Identity of the Fc Fragments of Pathological and Normal Human Immunoglobulin M†

Gerard Florent,‡ David Lehman, David Lockhart, and Frank W. Putnam*

ABSTRACT : The Fc fragment is a disulfide-linked polymer with a polypeptide molecular weight of about 270,000 that is composed of 10 identical polypeptide chains, each containing 251 amino acid residues representing the carboxyl-terminal portion of the constant region of the μ heavy chain of human immunoglobulin M (IgM). Partial amino acid sequences of the Fc region of five pathological human IgM proteins and of IgM from normal pooled human plasma have been determined to ascertain their structural identity with the Fc sequence previously established for another pathological IgM (Ou) by a combination of manual and automatic analysis. Fc fragments prepared by tryptic cleavage were not distinguishable by immunochemical and physicochemical methods and by peptide mapping. The amino acid composition of many tryptic and chymotryptic peptides was determined. Automatic amino acid sequence analysis was done on all the intact Fc proteins and on CNBr fragments of several Fc proteins. All the peptides analyzed and all the sequences determined could be placed by homology in the sequence previously established for the Fc region of IgM protein Ou. Trypsin at 60° cleaved at the same site in each IgM protein. Oligosaccharides designated C2-C5 appear to be attached to asparagine residues at four different sites in each Fcμ chain. For each oligosaccharide the site of attachment in each chain is apparently identical and the carbohydrate composition similar. Almost the entire Fc sequence of one IgM (Ga) was placed by homology to IgM Ou including about 100 residues with the sequenator and the rest by peptide composition. From 70 to 150 residues were determined with the sequenator in the other pathological Fc proteins and about 50 in the normal Fc, and many others through peptide composition. Altogether some 700 residues in the five pathological Fc fragments were tested for variation from the Ou Fc sequence, and no differences were found. Although we have not found the Fc fragments to be identical, we have been unable to establish any differences in serological activity, peptide maps, peptide composition, carbohydrate location, disulfide bridges, and amino acid sequence. Hence, we conclude that within the limits of detection and application of the methods and excluding possible minor allotypic variations, the Fc sequence is closely similar if not identical for most and possibly all human pathological μ heavy chains and the μ heavy chains of normal human IgM.

Although the complete covalent structure of one IgM immunoglobulin has been reported including the amino acid sequence of the κ light chain and the μ heavy chain and the location of all disulfide bonds and oligosaccharides (Putnam *et al.*, 1973), little information is available about the amino acid sequence of other IgM proteins other than partial sequences of the amino-terminal variable region (Capra, 1971; Wang *et al.*, 1971). Florent *et al.* (1974) have described studies on the primary structure of the V region and the switch point in a series of pathological human IgM proteins. This paper reports partial sequence analysis of the Fc region of five pathological IgM proteins and of normal IgM from pooled human plasma. This work was undertaken with three objectives: (i) to verify accessible portions of the Fc region of the μ chain sequence, (ii) to search for possible amino acid substitutions indicative of subclasses or of allotypes of the μ chain, and (iii) to adduce some concrete evidence for the generally accepted hypothesis that the C regions of μ heavy chains from pathological IgM immunoglobulins are identical in amino acid sequence with corresponding portions of the μ chain from normal IgM.

For this study IgM proteins were prepared from the plasma of five patients with Waldenström's macroglobulinemia, and normal IgM was prepared from plasma from a large pool of healthy donors. In each case the Fc fragment was obtained by limited cleavage with trypsin at 60° and purified by gel filtration. The Fc fragments were compared by immunochemical and physicochemical methods and also by peptide mapping. In several cases many peptides were eluted from the maps and were analyzed. All of the intact Fc proteins were subjected to automatic amino acid sequence analysis, and CNBr fragments of several of the Fc proteins were also analyzed with the sequenator. Although the intact IgM proteins differ in source and in some physical properties, no evidence was obtained for any substitutions in amino acid sequence in the extensive segments of the Fc region that were examined for the pathological μ chains and in the more limited portion studied in the normal μ chain. These results support the hypothesis that the C region sequence of pathological μ chains is a faithful transcription of the normal C region gene and suggest that the majority of pathological and normal μ chains belong to the same subclass.

Materials and Methods

Purification of IgM Globulins. The proteins studied include five κ type IgM specimens from patients with macroglobulinemia (Ou, Ga, Di, Ba, Dau)[1] and a preparation of normal IgM from pooled sera.[2] The Ou, Ga, Di, and Dau IgM globulins were prepared from the plasma according to the euglobulin precipitation method (Putnam *et al.*, 1967) followed by gel filtration on Sephadex G-200. The Ba IgM was not a euglobulin so after removal of the fibrinogen, the serum was applied di-

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‡ Present address: Recherche et Industrie Therapeutics, 1330 Rixensart, Belgium.

[1] Plasma from patients with macroglobulinemia was supplied by cooperating physicians as follows: Ou, Drs. John L. Fahey and William D. Terry, Clinical Center, National Cancer Institute; Ga, Dr. Samuel Murphy, Ohio State School of Medicine, Columbus, Ohio; Di, Dr. Thomas Newcomb, Veterans Administration, Washington, D. C.; Ba and Dau, Dr. John G. Harter, formerly of Massachusetts General Hospital, Boston, Mass.

rectly to the G-200 column. After gel filtration, the macroglobulin preparations were free of all contaminating proteins detectable by immunoelectrophoresis and immunodiffusion. The normal IgM was obtained as a partially purified preparation from the American National Red Cross[2] and was purified further on Sepharose.

Preparation of Fc Fragments. Fc fragments were prepared by limited tryptic cleavage at 60° (Plaut and Tomasi, 1970).[3] The IgM globulins were dissolved in 0.1 M Tris buffer–0.15 M NaCl (pH 8.1) in the presence of 0.0115 M $CaCl_2$ at a protein concentration of about 15 mg/ml. The solution was heated at 60° prior to the addition of Tos-PheCH_2Cl-trypsin at an enzyme to protein weight ratio of 1:50. The digestion was carried out for 45 min and stopped by addition of lima bean trypsin inhibitor (Worthington). The $(Fc)_{5\mu}$ fragment was isolated by gel filtration on a Sephadex G-200 column in 0.1 M Tris–0.15 M NaCl (pH 8.1). The first peak which came off the column in the void volume contained $(Fc)_{5\mu}$.

Reduction and Alkylation. The $(Fc)_{5\mu}$ preparation was reduced with 0.1 M mercaptoethanol for 50 min in a pH 8.1 buffer containing 0.1 M Tris–0.15 M NaCl–8 M urea. The alkylation was performed for 30 min with 0.2 M iodoacetamide. The samples were dialyzed against water to remove the excess of reagent.

Cyanogen Bromide Cleavage. The cyanogen bromide treatment was performed on the intact $(Fc)_{5\mu}$ fragment in 70% formic acid for 4 hr at room temperature at a ratio of 5 g of CNBr/g of protein. The excess of CNBr was removed by lyophilization. After reduction and alkylation as above, the cyanogen bromide fragments were separated by gel filtration on Sephadex G-100 in 6 M urea–0.05 M formic acid.

Physicochemical and Immunochemical Characterization of Proteins. Ultracentrifugation was done in the Spinco Model E analytical ultracentrifuge at 59,780 rpm. Antigenic analysis was performed by the agar diffusion method and by immunoelectrophoresis. The following rabbit antisera were used in this study: antisera specific for μ chain and for the κ light chain were obtained from Meloy Laboratories,[4] and antisera to the Fab and Fc fragments of Ou IgM prepared in our own laboratory.

Peptide Maps. Tryptic digestions were performed in 1% NH_4HCO_3 with Tos-PheCH_2Cl-trypsin with an enzyme:protein ratio of 1:50. The two-dimensional mapping of the tryptic digest was performed at pH 3.7 as described by Putnam and Easley (1965) with ninhydrin staining on one map and staining with trinitrobenzenesulfonic acid on another (Shinoda and Satake, 1961). Amino acid analysis of the trinitrobenzenesulfonic acid peptides was done with the Beckman Model 120 automatic amino acid analyzer after hydrolysis in 5.7 N HCl under vacuum at 105° for 20 hr.

Sequence Analysis. The sequencer runs were performed with the automatic (Beckman Model 890) protein sequencer. Both for large fragments and peptides, procedures based on Hermodson *et al.* (1972) were used. The anilinothiazolinone derivatives were converted into phenylthiohydantoins by incubation in 1 N HCl at 80° for 10 min. The PTH-amino acid residues were identified by gas chromatography on a DC-560 column before and after silylation and by amino acid analysis of hydrolysates of PTH-amino acids (Pisano and Bronzent, 1969). The hydrolysis was carried out under reduced pressure for 20 hr at 130° in 5.7 N HCl containing 1 drop of mercaptoethanol.

Prior to sequenator analysis amino-terminal groups of peptides were determined by the dansyl method and carboxyl-terminal groups by digestion with a mixture of carboxypeptidases A and B. Some sequences were partially determined by the manual dansyl-Edman method. For procedural details of the manual sequence analysis and end group determinations, see Moore and Putnam (1973) and Titani *et al.* (1970).

Nomenclature. Substantial evidence (Florent *et al.*, 1974) shows that μ heavy chains are divided into variable or V regions of different sequence and length and into constant or C regions that are believed to be of identical sequence and length except for possible allotypic or isotypic variations. The variation in length of the V region precludes a uniform continuous numbering system that begins at the amino terminus. For simplicity and ease of cross reference, we use the numbering system of the Fc sequence of the Ou μ chain (Putnam *et al.*, 1973) for the Fc fragments of the other μ chains described herein. Similarly, the number of methionine residues in the V_H region of μ chains may vary from two in Di to five in Ou (Florent *et al.*, 1974), whereas only six can theoretically be derived from Cμ. As illustrated in Figure 1 the theoretical CNBr fragments are designated F5 to F11 for the Ou μ chain. The same designations will be used here for the CNBr fragments of the Fc region. Thus, the C region of Fc is expected to yield CNBr fragments F8–F11 and a portion of F7. Other characteristics of the Fc region, the whole μ chain, and IgM are identified in Figure 1, which also shows the location of interchain and intrachain bridges and of the five oligosaccharides (C1–C5) referred to in the text. A later figure gives the amino acid sequence of Ou Fc as a base for comparison of the Fc sequence of the other IgM proteins. Fc as used in the text will refer to $(Fc)_{5\mu}$ as defined by Figure 1.

Results

Physical and Immunochemical Characterization of IgM. All of the IgM preparations including the normal IgM gave the characteristic physicochemical and immunochemical properties of human IgM immunoglobulins (Putnam *et al.*, 1967a,b; Metzger, 1970). In analytical ultracentrifugation the IgM preparations showed a predominant peak with an $s_{20,w}$ of about 19 S, varying proportions of minor components representing polymers of 22 S, 26 S, and higher, but no detectable 7S component. No lower molecular weight components were detected by acrylamide gel electrophoresis in the absence of reducing agents, but in the presence of mercaptoethanol or dithiothreitol all the IgM proteins dissociated into μ heavy chains, light chains, and a triplet of fast-moving bands attributable to J chain.[5]

[2] Partially purified IgM from pooled donor plasma was supplied by Drs. Milan Wickerhauser and Yu-Lee Hao, of the American National Red Cross Blood Research Center, Bethesda, Md. In later studies human IgM of 98% immunochemical purity prepared from normal pooled plasma was supplied gratis by Dr. H. G. Schwick of Behringwerke, Marburg, Germany.

[3] Dr. Akira Shimizu of Osaka University School of Medicine, Osaka, Japan, supplied immunochemically pure Fc from IgM Hir that had been prepared by incubation with trypsin at 25° for 24 hr after prior denaturation in 5 M urea.

[4] Dr. W. Frederick Hymes of Meloy Laboratories kindly gave us antisera not commercially available.

[5] Acrylamide gel electrophoresis for the detection of J chain was performed by Dr. Elizabeth Raff of our laboratory. Antiserum specific for J chain was kindly supplied by Dr. Jiri Mestecky, University of Alabama Medical Center, Birmingham, Ala. J chain was detected in the following IgM preparations: Ou, Di, Dau, and Ga and in Fc preparations of Ou and Di, and was isolated from IgM Ou and Di. J chain appears to have a blocked amino terminus because no reaction was obtained with the sequenator or by the dansyl method. Sequenator analysis of the CNBr reaction products of J chain was also negative. Hence, it is unlikely that J chain interfered with sequenator analysis of the Fc fragments or their CNBr cleavage products.

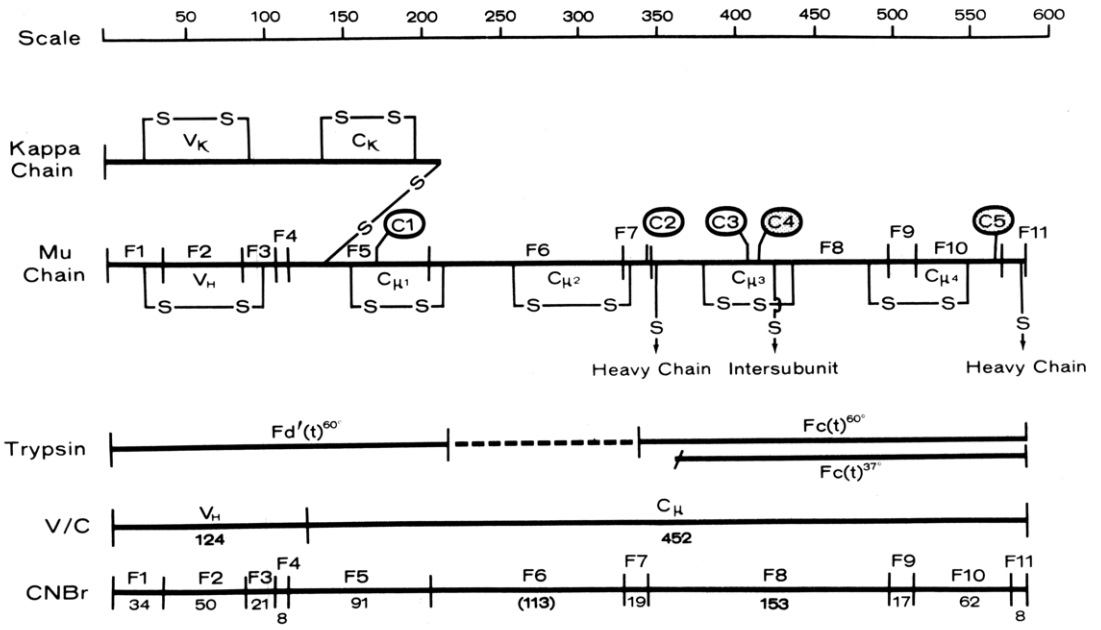

FIGURE 1: Schematic structure of the μ heavy chain and the κ light chain of IgM Ou showing: (1) the interchain and intrachain disulfide bridges, (2) the two homology regions of the light chain (V_k and C_k), and (3) the five homology regions of the μ heavy chain (V_H and $C_\mu 1$-$C_\mu 4$). The figure for the μ chain also shows the location of the five oligosaccharides (C1–C5), the points of cleavage by trypsin with the respective fragments (Fd and Fc), and the sites of cleavage by CNBr and the respective fragments (F1–F11). The scale indicates the number of amino acid residues in each chain and fragment. The Fc sequence established for the Ou μ chain (Putnam *et al.*, 1972a, 1973) has 251 residues joined in a single polypeptide chain (see a later figure). Ten such chains are linked through intrachain and intersubunit bridges to form the decameric Fc fragment derived by cleavage of intact IgM with trypsin at 60° (sometimes designated $(Fc)_5\mu$). The molecular weight of a single Fc chain, calculated from the amino acid composition and exclusive of the carbohydrate, is 27,600; thus, $(Fc)_5\mu$ has a molecular weight of 276,000 (from Putnam *et al.*, 1973; Copyright 1973 by the American Association for the Advancement of Science; reprinted with permission).

When tested in various combinations, all of the six IgM preparations (Ou, Ga, Di, Ba, Dau, and normal) gave a reaction of identity in immunodiffusion with antiserum to Ga Fc and with antiserum to Ou Fc (Figure 2). This apparent immunological identity with antisera to different Fc fragments suggests either (1) that there are no subclasses of human μ chains at least with respect to the Fc region or (2) that the five pathological proteins belong to the same subclass and that this is the predominant subclass in normal IgM.

Preparation of Fc Fragments. The optimum conditions for preparation of Fc fragments were determined by trial experiments on an analytical scale followed by analytical ultracentrifugation, gel filtration, immunoelectrophoresis, and acrylamide gel electrophoresis. As shown later by amino acid sequence analysis, the primary site of tryptic cleavage at 60° was identical in the five pathological IgM proteins, but they varied in susceptibility of the Fab region to further tryptic degradation. After gel filtration on Sephadex G-200 the tryptic digests of all of the IgM preparations except Ba gave an elution pattern similar to that shown for IgM Ga in Figure 3. The first peak contains $(Fc)_5\mu$, the second Fab, and the third, which was present in varying quantities, contained many peptides resulting from extensive tryptic degradation. Ultracentrifugation and immunoelectrophoresis performed on the tryptic digests prior to gel filtration showed the absence of uncleaved pentameric IgM. This was also indicated by the failure of the first peak to react with anti-κ antiserum. On the other hand, the Fab fragment of IgM Ba was completely digested to peptides, and the Dau Fab was also quite susceptible to tryptic degradation. A kinetic study of the tryptic cleavage of IgM Ba by ultracentrifugal analysis showed that the highest yield of Fab with this IgM protein was achieved with a very short digestion time, *i.e.*, from 10 to 15 min. The enhanced susceptibility of the Ba and Dau IgM preparations to extensive tryptic degradation is attributed to denaturation occurring during the period of several years storage of these sera in the frozen state.

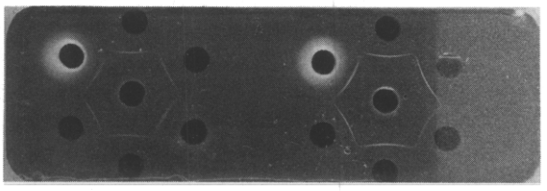

FIGURE 2: Reaction of immunological identity among five pathological IgM preparations and normal human IgM as shown by immunodiffusion against antisera to Fc fragments. Antiserum of Ga Fc was placed in the center well of the plate on the left and antiserum to Ou Fc in the center well of the plate on the right. The disposition of the IgM test preparations is the same on the right and the left. Reading clockwise from the top well, IgM preparations were placed in the six exterior wells as follows: Ou, Ga, Di, Ba, normal, and Dau. In all cases a line of identity is formed. The heavy precipitate around the well of IgM Dau results from the high concentration used for the test solution and the lower solubility of IgM Dau in the agar medium.

However, single amino acid substitution in the primary structure of these chains cannot be ruled out as the cause of the enhanced susceptibility. For example, the allotypic interchange of Met-222C and Thr-222C in rabbit γ chains is associated with a difference in resistance to papain cleavage owing to the attachment of oligosaccharide at Thr-222C (Smyth and Utsumi, 1967).

Preparation of CNBr Fragments of Fc. The CNBr fragments prepared from the reduced-alkylated Fc fragments of the IgM proteins were separated by gel filtration on Sephadex G-100 in 6 M urea–0.05 M formic acid as shown in Figure 4 for IgM Ga. Even with use of this dispersing agent some aggregate is present and emerges as the first peak. The second peak contains the F8 fragment, the third F10, and the shoulder of the third peak and the broad fourth peak represent a mixture of the smaller fragments F7, F9, and F11. The lower proportion

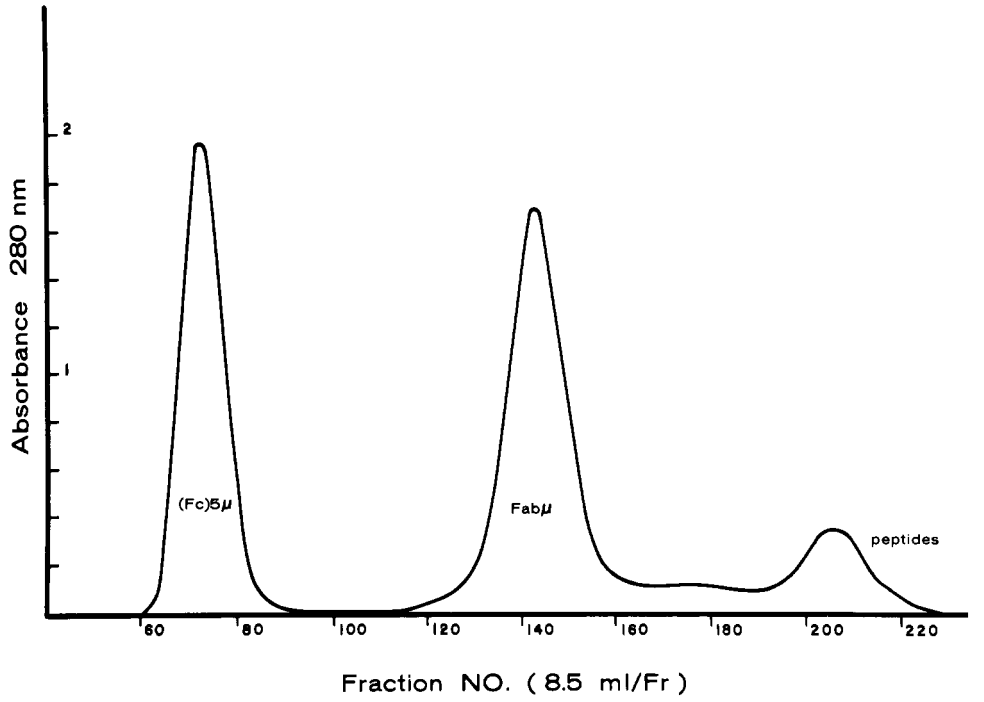

FIGURE 3: Gel filtration of a tryptic digest at 60° of IgM Ga. The gel filtration was done on Sephadex G-200 in the cold with a buffer of 0.1 M Tris-HCl-0.15 M NaCl (pH 8.1) with a column size of 5 cm and a fraction size of 8.5 ml.

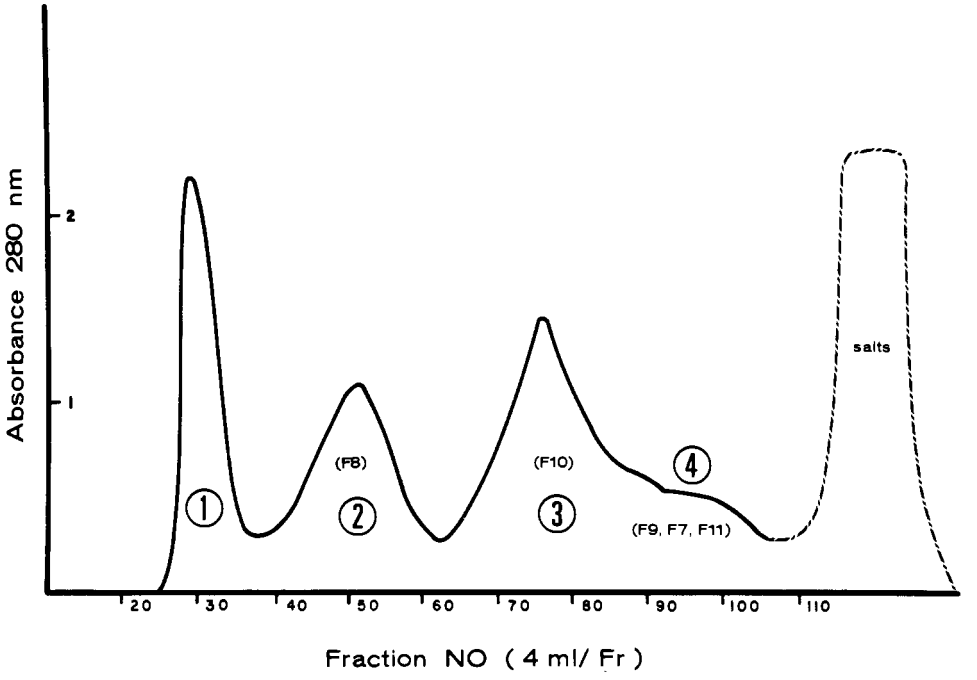

FIGURE 4: Gel filtration of CNBr fragments of reduced-alkylated Ga Fc on Sephadex G-100 in 6 M urea-0.05 M formic acid, column size 2.5 × 100 cm. The first peak contains aggregate, the second the F8 fragment, the third F10, and the fourth a mixture of F7, F9, and F11. (Nomenclature of CNBr fragments as for Ou (see Figure 1 and Putnam *et al.*, 1972a, 1973)).

of F8 than F10 results from the fact that most of the aggregated material consists of F8.

The nomenclature of the CNBr fragments corresponds to that used for IgM Ou (Putnam *et al.*, 1971, 1972a, 1973). Although the number of CNBr fragments expected from the μ chain of a pathological IgM may vary from 8 to 11 depending on the methionine content of the V region, the number obtained from the Fc region should be constant in the absence of isotypic or allotypic substitutions of methionine. In support of this hypothesis a similar gel filtration pattern was obtained for the CNBr fragments of all of the Fc fragments studied in this work.

Tryptic Peptide Maps. Peptide maps stained with ninhydrin were prepared for the Fc fragments of the five pathological proteins. These maps indicated an apparent identity for the five Fc fragments. To obtain better identification of the peptides trinitrobenzenesulfonic acid maps were prepared for the Fc fragments of Ou and Ba and the F8 fragment of Ga, and the

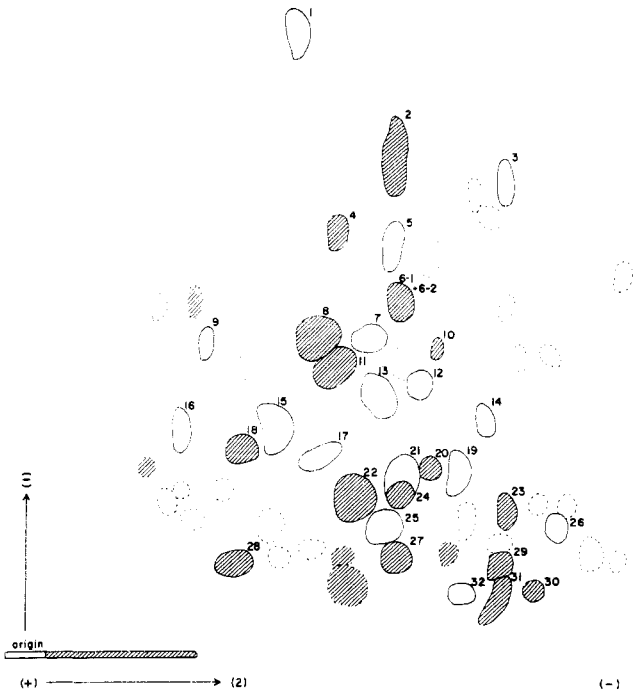

FIGURE 5: Peptide map of the Ou Fc fragment. The numbered peptides identified in the sequence of Ou Fc are shown in Figure 6. The same numbers apply to peptides from the Fc fragments of other IgM proteins. Spots with dash outlines represent faint peptides for which stoichiometric analyses could not be obtained. The shaded peptides were also identified in the peptide map of the Ga F8 fragments. The following peptides from the map of Ba Fc were isolated and analyzed: 6-2, 8, 11, 15, 21, 22, 25, 28, 29, and 31. Paper chromatography was done in the first direction (1) and electrophoresis in the second (2) at pH 3.7 (see the text). Glycopeptides are present in the shaded streak at the origin.

Tnp peptides were eluted and analyzed.[6] For the tryptic digest of Ou Fc a commercial lot of trypsin was used in which the chymotryptic activity was supposed to have been inactivated; however, in addition to the tryptic peptides many peptides attributable to chymotryptic cleavage were obtained. From their composition 32 peptides could be identified in the sequence of the Ou Fc. These are denoted by number in the peptide map of Figure 5, and their position is indicated in the sequence of Ou Fc given in Figure 6. Some peptide spots that are denoted by dashed lines in Figure 5 were too weak to give a good analysis after elution.

All of the peptides that gave stoichiometric analyses were identifiable with a specific sequence in Ou Fc. Of course, end groups and sequence data could not be obtained on the Tnp peptides, and the fit is based only on the composition and the assumed specificity of trypsin and chymotrypsin. Of the 32 peptides, 14 were tryptic peptides including two (peptides 24 and 26) that corresponded to an unusual split before proline in the sequence Gln-Thr-Ile-Ser-Arg-Pro-Lys. The expected tryptic peptide containing the Arg-Pro-Lys sequence was also recovered (peptide 31). Nineteen peptides corresponding to chymotryptic splits in the Ou Fc sequence were identified by the method. All of these are compatible with the usual specificity of chymotrypsin. Areas of sequence that were not identified by the trinitrobenzenesulfonic acid method were generally large and hydrophobic (for example, the sequence from position 347 through 358), or they contained carbohydrate and consequently only streaked at the origin of the peptide map. The missing glycopeptide sequences include the amino-terminal and carboxyl-terminal portions of Fc and also the sequence from Thr-392 through Trp-419, which contains two oligosaccharides.

In the same manner, a trinitrobenzenesulfonic acid map was prepared of the tryptic digest of the first large CNBr fragment (F8) of Ga Fc. Again both tryptic and chymotryptic peptides were obtained.[6] However, all of the 14 peptides derived from the Ga F8 region were identifiable with peptides derived from the Ou F8 region. With the exception of the Cm-cysteine residues, which were low probably because of oxidative destruction, the stoichiometry of these peptides was in good agreement with that expected from the Ou Fc sequence.

A new lot of trypsin was used to prepare the tryptic digest of the Ba Fc fragment, and 10 Tnp peptides corresponding to the specificity of trypsin were isolated, *i.e.*, peptides 6-2, 8, 11, 15, 21, 22, 25, 28, 29, and 31 of Figure 5.[6] All of these also can be placed in the sequence of Ou Fc (Figure 6).

Sequenator Analysis of the Fc Region

The automatic sequence analysis of the intact Fc fragments and of the CNBr fragments and peptides obtained therefrom is discussed in the order of the sequence beginning at the amino terminus of Fc, rather than individually for each protein. The results are illustrated by comparison to the sequence published for Ou Fc (Putnam *et al.*, 1972a, 1973).

Amino-Terminal Portion of the Fc Fragments of Pathological and Normal IgM Proteins. Sequenator analysis using the protein program was performed on the intact Fc fragments of the IgM proteins from patients Ou, Ga, Ba, Di, and Dau and on the Fc from normal pooled IgM. The results[6] are summarized in Figure 7. A number of runs were made on the Ou Fc; in the most successful one, the residues were identified for 35 steps. This sequence had been determined independently by the manual method on a series of peptides as will be described elsewhere in the full documentation of the complete sequence of the Ou μ chain. This enabled identification of certain residues, such as the asparagine at step 7, which were not determined by the automatic method.

Sequenator analysis of the Fc fragment of IgM Ga likewise was successful for 35 cycles. The sequence obtained was identical with that for the Ou Fc except that some of the amino acids which give a low response in gas chromatography (such as the dicarboxylic acids, and serine and arginine) were not identified. Sequenator analysis of Ba Fc for 20 steps and of Di Fc for 30 steps likewise showed no difference from the sequence of the Ou and Ga Fc fragments. However, sequenator analysis of Dau Fc and of normal Fc gave a double sequence. Although only glycine was identified in the first cycle, leucine and valine were present in the second cyle and two residues not attributable to overlap were identified at many steps thereafter. The same sets of residues were present in the double sequence for the Dau Fc and the normal Fc. Comparison with the sequence previously established for the whole Ou μ chain showed that one sequence was attributable to the amino terminus of Fc and the other to the sequence beginning at Gly-446 in the Ou μ chain. Since Gly-446 is preceded by a lysine residue, we attributed the sec-

[6] Eight tables of analytical data documenting the results presented herein have been submitted to the reviewers for examination but are not published in this article. Copies of these tables will be made available on request. The tables omitted are: (I) Identification of Tnp peptides in Fc and CNBr fragments; (II) Amino acid composition of the tryptic and chymotryptic peptide from CNBr fragment Ga F8; (III) Amino acid composition of the tryptic peptides of Ba Fc from the TNBS map; (IV) Sequenator analysis of Fc fragments of pathological and normal Fc fragments; (V) Sequenator analysis of F8 fragments of pathological IgM proteins: (VI) Sequenator analysis of insoluble tryptic peptides from IgM Ga F8; (VII) Sequenator analysis of F10 fragments of pathological IgM proteins; (VIII) Amino acid composition of glycopeptides from Ga F10 and of a tryptic peptide from Ga F8.

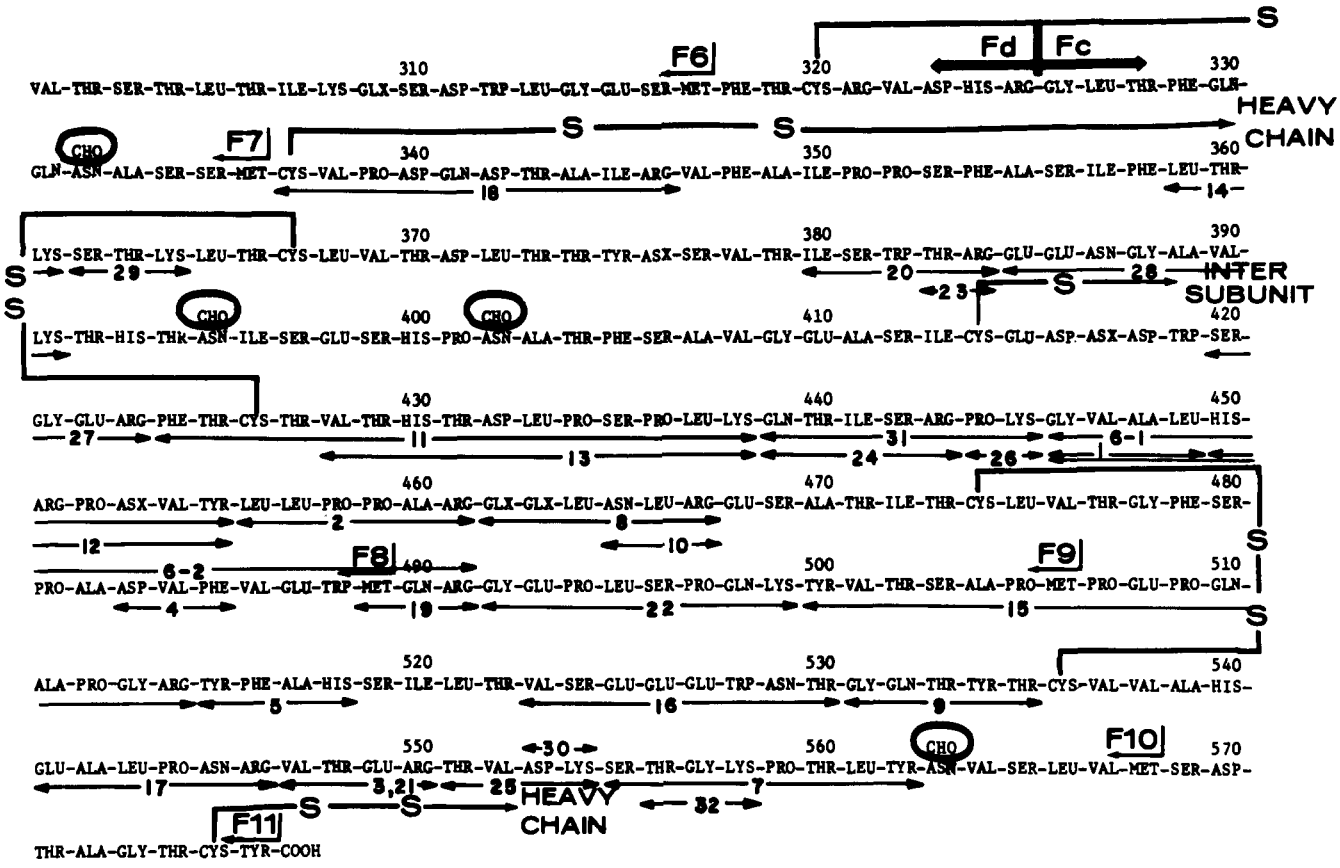

FIGURE 6: Amino acid sequence of the Fc fragment from IgM Ou (from Putnam *et al.*, 1973). The position in the sequence is indicated by arrows for peptides denoted by number in the peptide map of Figure 5.

ond sequence to an adventitious tryptic cleavage at the Lys-Gly bond during the preparation of the Fc fragments by tryptic digestion at 60°. We have observed such secondary cleavages on several other occasions. It is uncertain whether this secondary cleavage is a characteristic only of some pathological proteins such as the Dau IgM and of similar molecules in the normal IgM, or whether this Lys-Gly bond is exposed on the surface of native IgM or denatured IgM. The results for all sequencer analyses of Fc fragments are summarized in Figure 7.

Sequenator Analysis of F8 Fragments. Automatic sequence analysis[6] was performed for 23 steps on the F8 fragment of Ou and for 34 steps on the F8 fragment of Ba. Since the sequenator analysis of the Ba F8 went farther than for the Ou F8, the sequence for the latter is given from step 24 through step 34 as determined by manual analysis of peptides from Ou F8 and the whole μ chain (Putnam *et al.*, 1972a, 1973). The amino acid sequence of the first 34 residues of the F8 fragments of these two IgM proteins is identical in all positions that could be established by sequenator analysis. The only positions not verified are the basic amino acids, Arg-346 and Lys-361. However, Lys-361 must be present in Ba Fc because a tryptic peptide (peptide 29), which is Ser-Thr-Lys, was isolated from the Ba Fc fragment.

Arg-346 was not identified in the sequenator analysis of the amino terminus of any of the Fc fragments, but its presence in Ga Fc was indicated by isolation of peptide 6-3, which is Ile-Arg. It should be noted that although sequenator analysis of Ba Fc stopped at Arg-346, the analysis of Ba F8 overlaps that of the amino terminus of Ba Fc and extends it to a total of 45 residues; that is, the combined sequenator analyses of Ba Fc and Ba F8 extend from the amino terminus of Fc (Gly-326) through Thr-370 with no evidence for any difference in sequence from the Ou μ chain over this entire region.

To extend the comparative sequence data, the F8 fragment of Ga was digested with trypsin, and some of the tryptic peptides were isolated by preparative paper electrophoresis and chromatography. Sequencer analysis of one preparation of poorly soluble material showed it to be an almost equimolar mixture of two tryptic peptides, one corresponding to positions 365–382 of the Ou μ chain, and the other to positions 468–489. The double sequence obtained[6] was interpreted as follows: the first sequence overlaps the sequencer data on the amino terminus of the F8 fragment of Ba for six steps up to position 370 and extends the comparative sequence data up to position 382. The second sequence initiates at position 468 and continues for 19 steps up to position 486 (Figure 7). Although double sequence data of this type can be interpreted by the clear relationship to the sequence of the Ou μ chain, the results are supportive rather than conclusive for the identity of these segments of the two μ chains.

Sequenator Analysis of F10 Fragments. Sequenator analysis was successful for 42 cycles on the F10 fragment from Ou and confirmed manual degradation data on many peptides for this fragment.[6] Single runs in the sequenator on the F10 fragments of Ga, Ba, and Dau gave results the same as those for Ou for 22, 32, and 20 cycles, respectively (Figure 7).

Miscellaneous Peptides from the Di μ chain. In separate experiments designed to purify glycopeptides[7] several tryptic peptides corresponding to residues 547–550 and 551–554 were isolated from the Di μ chain and also a series of glycopeptides.

[7] Isolation and sequence analysis of tryptic and thermolysin glycopeptides from the Di μ chain were done by Dr. Akira Shimizu. Incidental to this work a few tryptic peptides that lacked carbohydrate were obtained and sequenced by manual procedures. We thank Dr. Shimizu for use of these data.

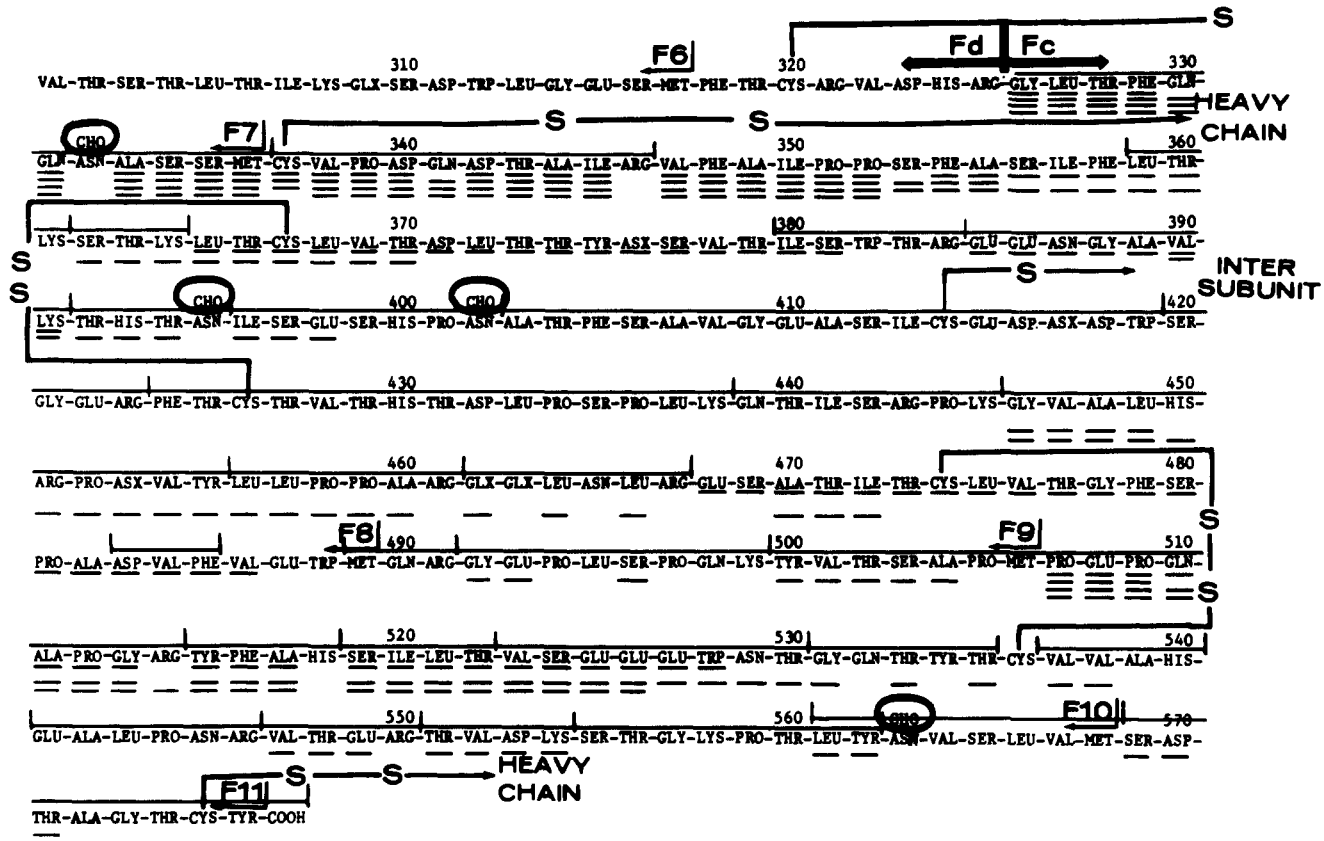

FIGURE 7: Sequence analysis of the Fc fragments of IgM Ga, Di, Ba, and Dau and of normal IgM. The sequence given is that of the Fc fragment of the Ou μ chain (Putnam *et al.*, 1972a, 1973) and is identical with that of Figure 6. Results obtained by sequenator analysis of fragments and peptides described in the text are indicated by lines (——) under each residue determined for each of the four Fc fragments. The order of the lines under each residue is: Ga, Di, Ba, Dau. Thus, the N-terminal sequence Gly-Leu-Thr-Phe was determined in all four Fc fragments, but Gln-330 though detected in Di, Ba, and Dau was not detected in Ga—probably owing to the poor response of PTH-glutamine in gas chromatography. The divided lines above the sequence indicate some of the peptides isolated from one or more of the Fc fragments by the Tnp-peptide method or other methods (see Figure 6 and text for details).

These were sequenced manually and the data are included in Figure 7. Partial sequence data were also obtained on the F9 fragment of the Di μ chain by Dr. Akira Shimizu and also on the tryptic peptide of Di corresponding to positions 446–461 (Figure 7). The COOH-terminal CNBr fragment F11 was also isolated from the Di μ chain by Dr. Shimizu.

Glycopeptides

Previous work (Shimizu *et al.*, 1971) had indicated that five oligosaccharides (designated C1–C5) were located at homologous sites in the Ou and Di μ chains. Three of these (C1–C3) are of complex carbohydrate composition; these contain fucose, mannose, galactose, glucosamine, and sialic acid, whereas the two "simple" oligosaccharides (C4 and C5) contain only mannose and glucosamine. Although C1 is located at Asn-170 in the Ou μ chain and at the identical site in the Di μ chain, C2–C5 are all in the Fc fragment. One objective of this work was to ascertain whether the same five oligosaccharides are present at homologous sites in other human μ chains.

Analysis of the Fc fragments of Ou, Di, Ga, and Ba by Dr. John Clamp[8] indicated that fucose, mannose, galactose, glucosamine, and sialic acid are present in approximately the same molar ratios for each IgM protein, *i.e.*, in a ratio of about 1:10:3:7:1.5–4. However, the absolute amounts of sugar found differ, as does the sialic acid content. Hurst *et al.* (1973) found five oligosaccharides analogous to C1–C5 in the Dau μ chain, and C2–C5 were located in the Dau Fc fragment.

Glycopeptide C2. This glycopeptide is located at the beginning of Fc in the sequence Gly-Leu-Thr-Phe-Gln-Gln-Asn(CHO)-Ala-Ser-Ser-Met. This is the C-terminal portion of CNBr fragment F7 in Ou, and this glycopeptide was also isolated by CNBr cleavage of Di Fc by Shimizu *et al.* (1971) and of Dau Fc by Hurst *et al.* (1973). The same sequence was observed by sequenator analysis in the Fc fragments of the five μ chains we studied, and also for Hir Fc and normal Fc (Figure 7). In all cases the Asn residue was blank in the sequencer analysis because of the presence of carbohydrate. We conclude that C2 is present in the same sequence in most or all normal and pathological human μ chains.

Glycopeptide C3. A thermolysin peptide with the sequence Val-Lys-Thr-His-Thr-Asx(CHO) was isolated from the Fc fragments of Ou and Di by Shimizu *et al.* (1971) and was placed in the sequence Thr-Asx(CHO)-Ile-Ser-Glu-Ser in Ou (Putnam *et al.*, 1972a, 1973). Hurst *et al.* (1973) isolated a similar glycopeptide from Dau. However, they give the sequence Thr-Asx(CHO)-Ile-Ser(Glx,Ala), which differs by the unplaced alanine residue in parentheses. We are attempting to isolate this peptide from Dau to clarify this possible difference.

Glycopeptide C4. A thermolysin glycopeptide with the sequence Ile-Ser-Glx(Ser, His, Pro, Asx, CHO) was isolated from Ou and Di by Shimizu *et al.* (1971) and was placed in the

[8] Carbohydrate determinations of whole IgM, Fab, and Fc fragments, of CNBr fragments and of various peptides were done by Dr. John R. Clamp, Department of Medicine, University of Bristol Medical School, England, by the method of gas chromatography (Bhatti and Clamp, 1971). These analyses, including a study of the effect of conditions of preparation on the carbohydrate content of glycopeptides, will be reported elsewhere.

sequence Ile-Ser-Glx-Ser-His-Pro-Asn(CHO)-Ala-Thr in the Ou μ chain (Putnam *et al.*, 1972a, 1973). For Dau, Hurst *et al.* (1973) report a glycopeptide C4 in a similar sequence Glx-Ser-*Asx*(CHO)-Pro-*Thr*-Ala-*His;* however, the three residues italicized are in a different sequence from that which we reported. We are undertaking further studies on Dau to clarify this discrepancy.

Glycopeptides C3 and C4. No attempt was made to isolate small thermolysin glycopeptides containing oligosaccharides C3 and C4 from either Ga or Ba Fc. However, a succinylated tryptic peptide containing 39 residues and presumably both C3 and C4 was prepared from Ga Fc and Ba Fc. Amino acid analyses were done for two separate preparations of Ga F8. Except for an unexplained discrepancy in the lysine values, the analyses are in good agreement[6] with the composition expected for positions 385–423. A minimum of 2.5 residues of glucosamine was present by amino acid analysis. All five sugars expected for C3 and C4 were present by gas chromatographic analysis; however, the molar ratios relative to the peptide were lower than expected.[8]

Glycopeptide C5. An oligosaccharide was present in the F10 fragment of all five μ chains. Thermolysin glycopeptides containing the simple oligosaccharide C5 and having the sequence Leu-Tyr-Asn(CHO)- were isolated from Ou and Di Fc (Shimizu *et al.*, 1971). Overlapping peptides were obtained that fitted into the overall sequence Leu-Tyr-Asn(CHO)-Val-Ser-Leu-Val-Met. Subsequently, Hurst *et al.* (1973) reported isolation from Dau IgM of a glycopeptide corresponding to C5 which had the sequence Leu-*Thr*-Asx(CHO)-Val-Ser-Leu; this differs from our glycopeptides by the apparent substitution of threonine for tyrosine. We are investigating this discrepancy.

Preparations of F10 from Ga and Ba contain carbohydrate.[6,8] Sequencer analysis of F10 from Ga and Ba for 22 and 32 steps, respectively, showed no difference from the sequence reported for Ou F10 (Putnam *et al.*, 1972a, 1973), but the possible substitution of tyrosine for threonine at the 56th position in this 62-residue CNBr fragment was not investigated directly in Ga and Ba F10. Gas chromatographic analysis of these CNBr fragments showed the presence of mannose and glucosamine in the ratio of about 3:1 as expected for a simple oligosaccharide and only trace amounts of fucose and sialic acid. However, the amount of galactose present in Ga F10 was greater than expected for a simple oligosaccharide.

In several instances anomalous peaks and low values were observed in gas chromatography of sugars from oligosaccharides in CNBr fragments. We attribute this to degradation of the oligosaccharides by the rigorous conditions used for preparation of the CNBr fragments.[8] Also, because the peptides containing oligosaccharides are large and difficult to separate one from another, further study is being made of methods to purify glycopeptides from the μ chain.

Discussion

This work has extended sequence analysis of the human μ heavy chains to the Fc fragments of four more pathological IgM proteins (Ga, Di, Ba, and Dau) and to IgM from pooled normal plasma (normal). In the areas of sequence covered no difference was observed from the complete sequence we earlier reported for the Fc fragment of IgM Ou (Putnam *et al.*, 1972a, 1973). The segments of sequence covered include: (1) almost the entire Fc sequence for Ga (about 100 residues in the sequenator and all but a few of the remaining residues through Tnp peptides or other peptides that were placed only by amino acid composition and homology, (2) more than 100 residues of Di Fc of which 62 were determined with the sequencer, (3) about 150 residues of Ga Fc, divided almost equally into those determined by the sequencer and those in peptides placed by amino acid composition and homology, (4) some 70 residues in Dau Fc determined by the sequencer, and (5) some 50 residues in normal Fc determined by the sequencer. Altogether some 700 residues in the five Fc fragments were tested for variation from the Ou Fc sequence, and no differences were found. However, the qualification must be made that only half of the 700 residues were sequenced, and that these residues are located in similar accessible regions of the sequence. Furthermore since quantitative estimation of some residues such as hydroxyamino and basic amino acids is difficult at later cycles with the sequenator, single substitutions cannot be completely excluded. Nonetheless, these results argue strongly against the existence of subclasses of the human μ chain such as had been proposed by Franklin and Frangione (1968) on the basis only of peptide maps. If subclasses exist, major differences in primary structure must be confined to the Fd rather than the Fc section of the constant region[9,10] or one subclass is predominant.

The hypothesis that most or all human μ chains have identical primary structure in the C region is supported by our evidence for the presence of five oligosaccharides (C1–C5) each of which is attached to an homologous site in the Ou, Ga, Ba, and Di μ chains. We and Hurst *et al.* (1973) have evidence for the presence of the five oligosaccharides at homologous sites on the Dau μ chain. However, several of the glycopeptides reported by Hurst *et al.* differ in the arrangement of the sequence (C1 and C4) and in one instance (C5) there is a possible single amino acid substitution. Hurst *et al.* give no data on the amino acid composition or sequence determination of their glycopeptides and cite no overlapping peptides. We attribute these minor discrepancies to technical difficulties in the sequence determination of glycopeptides rather than to allotypic or isotypic variation. However, we are reinvestigating the Dau glycopeptides.

Additional support for the hypothesis that most or all human μ chains have an identical amino acid sequence in the C region is given by data of other workers on the sequence of disulfide bridge peptides. The partial sequences given by Frangione *et al.* (1971) for four interchain bridge peptides and eight intrachain bridge peptides from the constant region of yet another μ chain can be fitted into our complete sequence of the human μ chain. The same is true for the partial sequences summarized by Metzger (1970) and obtained by various workers for other μ chains. The correspondence in sequence around the intrasubunit disulfide bridge (Cys-414 in Figure 6) of the Ou μ chain with the bridge peptide isolated by Miekka and Deutsch (1970) is important because this peptide though isolated from another of

[9] One J (joining) chain of about 15,000 molecular weight is reported to be attached to each IgM polymer, whether a pentamer or higher. The J chain, which may have the function of initiating polymerization, was once thought to be linked to the intersubunit bridge at Cys-414 in Figure 6. However, recent work on IgA indicates J chain is joined to the COOH-terminal half-cystine in the human α heavy chain (Mendez *et al.*, 1974; Mestecky *et al.*, 1974). Since the COOH-terminal tetrapeptide sequences of the μ and α chains are identical (*i.e.*, Gly-Thr-Cys-Tyr), this sequence may also be the site of attachment of the J chain to the μ chain. Although J chain was demonstrable in the intact Fc fragments by use of acrylamide gel electrophoresis or specific antisera,[5] it was removed by reduction alkylation and subsequent preparation of CNBr fragments.

[10] We have reported extensive data on the 80 residues of the Fd′ sequence of Ga and Di from the beginning of the C region through Met-204 and have found no variation there from the Ou μ chain (Florent *et al.*, 1974). Nor have we yet found differences in the uncompleted study of the segment from Met-204 to the beginning of Fc (Gly-326).

our proteins (Ga) was not sequenced because of the small amount obtained.

On the other hand, there is no agreement between our findings and those of Razafimahaleo and Bourrillon (1970) who reported the isolation and amino acid analysis of eight fragments obtained by CNBr cleavage of a human μ chain. None of the CNBr fragments that we sequenced correspond to any of theirs in amino acid composition. For six of their fragments they propose a COOH-terminal sequence deduced only from digestion with carboxypeptidase A, but none of their sequences correspond to any we have determined for the 11 CNBr fragments of the Ou μ chain or any fragments of other μ chains.

Tryptic cleavage of undenatured IgM at 60° appears to result uniformly in cleavage at the Arg–Gly bond at positions 325–326 in the μ chain (Figure 7) to yield an Fc fragment beginning with the sequence Gly-Leu-Thr-Phe-Gln-Glx-Asx-(CHO) . . . This is the case for the five pathological IgM proteins we have studied and also for normal IgM. The same site of cleavage was found by Shimizu *et al.*[11] after tryptic cleavage at 25° for 2–24 hr after preincubation with 5 M urea. The Fc obtained from IgM Hir was sequenced by the automatic method for 28 cycles with results identical with those shown in Figure 7 (Shimizu *et al.*[11]). A similar preparation of Fc from Ga had N-terminal glycine in the sequenator but further analysis was unsuccessful because of a technical error.

The specific cleavage to produce Fc probably results from a slight opening of the pentameric molecule which exposes a trypsin-sensitive bond. The proximity of oligosaccharide C2, which must be at the surface, and of an interchain disulfide bridge probably affect the conformation aiding exposure of the bond but retarding further degradation of the Fc fragment. The ten chains in the Fc fragment are tightly held together in symmetrical array by two interchain disulfide bridges between each chain in each of the five pairs, and each pair is joined to two other pairs by an intersubunit bridge. However, occasionally adventitious tryptic cleavage may take place at other sites in Fc as occurred after Lys-445 in Dau Fc and normal Fc (see analysis of F8 above). This bond is midway between the Cμ4 and Cμ5 domains and thus is probably somewhat exposed. All IgM proteins we have studied are degraded by trypsin at 60° in the Cμ2 region between Fab and Fc. The Fab fragments of some such as Ba and Dau are also extensively degraded by trypsin at 60°. At 37° trypsin gives a dimeric $(Fab\mu)_2$ (Beale and Buttress, 1969), which probably extends through Arg-346, and a dimeric Fcμ that appears to begin at Val-347 (Paul *et al.*, 1971). However, the yield of both fragments is small, and sequence data are not available.

Although there is much suggestive evidence, there is remarkably little direct proof for the now widely accepted dogma that the C regions of Bence-Jones proteins and of pathological heavy chains are identical in amino acid sequence with the C regions of the homologous light and heavy chains of normal human immunoglobulins. This concept originated in the finding (Putnam, 1962) that many of the peptides identifiable by peptide maps in Bence-Jones proteins and myeloma globulins are present in normal human γ globulin, a result which suggested that portions of the structure are common to normal and pathological immunoglobulins. Complete sequence determinations of κ and λ Bence-Jones proteins established the division of light chains into a variable N-terminal region and a constant C-terminal region (Putnam *et al.*, 1966, 1967b). Hundreds of subsequent complete or partial sequence analyses have generalized this finding to all light and heavy polypeptide chains of human and animal immunoglobulins. Majority sequence analysis of small segments of the V regions of normal light or heavy chains has been done in a few instances, and the results support the hypothesis that the V regions of the pathological chains reflect the enormous variability in sequence of the normal chains (Niall and Edman, 1967; Milstein, 1967; Capra *et al.*, 1973). However, except for identity in the amino acid composition of the C-terminal tryptic peptides of Bence-Jones proteins and normal light chains (Milstein, 1966; Putnam *et al.*, 1963), there is very little direct evidence for the identity in sequence of the C regions of normal and pathological immunoglobulin polypeptide chains. The results we report here on the apparent identity of the N-terminal sequence of the Fc fragments of normal and pathological IgM proteins are the first data of which we are aware that show that extensive segments of the sequence of normal and pathological heavy chains are identical—at least within the limits of detection by the automatic sequencing technique. By techniques similar to those described in this article we are extending this comparison to other segments of the C regions of normal and pathological μ chains.[12]

Wells *et al.* (1973) have reported the first allotypic marker on human serum IgM and have designated it Mm1. Because the marker requires both μ chains and light chains in association for its full expression, it has not been possible to localize Mm1 on the μ chain. However, it is believed that Mm1 is located in the Fc region of the μ chain. Four of our five IgM proteins were tested for this marker by Dr. J. V. Wells of the University of California School of Medicine, San Francisco, Calif. All four proteins (Ou, Dau, Ga, and Di) were negative for the Mm1 marker. Hence we were unable to correlate the incidence of this marker with the amino acid sequence of the Fc fragment.

Addendum

After this manuscript was completed, the report of Watanabe *et al.* (1973) on the complete amino acid sequence of the human μ chain Gal appeared. These workers had published no previous data on μ chain sequence. However, their sequence for the Fc region essentially confirms the sequence we earlier published for Fc (Putnam *et al.*, 1972b) and essentially confirms our sequence for the entire constant region (Putnam *et al.*, 1973). The differences are largely technical and relate to the order of several amino acids. For example, their sequence omits the second threonine in the sequence Leu-Thr-Thr-Tyr beginning at position 372. However, our sequence for Ou Fc was confirmed by sequencer analysis of Ga Fc (see Figure 7). A second difference is their order of the glutamic acid residues in the sequence we give as Glu-Glu-Asn-Gly beginning at position 385; again, this sequence was confirmed in Ga (Figure 7). Minor technical problems also exist about the order of the dicarboxylic acids at Trp-419 and Trp-488. However, this independent study strongly supports our hypothesis about the iden-

[11] Shimizu, A., Watanabe, S., Yamamura, Y., and Putnam, F. W., submitted to *Immunochemistry*.

[12] We have not made extensive studies of the physical properties of the Fc fragments of pathological and normal IgM proteins; however, the behavior of the various Fc fragments appears similar in solubility, gel filtration, and ultracentrifugation despite considerable differences in the solubility properties of individual IgM proteins which may behave as cryoglobulins, pseudoglobulins, or euglobulins. Some of these differences may be due to variations in carbohydrate content as well as variation in amino acid sequence. The Fc fragments are soluble in distilled water and thus are not responsible for the euglobulin properties of many IgM proteins. We have not yet been able to crystallize the Fc fragments.

tity in sequence of the Fc region of normal and pathological human μ chains.

References

Beale, D., and Buttress, N. (1969), *Biochim. Biophys. Acta 181,* 250.

Bhatti, T., and Clamp, J. R. (1971), *Biochim. Biophys. Acta 229,* 393.

Capra, J. D. (1971), *Nature (London), New Biol. 230,* 61.

Capra, J. D., Wasserman, R. L., and Kehoe, J. M. (1973), *J. Exp. Med. 138,* 410.

Florent, G., Lehman, D., and Putnam, F. W. (1974), *Biochemistry 13,* 2482.

Frangione, B., Prelli, F., Mihaesco, C., and Franklin, E. C. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1547.

Franklin, E. C., and Frangione, B. (1968), *Biochemistry 7,* 4203.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11,* 4493.

Hurst, M. M., Niedermeier, W., Zikan, J., and Bennett, J. C. (1973), *J. Immunol. 110,* 840.

Mendez, E., Prelli, F., Frangione, B., and Franklin, E. C. (1974), *Biochem. Biophys. Res. Commun.* (in press).

Mestecky, J., Schrohenloher, R. E., Kulhavy, R., Wright, G. P., and Tomana, M. (1974), *Proc. Nat. Acad. Sci. U. S.* (in press).

Metzger, H. (1970), *Advan. Immunol. 12,* 57.

Miekka, S. I., and Deutsch, H. F. (1970), *J. Biol. Chem. 245,* 5534.

Milstein, C. (1966), *Proc. Roy. Soc., Ser. B 166,* 138.

Milstein, C. (1967), *Nature (London) 216,* 330.

Moore, V., and Putnam, F. W. (1973), *Biochemistry 12,* 2361.

Niall, H. D., and Edman, P. (1967), *Nature (London) 216,* 262.

Paul, C., Shimizu, A., Köhler, H., and Putnam, F. W. (1971), *Science 172,* 69.

Pisano, J. J., and Bronzent, T. J. (1969), *J. Biol. Chem. 244,* 5597.

Plaut, A. G., and Tomasi, T. B., Jr. (1970), *Proc. Nat. Acad. Sci. U. S. 65,* 318.

Putnam, F. W. (1962), *Biochim. Biophys. Acta 63,* 539.

Putnam, F. W., and Easley, C. W. (1965), *J. Biol. Chem. 240,* 1626.

Putnam, F. W., Florent, G., Paul, C., Shinoda, T., and Shimizu, A. (1973), *Science 182,* 287.

Putnam, F. W., Kozuru, M., and Easley, C. W. (1967a), *J. Biol. Chem. 242,* 2435.

Putnam, F. W., Migita, S., and Easley, C. W. (1963), *Protides Biol. Fluids, Proc. Collog. 10,* 93.

Putnam, F. W., Shimizu, A., Paul, C., and Shinoda, T. (1972a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 193.

Putnam, F. W., Shimizu, A., Paul, C., Shinoda, T., and Köhler, H. (1971), *Ann. N. Y. Acad. Sci. 190,* 83.

Putnam, F. W., Shinoda, T., Shimizu, A., Paul, C., Florent, G., and Raff, E. (1972b), *Specific Receptors Antibodies, Antigens, Cells, Int. Convocation Immunol., [Proc.], 3rd,* 40.

Putnam, F. W., Shinoda, T., Titani, K., and Wikler, M. (1967b), *Science 157,* 1050.

Putnam, F. W., Titani, K., and Whitley, E. J. Jr., (1966), *Proc. Roy. Soc., Ser. B 166,* 124.

Razafimahaleo, E., and Bourrillon, R. (1970), *Biochim. Biophys. Acta 200,* 89.

Shimizu, A., Putnam, F. W., Paul C., Clamp, J. R., and Johnson, I. (1971), *Nature (London), New Biol. 231,* 73.

Shinoda, T., and Satake, K. (1961), *J. Biochem. 50,* 293.

Smyth, D. S., and Utsumi, S. (1967), *Nature (London) 216,* 332.

Titani, K., Wikler, M., Shinoda, T., and Putnam, F. W. (1970), *J. Biol. Chem. 245,* 2171.

Wang, A. C., Fudenberg, H. H., and Pink, J. R. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1143.

Watanabe, S., Barnikol, H. U., Horn, J., Bertram, J., and Hilschmann, N. (1973), *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1505.

Wells, J. V., Bleumers, J. F., and Fudenberg, H. H. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 827.