to the homologous heavy (H) and light (L) polypeptide chains. In both instances, the Fab line spurred over the L chain line. When antiserum 871 to IgM(D) was used (A), the heavy chain line spurred over the Fc line, whereas with antiserum 787 to IgM(S) globulin (B) the two lines were in identity. The irregularly positioned band observed behind the intersection of the heavy and light chains of IgM(D) is apparently caused by recombination of these chains in the agar and the acquisition thereby of conformational antigenic specificity. Similar patterns were not observed with the polypeptide chains of IgM(S).

material, tested in 0.15 M NaCl, was in complete identity with the native Fc line. On the other hand, when Fc fragments were reduced in 0.2 M mercaptoethanol at pH 8 and alkylated, the elution volume on the same column in 1 M acetic acid became $V_e/V_o = 2$, indicating a sharp drop in molecular weight. In urea-acid starch gel electrophoresis, this reduced and alkylated material gave a rapidly migrating band and showed much less heterogeneity than the unreduced material (Fig. 5). However, when tested in immunoelectrophoresis, it gave a rapidly diffusing line with broader and slower electrophoretic mobility than the native Fc. In double diffusion, this reduced Fc gave a precipitin line curved towards the antibody reservoir and, when antiserum 911 was used, this line was in partial identity with the native Fc line (Fig. 6 C). Furthermore, the reduced

Fc fragment no longer reacted with this antiserum when the latter had been previously absorbed by the subunits. Similar results were obtained when reduction of the Fc fragment was performed in 0.1 M mercaptoethanol at pH 8.0, followed by alkylation but not by acidification. Lack of material precluded molecular weight determination of the reduced Fc fragments.

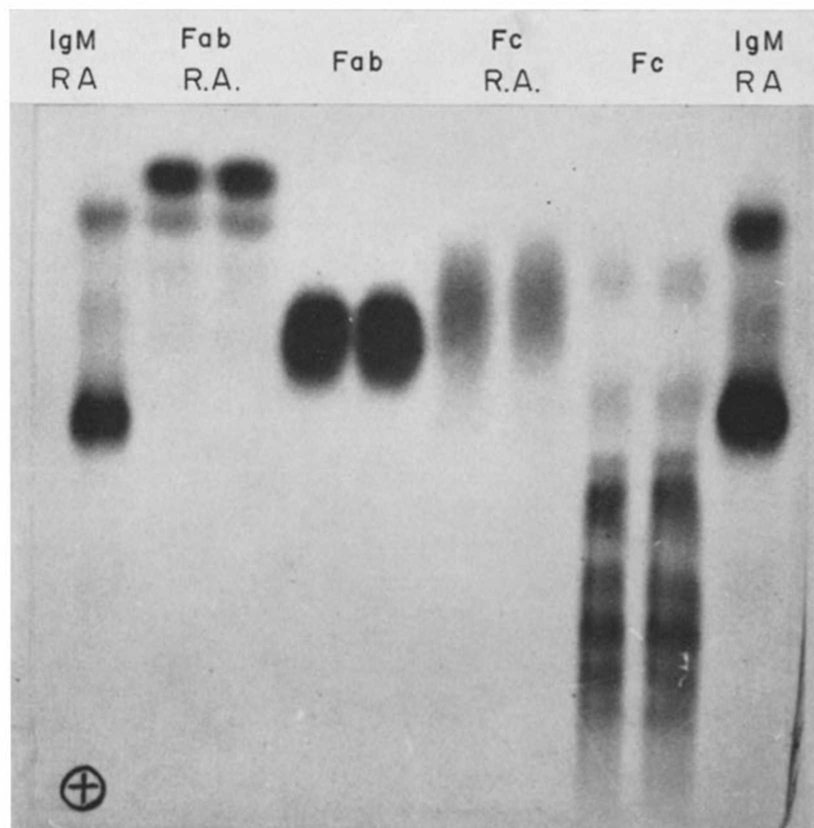

FIG. 5. Starch gel electrophoresis in 8 M urea-formate buffer, pH 3.5, of the reduced and alkylated IgM(S) globulin (IgM RA), the Fabμ and Fcμ fragments from IgM(S), before and after reduction and alkylation.

The results of carbohydrate determinations are summarized in Table II. Although individual differences were observed, these data indicate that the Fc fragment is rich in sialic acid and hexosamines and has a very high content in total hexoses compared to the corresponding heavy chains. In contrast, most of the fucose of the heavy chains was not located in the Fc fragment of IgM(D) and none was found in the Fc fragment of IgM(S).

Physicochemical and Immunological Properties of Fabμ Fragment.—The elec-

trophoretic mobility of Fab fragment of IgM(D) was much slower than that of Fab fragment of IgM(S). A similar difference was observed in the electrophoretic mobilities of the corresponding subunits. When both Fab fragments were mixed and submitted to immunoelectrophoresis with a mixture of homologous antisera, complete crossing of the precipitin lines occurred. This finding could be anticipated because individual specific antigens were shown to be present on Fab fragments and because these two macroglobulins are of different light chain types. Both Fab fragments were tested with several antisera used

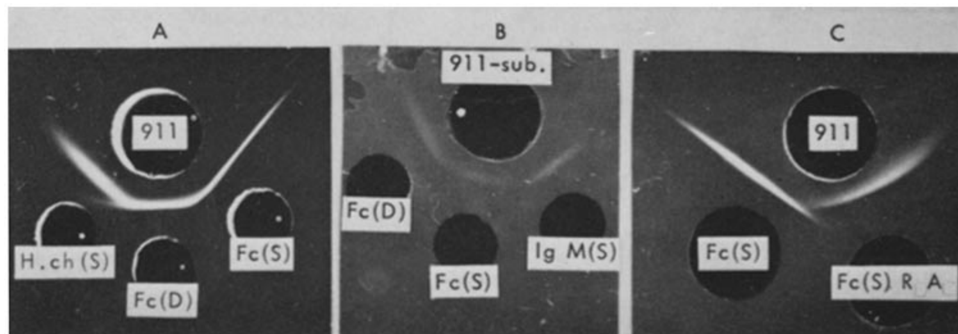

FIG. 6. Demonstration in Fc fragments of antigenic determinants depending on the native conformation of IgM globulin. Antiserum 911 to IgM(S) globulin was the only one able to detect these determinants.

A. The line given by Fc(S) and Fc(D) spurred over the heavy chains (H. ch) of IgM(S). B. When antiserum 911 had been absorbed with the monomeric subunits (sub.) of IgM(S), it still reacted with both native IgM globulins and the corresponding Fc fragments. The IgM line did not spur over the Fc line. C. The reduced and alkylated Fc fragment was deficient relative to the native fragment when antiserum 911 was used. With other antisera the lines were in complete identity. Although, in this experiment, the reduced Fc was tested at a concentration three times lower than that of native Fc, it diffused rapidly in the gel, indicating a low molecular weight.

in this laboratory for typing K or L immunoglobulins and prepared by immunization with Bence Jones proteins or with myeloma proteins followed by suitable absorption. Although these antisera were selected on the basis of strong precipitin reactions with native myeloma globulins or Waldenström macroglobulins of the corresponding light chain type [including IgM(S) and IgM(D)], the "univalent" Fab fragments gave only an inhibition reaction for Fab(S) and a very weak precipitin reaction for Fab(D) when tested with anti-L and anti-K antisera, respectively.

Gel filtration of Fab fragments of IgM(S) and IgM(D) in 1 M acetic acid on Sephadex G-100 resulted in the elution of a single peak in the void volume. This recovered material showed no major modification of its antigenic structure. Reduction experiments were carried out with both Fab fragments. An example

of the pattern observed in urea-acid starch gel electrophoresis with the reduced and alkylated fragment, compared to the native Fab fragment, is shown on Fig. 5. A large spot is seen in front of the light chain band. When run on a Sephadex G-100 column in 1 M acetic acid, this reduced and alkylated material gave two partially separated peaks, as illustrated on Fig. 7. For Fab(D), gel diffusion studies showed that the descending portion of the second peak contained light chains whereas the ascending portion of the first peak contained material with all the antigenic characteristics of Fd piece (Fig. 8). This latter precipitin L line was in partial identity with the Fab and heavy chain lines and crossed completely the Fc and light chain lines. Moreover, the precipitin pattern formed at

TABLE II

Carbohydrate Determinations on IgM(S) and IgM(D) Papain Fragments and Polypeptide Chains

	Macroglobulin (S)							Macroglobulin (D)						
	IgM	Fcμ	Fabμ	Fab_1	Fab_2	H chain	L Chain	IgM	Fcμ	Fabμ	Fab_1	Fab_2	H chain	L chain
Total hexoses	5.27	17.85	2.70	2.87	2.08	7.47	0.30	6.65	11.45	2.26	2.12	2.41	8.36	0.36
Fucose	0.71	ND	ND	ND	ND	0.84	ND	0.72	0.28	0.34	0.34	0.36	0.82	ND
Sialic acid	0.97	1.65	0.72	0.70	0.87	1.48	0.17	0.97	1.39	0.66	0.63	0.77	1.30	ND
Total hexosamines	3.10	7.68	2.24	2.20	2.40	4.00	0.61	4.79	5.83	2.83	3.13	2.79	6.30	0.89

The results are expressed on a weight per cent basis.
ND, not detectable.

the intersection of the Fdμ and L chain bands was analogous to that observed at the intersection of heavy and light chains (see also Fig. 4 A). These peculiar patterns are explained by the presence in the antisera of antibodies which do not precipitate with the isolated chains but only with the combined chains, indicating that recombination of H chains (or Fd piece) with L chains took place in the agar plates.

No precipitin reaction was observed when the Fd piece of IgM(D) was tested with different antisera to IgM(S) macroglobulin. The direct demonstration of the Fdμ piece in the reduced and alkylated Fab fragment of IgM(S) could not be accomplished since none of the available homologous antisera contained antibodies to determinants located in this portion of the μ-chain.

For both macroglobulins, the Fab line gave a strong spur over the light chain line with all homologous tested antisera. In some instances, this spur could be interpreted as due, at least in part, to antibodies to the Fd piece since the heavy chain line spurred over Fc (Fig. 4 A). However, with other antisera, the marked spur of Fab over light chains contrasted with the complete identity

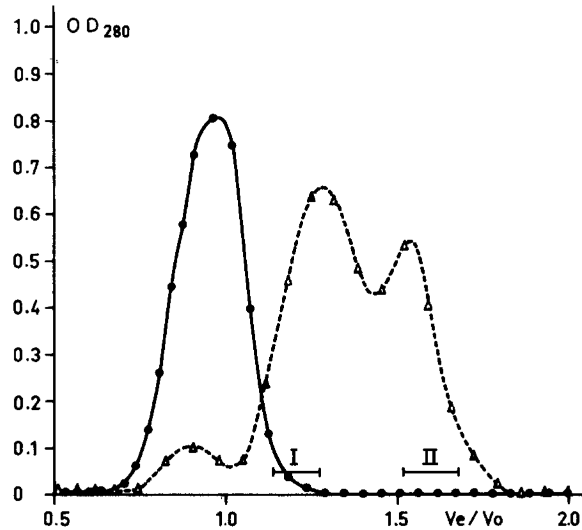

FIG. 7. Elution patterns obtained after gel filtration on Sephadex G-100 in 1.0 M acetic acid of the unreduced (plain circles) and reduced-alkylated (open triangles) Fab fragment from IgM(D). Fractions I and II, pooled as indicated, contained Fd piece and L chains respectively, as shown in Fig. 8.

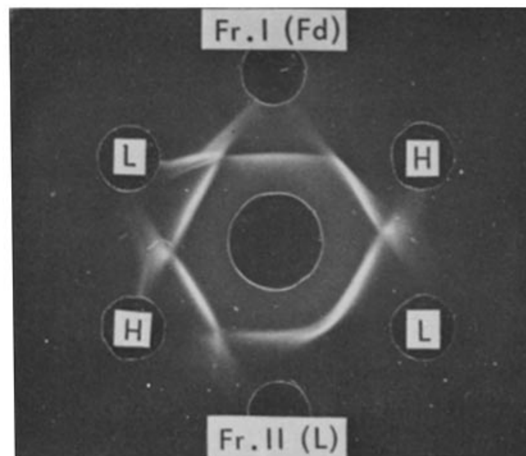

FIG. 8. Antigenic relationship between the heavy (H) and light (L) chains of IgM(D) and the fractions I and II obtained after gel filtration of the reduced and alkylated Fab fragment of IgM(D) (see Fig. 7). Whereas fraction II contained light chains, fraction I contained the Fd piece, which was deficient relative to heavy chains and had no antigenic determinants in common with light chains. The recombination in the agar of this Fd piece and the light chains was indicated by the pattern observed behind the intersection of the two lines, similar to that observed at the intersection of the heavy and light chain lines in this experiment and in Fig. 4 A.

between the Fc and heavy chain lines (Fig. 4 B). Further experiments were performed with these latter antisera and some results are illustrated in Fig. 9. After absorption by homologous heavy chains in slight antigen excess, these antisera still showed an obvious spur of Fab over light chains. When these antisera were absorbed by light chains, the Fab line crossed the heavy chain line without appreciable diminution in intensity. Moreover, when those antisera, which did not contain antibodies to the Fd piece or to the portion of μ-chain destroyed by papain (see below), were absorbed by light chains and Fc fragment, they still gave a strong precipitin line with Fab fragment in partial

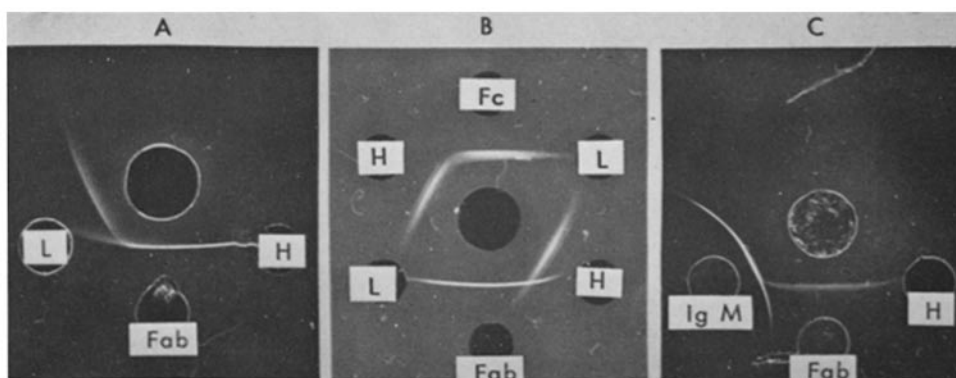

FIG. 9. Conformational antigenic determinants of Fab fragment. Antiserum 787B to IgM(S) globulin was used in these experiments.

A. When this antiserum was absorbed with homologous heavy chains, the Fab line spurred over the light chain line. B. When this antiserum was absorbed by homologous light chains, the Fab line crossed the heavy chain line without diminishing in intensity. C. When this same antiserum was absorbed by homologous light chains and Fc fragment, a precipitin line was observed with Fab whereas no reaction was obtained with heavy chains. The IgM line spurred over the Fab line.

identity with the IgM line, whereas no precipitation occurred with heavy chains. These results indicate that the antigenic specificity of Fab fragment is partly dependent on the presence of the combined chains. Moreover, in this last experiment where an antiserum reacting neither with the isolated chains nor with the native Fc fragment was used, the strong spur of IgM over Fab (Fig. 9 C) points to the possible importance of the native conformation in the Fab region for the antigenic specificity of the intact molecule.

Ultracentrifuge analysis showed that the extrapolated sedimentation coefficient of Fabμ(S) was $s^{\circ}_{20,w} = 3.84 \pm 0.05$ and that of Fabμ(D) was $s^{\circ}_{20,w} = 3.80 \pm 0.05$. Although the Schlieren patterns gave one symmetrical peak for both preparations, sedimentation equilibrium measurements revealed considerable mass heterogeneity. The values for $\bar{M}w$ app. were highly dependent on concentration and on velocity. This polydispersity was somewhat reduced in

0.1 M tris-HCl buffer, 1 M in NaCl. The Fabμ(S) fragment had an estimated $\bar{M}w$ app. = 46,000 with an $\bar{M}z$ app. = 60,000. The Fabμ(D) fragment had an estimated $\bar{M}w$ app. = 43,000 with an $\bar{M}z$ app. = 61,000.

Zone electrophoresis on starch blocks or in agar resolved both Fabμ fragments into two subfractions. The difference in electrophoretic mobility between these two subfractions was more pronounced for Fabμ(D). The more cathodic Fabμ_2 and the more anodic Fabμ_1 subfractions represented 24% and 76% of the whole Fab(S) fragment, while they represented 60% and 40% respectively for Fab(D). For both Fab fragments, these two subfractions could not be

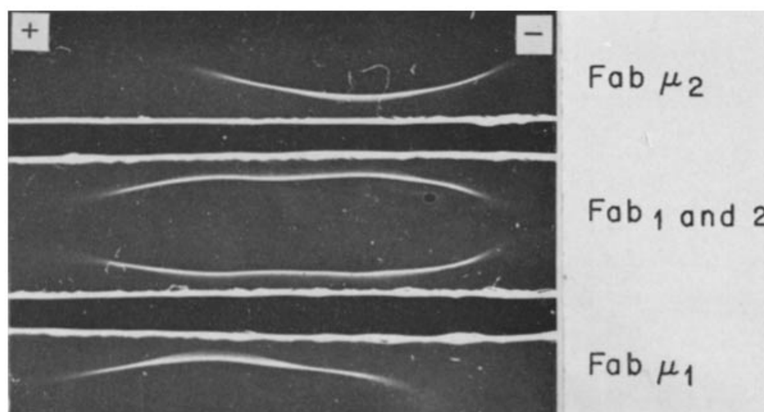

FIG. 10. Demonstration of two electrophoretic subfractions in the Fab fragment of IgM(D). These two subfractions could not be distinguished immunologically. In this experiment, the duration of electrophoresis was longer than in usual conditions.

distinguished immunologically in immunoelectrophoresis (Fig. 10) and Ouchterlony gel diffusion or by cross-absorption experiments. Similar electrophoretic subfractions were not detected when the corresponding subunits were submitted to immunoelectrophoretic analysis. The results of carbohydrate determinations on both Fab fragments and their subfractions are summarized in Table II.

Analysis of Papain Digests of IgM Globulins and Their Monomeric Subunits at Different Stages.—The papain digests obtained at earlier stages of degradation were studied by immunoelectrophoretic analysis. Some of these samples (2 and 4 hr) were run on Sephadex G-200 columns and the fractionated material was tested by gel diffusion and immunoelectrophoresis.

For both macroglobulins, a faint Fc line was detected in the 1 hr unfractionated sample. The amount of Fc fragment raised progressively at 2 and 4 hr but still remained lower than in the 24 hr sample. The Fc fragment recovered from the 2 hr sample had electrophoretic and antigenic properties similar to those of

the 24 hr Fc fragment. The Fab fragment was present in fair amount in the 1 hr samples and its yield raised slowly in the subsequent samples (29% at 2 hr and 37% at 4 hr), while the amount of small peptides was 35% at 2 hr and 43% at 4 hr. Several intermediary split products migrating between Fab and Fc fragments and sharing antigenic determinants of both fragments were demonstrated by immunoelectrophoretic analysis in the 2 hr samples (Fig. 11) and were eluted in the void volume of Sephadex G-200 columns. This partially digested material was present in lesser amount in the 4 hr samples than in the 2 hr samples and was no longer detected in the 24 hr samples.

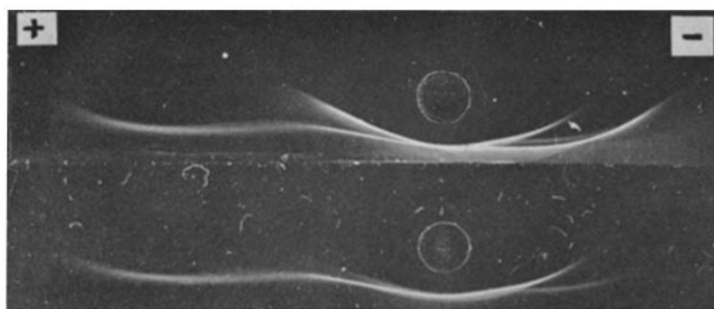

FIG. 11. Immunoelectrophoretic analysis of the papain digest of IgM(S) obtained after 2 hr of degradation and tested with unabsorbed antiserum to IgM(S) in the upper photograph and with the same antiserum absorbed with Fab fragment in the lower photograph. Fab and Fc fragments were present at this stage of digestion, together with several intermediary split products.

The immunoelectrophoretic pattern of the split products obtained after 72 hr of digestion was quite similar to that of the 24 hr samples. Moreover, when purified Fc or Fab fragments were submitted to further papain digestion for 48 hr, their electrophoretic and antigenic properties were unaltered.

The papain digests of the monomeric subunits of IgM(S) after 1, 2, 4, and 24 hr of digestion were studied by immunoelectrophoretic analysis. The prominent finding was that a fast migrating component, similar but not identical to the Fc fragment, was present in the 1 hr sample but was no longer detectable in subsequent samples. This component was less anodic than the Fc fragment and its broad electrophoretic mobility was strikingly similar to that of the reduced and alkylated Fc fragment. Furthermore, this component was antigenically deficient when compared, by means of antiserum 911, with the Fc fragment from native IgM(S) globulin (Fig. 12). Intermediary degradation products were present in relatively low amount in the 1 hr sample and practically disappeared in further samples in which only Fab fragment was found.

Observations on the Heavy Chain Determinants Destroyed by Papain Digestion.—In view of the high yield of small peptides produced by papain diges-

tion, experiments were performed to determine if some antisera reacted with antigenic structures located on the destroyed portion of the IgM molecule. One antiserum to IgM(S) and one antiserum to IgM(D) were absorbed by homologous Fab and Fc fragments. They were still able to precipitate with the corresponding native IgM globulin and its monomeric subunits. The two precipitin lines were in complete identity, although, with the same unabsorbed antisera, IgM spurred over the subunits. Both absorbed antisera were shown to react with the homologous heavy chains. Whereas μ-chains of IgM(D) gave a weak precipitin line in complete identity with the IgM and subunit lines, the μ-chains of IgM(S) strongly inhibited the precipitin reaction with the native IgM or its subunits.

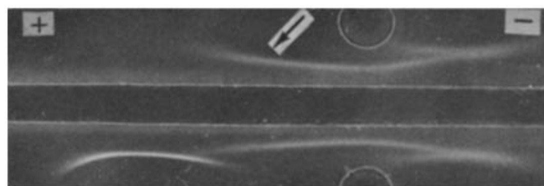

FIG. 12. Immunoelectrophoretic analysis of the Fc-like fragment produced at early stages of papain digestion of IgM monomeric subunits. Antiserum 911 to IgM(S), previously absorbed by Fab(S) fragment, was used in this experiment. Upper well, 1 hr digest of IgM(S) subunits; lower well, the same sample mixed with the 24 hr digest of native IgM(S). The Fc-like fragment (arrow) is deficient compared to the "native" Fc fragment.

DISCUSSION

The present report demonstrates that the degradation of human IgM globulins by papain is a progressive process resulting in the production of a high proportion of small peptides and leading to the formation of two distinct fragments which are resistant to further papain digestion. Since these two fragments share many characteristics with the well-documented Fab and Fc fragments of IgG globulins, they were designated Fabμ and Fcμ. However, this study indicates that these IgM fragments, and especially the Fcμ fragment, display a number of properties which differ from those of the IgG fragments.

Similar to the Fc fragment of IgG globulins, the Fcμ fragment represents segments of the heavy chains, has a fast electrophoretic mobility, and is rich in carbohydrate. Its heterogeneity in starch gel electrophoresis is reminiscent of previous findings with Fc fragments of IgG myeloma proteins (16, 17, 18). The main distinctive feature of the Fcμ fragment is its large size, dependent on covalent forces since no major modification occurred in dissociating agents. Its molecular weight is not compatible with an Fc fragment derived from a single monomeric subunit. That the Fc fragment is made up of μ-chain pieces

of several disulfide-linked subunits is demonstrated by its appreciable drop in molecular size, as well as by the modification showed by antigenic analysis after reduction in mild conditions even without subsequent acidification. The immunological findings are of particular significance since they indicate the presence in the unreduced Fc fragment of some antigenic properties dependent on the native structure of the IgM globulin. This configurational specificity disappeared when the Fc fragment or the IgM globulin were reduced at the subunit level. This contention is supported by the properties of the Fc-like fragment obtained from IgM subunits which has electrophoretic and antigenic characteristics similar to those of the reduced Fcμ fragment and, as recently demonstrated (19), a sedimentation coefficient of 2.9S. Our findings that this Fc-like fragment rapidly disappears when IgM subunits are digested by papain are in agreement with those of Onoue et al. (19) and indicate that the native conformation of Fc fragment is necessary to confer resistance to papain proteolysis. In view of these data, it appears reasonable to assume that small pieces of heavy chains belonging to all the subunits contribute to the structure of the Fcμ fragment. However, since molecular weight determinations are not yet available, the present results cannot definitely rule out the possibility that a more restricted number of subunits (at least 3) are involved. The small pieces forming the Fc fragment probably represent the carboxy-terminal end of the heavy chain. This hypothesis is consistent with recent data on the location of a disulfide bond at the carboxy terminus of μ-chains (20, 21).

Although slight individual differences were observed, the yield of the Fc fragment was, for both macroglobulins, lower than expected if one assumes that each IgM molecule produced one Fc fragment. Moreover, the amount of Fc fragment formed at early stages of papain degradation was lower than that found at the end of the process. These findings strongly suggest that an appreciable proportion of IgM molecules underwent degradation without the formation of a stable Fc fragment. Since papain digestion of the monomeric subunits does not yield a stable Fc-like fragment, the possibility exists that the small amount of cysteine used for the activation of the papain disrupted the intersubunit disulfide bonds of some molecules, thus preventing the formation of the expected amount of native Fc fragment. Another possibility for explaining the low yield of Fc fragment is an unequal susceptibility to proteolysis among these presumably homogeneous molecules, due for instance to a variation in the distribution of carbohydrate moieties. Finally, the present experiments do not rule out the possibility of an artefactual increase in molecular weight due to disulfide interchanges or to some degree of aggregation by strong noncovalent bonds.

Previous findings have indicated that papain digestion of IgM globulins results in the formation of a slow-migrating fragment with a sedimentation coefficient of approximately 3.5S (22, 23). The Fabμ fragment isolated in the

present work bears many similarities to the Fab fragment of IgG globulins, since it consists of one light chain and one Fd piece. Its low ability to precipitate when combined with anti-K or anti-L antibodies, compared to the native IgM, is presumably due to its antigenic "univalency" (24) since the divalent fragment obtained after mild pepsin digestion strongly precipitates with the same antisera.[2] It should be emphasized that the isolated Fd piece of IgM(D), as well as the purified μ-chain, was shown to recombine in the agar plates with the isolated light chains. The relatively low content in carbohydrate (as compared to the native IgM) and the estimated molecular weight of Fabμ fragment are analogous to those of its IgG counterpart. Its yield is consistent with the existence of two fragments derived from each subunit, if allowance is made for the complete digestion of a small proportion of IgM(S) molecules. Most of the features above described are analogous to those of the fragment C formed during tryptic digestion and studied by Miller and Metzger (25). Additional similarities between Fabγ and Fabμ fragments can be pointed out. As with myeloma proteins, the electrophoretic mobility of each IgM subunit is reflected in that of its Fab fragment. In contrast to Fc fragments, Fab fragments were shown to possess individual specific antigens (26), similarly to previous findings with myeloma proteins (27, 28).

In contrast to current data on the Fab fragment of myeloma proteins, Fab fragments of both IgM globulins showed two subpopulations with distinct electrophoretic mobilities. No antigenic differences between these two subpopulations were detected and only small differences in carbohydrate contents were observed. The finding of two subpopulations is consistent with recent data showing chromatographic heterogeneity on DEAE-cellulose of Fab fragments derived from IgM subunits (19). Its meaning remains to be determined. It is highly improbable that these two Fab subfractions are derived from two populations of the original IgM molecules since we are dealing with Waldenström type macroglobulins showing a high degree of electrophoretic homogeneity. Although suggested by early data (29), the hypothesis that this phenomenon might be a reflection of electric charge heterogeneity at the subunit level is not supported by the present experiments. Another possibility is that this heterogeneity in electric charge reflects random proteolytic processes, although discrete subpopulations were repeatedly obtained in constant ratios for each macroglobulin. In view of the unexplained monovalency of the subunits of IgM antibodies (30, 31), a study of the antigen-combining activity of the Fab fragments derived from a homogeneous IgM globulin with antibody activity and of their two electrophoretic subpopulations seems warranted.

Another distinctive feature of Fabμ and Fabγ fragments is related to the nature of the noncovalent bonds involved in their conformation. Although the present experiments showed that, as Fabγ, the Fabμ fragment is resistant

[2] Mihaesco, C., and M. Seligmann. Peptic split products of human IgM globulins. Submitted for publication.

to further papain digestion at neutral pH, and although recent comparative physical data (32) indicate similarities between those fragments, structural differences between the two fragments were nevertheless revealed by enzymatic digestion at acid pH (26). Whereas the digestion of Fabγ fragment by pepsin at pH 4.5 yielded only 10% of small peptides in 24 hr, the Fabμ fragment was completely digested by pepsin under the same conditions. Moreover, papain digestion of IgM(S) globulin, performed at pH 4.5 for 24 hr, led to the production of 100% of small peptides.

The high yield of small peptides obtained after papain digestion at neutral pH, even at early stages, suggests the presence of large areas of unfolded structures in the IgM molecule. The roughly constant ratio of small peptides to Fab fragments at different stages of the proteolytic process suggests the absence of a population of IgM molecules with a high degree of resistance to papain. This interpretation is supported by the results of experiments not included in this report, in which papain in the absence of cysteine was employed. When the undigested material obtained in these mild conditions was again submitted to the same enzymatic treatment, it yielded identical amounts of Fab and small peptides. It is of interest that the fucose residues of IgM(S) were exclusively located on the portion of heavy chains destroyed by papain since they were practically absent on the Fab and Fc fragments. There is strong suggestive evidence that this presumably loose segment of μ-chains is situated between the Fd piece and the polypeptide piece included in the Fc fragment. Although the small peptides were not studied in the present investigation, the presence of antigenic determinants located on this "papain-sensitive" portion of the heavy chains was demonstrated by the experiments using antisera absorbed by both Fab and Fc fragments. Furthermore, these absorbed antisera were shown to react with divalent fragments obtained after mild digestion with pepsin and containing a larger portion of μ-chains than contained by Fab.[2]

These and other experiments provide useful information on the antigenic analysis of μ-chains since they delineate three sets of antigenic determinants: (*a*) those contained in the presumably carboxy-terminal Fc fragment, which were shown to be identical in the two studied macroglobulins; (*b*) those belonging to the Fd piece, which did not cross-react with the available heterologous antisera; individual specific antigenic determinants of IgM(D) were demonstrated in Fd piece (26); (*c*) those located in the mid-part of the chain which were detected in the subunits and in the pepsin divalent fragments.[2] Some of these latter determinants were common to the two studied macroglobulins.

Previous data have shown that mild reduction of IgM globulins resulted in the loss of some antigenic determinants (33, 34). The results reported above point to the importance of conformational antigenic specificity at various levels and in different regions of the IgM molecule. It should be emphasized that the conformation-dependent antigens within the Fc fragment and the

corresponding region of the native IgM molecule were shown to be identical in both macroglobulins herein studied. Conformational specificity is also of considerable importance in the Fab region at two different structural levels. Some of the Fab antigenic determinants gave precipitin reactions only when light chain and Fd piece were combined. Recent findings have outlined the importance of this interaction in establishing the antigenic structure of the immunoglobulin molecules (35, 36) and the individual antigenic specificity of IgG myeloma proteins (27, 28). Furthermore, it was shown in the present study and in other experiments that some of the antigenic specificity of the Fab region requires the native conformation of the IgM molecule, recalling previous observations from this laboratory (37). The importance of the native structure in determining the individual antigenic specificity of Waldenström type macroglobulins has been indicated by recent reports (26, 27, 38). On the other hand, when antisera absorbed in order to detect only the antigenic determinants of the mid-part of the μ-chain were used, conformational specificity was not detected at the level of the native IgM molecule.

These immunologic findings together with the papain digestion data clearly indicate that Fab and Fc fragments represent relatively compact regions of the IgM molecule held together by looser heavy chain areas. The high yield of small peptides, mainly derived from this latter region, does not allow us to infer a molecular model for IgM globulins from the present data. However these data could be consistent with the topologic models of Miller and Metzger (25, 39) and with recent electron microscopy findings (40).

SUMMARY

Papain digestion of two Waldenström IgM globulins produced a high amount of small peptides and resulted in the formation of two end products, the Fabμ and Fcμ fragments.

The Fcμ fragment is characterized by a fast electrophoretic mobility, a high content in carbohydrate, and a high molecular weight. It was demonstrated that this fragment is made of heavy chain pieces belonging to several disulfide-linked monomeric subunits, presumably representing the carboxy-terminal end of the μ-chains. Fc fragments from the two macroglobulins could not be distinguished immunologically. An appreciable proportion of IgM molecules apparently underwent degradation without the formation of a stable Fc fragment. An Fc-like fragment, analogous to the reduced Fc fragment, was obtained at early stages of papain digestion of the IgM subunits.

The Fabμ fragment, with slow and individually distinct electrophoretic mobility, bears many physicochemical and immunological similarities to the Fabγ fragment. It consists of one light chain and one Fd piece, both of which were isolated. The interaction of these two constituents was demonstrated by gel diffusion studies. Fab fragments of both IgM globulins were resolved into two subpopulations with different electric charges.

In addition to these fragments, intermediary split products were observed at early stages of the degradation process, together with a high yield of small peptides mainly derived from the papain-sensitive region of the heavy chains. Immunologic data strongly suggested that this segment of μ-chains is situated between the Fd piece and the portion included in the Fc fragment.

Several experiments indicated the importance of conformational antigenic specificity in both Fab and Fc regions of the IgM globulins.

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