Fragments Produced by Digestion of Human Immunoglobulin G Subclasses with Pepsin in Urea

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It was previously shown that digestion of human IgG1/ κ myeloma proteins with pepsin in the presence of 8M-urea produces fragments which differ from other proteolytic fragments of IgG, including those produced by peptic digestion in aqueous buffers. The two large urea/pepsin fragments each consist of three peptides, and together account for all of the constant region of the light chains and most of the constant region of the heavy chains. Myeloma proteins of subclasses IgG2, IgG3 and IgG4 with κ light chains were digested with pepsin in 8M-urea, and the resulting fragments compared with those produced from IgG1/ κ proteins. Gel filtration, starch- and polyacrylamide-gel electrophoresis and sequence analysis have shown that the peptides from each subclass are analogous with those from IgG1. A brief investigation of the products of urea/pepsin digestion of myeloma proteins with λ light chains has shown that in these proteins light-chain cleavage occurs at residue leucine-182, instead of, or as well as, at residue 117, where cleavage takes place in κ chains. Comparison of sequences around sites of urea/pepsin cleavage has shown that pepsin has quite restricted specificity under these conditions.

Digestion of IgG* by pepsin in the presence of urea has been shown to result in a pattern of cleavage totally different from that produced by digestion of IgG by pepsin in aqueous buffers (Parr *et al.*, 1976a,b). Fragment F(ab')₂, the major product of peptic digestion in aqueous buffers, consists of both light chains and the V_H, C_H1 and hinge regions of both heavy chains with all inter- and intra-chain disulphide bonds intact (Nisonoff *et al.*, 1960; Utsumi & Karush, 1965; Heimer *et al.*, 1967). Peptic digestion of IgG1/ κ in 8M-urea produces two large fragments, Fb'₂ and upFc, which together contain the C_L, C_H1, C_H2 and most of the C_H3 domains. The variable regions of both light and heavy chains are not recovered; they are presumably more susceptible to proteolytic attack under these conditions, and so are lost as small peptides.

Digestion with pepsin in the presence of 8M-urea has now been carried out by using IgG of subclasses 2, 3 and 4, with κ light chains, to compare the digestion products of these proteins with those of IgG1/ κ proteins.

A partial investigation of the products of urea/pepsin digestion of immunoglobulins with λ light chains has also been undertaken.

* Abbreviations: IgG, immunoglobulin G; IgG1/ κ , immunoglobulin with heavy chains of the γ 1 subclass and κ light chains; V_L, C_L, variable and constant domains of the light chain; V_H, C_H1, C_H2, C_H3, variable and constant domains of the heavy chain; fragment Fc, C-terminal half of the heavy chain; SDS, sodium dodecyl sulphate.

Materials and Methods

Immunoglobulins G of subclasses 2, 3 and 4 with κ light chains from patients with multiple myeloma were used in this study. IgG2(Zie) and IgG3(Pav) were cryoglobulins, which precipitated from the serum on cooling. This property was utilized in their purification. The sera were stood in ice for 30 min, and the resulting precipitates washed with ice-cold phosphate-buffered saline (0.15M-NaCl/0.039M-Na₂HPO₄/0.011M-NaH₂PO₄, pH 7.3) at least three times, until they were seen to migrate as single bands when examined by electrophoresis on cellulose acetate strips. IgG3(Pla) and IgG4(Mach) were prepared by electrophoresis in an LKB 5800 Porath column (Porath, 1954) in 0.2M-Tris/0.03M-boric acid/8.4mM-EDTA buffer, pH 8.9. In addition, two proteins with λ light chains, IgG1(Byn) and IgG2(Tuf) were prepared electrophoretically by the method of Porath (1954).

The times required for preparative digestion of the various immunoglobulins were determined from the results of time-course studies on small amounts of each protein. Urea/pepsin digests were done at 22°C, at pH 3.6, at an enzyme/substrate ratio of 1:100 (w/w). Samples were dialysed against 0.1M-sodium acetate buffer, pH 4.3; immediately before adding the enzyme, sufficient solid urea was added to make the urea concentration 8M, and the pH was lowered to 3.6 with HCl. Digestion times ranged from 45 min to 60 min. The digestion was stopped by raising the pH to 9.0 with 2M-Tris/HCl, pH 9.0, and 1M-NaOH.

Other methods used to prepare and characterize fragments Fb'_2 and upFc from human IgG myeloma proteins were described previously (Parr *et al.*, 1976a,b). For IgG3 proteins, the column (1.8cm $\times$ 210cm) of Sephadex G-50 used to separate the products of reduction and alkylation of fragment Fb'_2 was replaced by a column (2.6cm $\times$ 90cm) of Sephadex G-100. The same elution buffer, 6M-urea/0.1M-sodium acetate, pH5.0, was used as for the other subclasses. Cellulose acetate electrophoresis was performed by using the Beckman Microzone system (Beckman Instruments, Toronto, Ont., Canada).

Manual sequence determinations were done by Edman degradation, followed by dansylation, as described by Percy & Buchwald (1972).

Results

Preparation of fragments Fb'_2 and upFc

Digestion times for the various immunoglobulins ranged from 50 min to 90 min. The Sephadex G-150 elution patterns of digests of the four subclasses were very similar (Fig. 1), the most significant

difference being that the peak containing fragment Fb'_2 from the IgG3 samples was eluted from the column earlier than the same peak from the other subclasses. Examination of the digestion products by starch-gel electrophoresis showed that Fb'_2 fragments from IgG3 proteins Pav and Pla migrated markedly more slowly than Fb'_2 fragments from IgG1(Tem), IgG2(Zie) or IgG4(Mach) (Plate 1a).

Reduction and alkylation of fragments

When peptide mixtures resulting from reduction and alkylation of the Fb'_2 fragments were examined by electrophoresis in SDS/polyacrylamide gels, the bands obtained from Fb'_2 fragments from IgG2(Zie) and IgG4(Mach) had the same mobility as those obtained previously from fragment Fb'_2 of IgG(Tem), an IgG1/ κ myeloma protein. An extra band of lower mobility was found after electrophoresis of reduced and alkylated Fb'_2 fragments from IgG3(Pav) and IgG3(Pla) (Plate 1b). Reduction and alkylation of the upFc fragments from IgG2(Zie), IgG3(Pav) and IgG4(Mach) resulted in peptide mixtures which had the same mobility as those from fragment upFc(Tem) (Plate 1c).

Fig. 1. Separation of the products of urea/pepsin digestion of IgG subclasses 1-4. The column (5cm $\times$ 90cm) was packed with Sephadex G-150 and eluted with 0.1M-sodium acetate buffer, pH5.0, containing 6M-urea. The flow rate was 48 ml/h and 400 ml was eluted before fraction collecting began; 8 ml fractions were collected. A, fragment Fb'_2 ; B, fragment upFc.

EXPLANATION OF PLATE I

Electrophoresis of various products of urea/pepsin digestion of IgG subclasses

(a) Starch-gel electrophoresis of urea/pepsin digests, carried out at 22°C in 8M-urea/0.1M-sodium formate buffer, pH3.6, by the method of Smithies *et al.* (1962). The buffer was made up without 2-mercaptoethanol; (b) SDS/polyacrylamide-gel electrophoresis of totally reduced and alkylated Fb'_2 fragments. In subclass 3 the band of intermediate mobility contained only peptide I; (c) SDS/polyacrylamide-gel electrophoresis of totally reduced and alkylated upFc fragments. Electrophoresis in (b) and (c) was done by the method of Weber & Osborn (1969). (1) IgG1(Tem); (2) IgG2(Zie); (3) IgG3(Pav); (4) IgG4(Mach).

Table 1. *N-Terminal sequences of peptides derived from fragments Fb'2 and upFc*

Sequences are compared with those of equivalent peptides from IgG(Tem). Numbers above the sequences are according to IgG(Eu) (Edelman *et al.*, 1969). — indicates identity of sequence with that of peptide from IgG(Tem) (Parr *et al.*, 1976a,b); [...], identification of residue uncertain; N.D., not determined.

Fragment	Peptide	Protein	Chain	
Fb'2	I	Tem	κ	117 Ile-Phe-Pro-Pro-Ser-Asp-Glu-Gln-Leu-Lys
		Zie	κ	_____
		Pav	κ	*_____
		Mach	κ	*_____
	II			180
		Tem	γ1	Tyr-Ser-Leu-Ser-Ser-Val-Val-Thr-Val-Pro
		Zie	γ2	_____
		Pav	γ3	_____
		Mach	γ4	_____
	III			113
		Tem	γ1	Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly
		Zie	γ2	—Val—[...]—
		Pla	γ3	_____
		Mach	γ4	_____
upFc	Aa			253
		Tem	γ1	Ile-Ser-Arg-Thr-Pro-Glu-Val-Thr-Cys-Val
		Zie	γ2	_____N.D._____
		Pav	γ3	_____N.D._____
	Ac			307
		Tem	γ1	Thr-Val-Leu-His-Gln-Asp-Trp-Leu-Asn-Gly
		Zie	γ2	_____Val_____
		Pav	γ3	_____
		Mach	γ4	_____
	B			411
		Tem	γ1	Thr-Val-Asp-Lys-Ser-Arg-Trp-Gln-Gln-Gly
		Zie	γ2	_____N.D._____
		Pav	γ3	_____
		Mach	γ4	_____Glu_____

* Sequence determined manually.

Molecular weights

Molecular weights of the peptides, when determined by comparison of their mobilities in SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) were found to be the same as those from IgG(Tem), except for the large peptides from Fb'2 fragments derived from IgG3(Pla) and IgG3(Pav). The mobilities of the large peptides from fragments Fb'2(Pav) and Fb'2(Pla) were 0.53 and 0.52 respectively, relative to Bromophenol Blue. These mobilities were compared with those of several standard markers of known molecular weights: (i) IgG heavy chain, mol.wt. 50000, 0.24; (ii) ovalbumin, mol.wt. 43500, 0.29; (iii) chymotrypsinogen, mol.wt. 25000, 0.47; (iv) ribonuclease, mol.wt. 13700, 0.67. The apparent mol.wts. of the large peptides were 20900 and 21400.

Sequence analyses

The results of *N*-terminal sequence analyses of peptides from each IgG are shown in Table 1, and compared with those of peptides from IgG1(Tem) (Parr *et al.*, 1976a,b). Fig. 2 illustrates the positions of the six peptides in the IgG1 molecule.

Immunoglobulins with λ light chains

Results of SDS/polyacrylamide-gel electrophoresis and *N*-terminal determinations indicated that peptides Aa, Ac and B from fragment upFc of IgG2/λ(Tuf) were the same as those from other upFc fragments. When fragment Fb'2(Tuf) was reduced and alkylated, the elution profile obtained from the Sephadex G-50 column was different from those of reduced and alkylated Fb'2 fragments from the other proteins studied. A peptide which was eluted from

Fig. 2. Schematic representation of the positions of fragments Fb'_2 and $upFc$, and peptides derived from them (I, II, III and Aa, Ac, B) in the IgG1/ κ molecule

Residues marked are the *N*-terminals of the peptides, except for residues leucine-174 and aspartic acid-376, which are the *C*-terminal residues of peptides III and Ac respectively. N_L , N_H , *N*-termini of light and heavy chains; C_H , *C*-terminus of heavy chain.

the column in the same position as peptide II₄ from the other proteins was found to have the same mobility in SDS/polyacrylamide-gel electrophoresis as peptide II, and also have *N*-terminal tyrosine. This peptide was presumed to be analogous with peptide II from proteins with κ chains. Peptides analogous with peptides I and III were not identified in the column eluate, but a small peptide with *N*-terminal leucine was purified. The *N*-terminal sequence of this peptide was Leu-Ser-Leu-Thr-Pro-Glu-Gln-Trp-Lys-Ser-, which corresponds to the sequence of λ Bence-Jones protein New, beginning at residue 182 (Langer *et al.*, 1968). The only difference found was at residue lysine-9 of the peptide, which corresponds to residue arginine-190 of protein New. This position has been shown to be the site of the Oz antigenic marker. In Oz(+) chains residue 190 is lysine, whereas in Oz(-) chains the lysine is replaced by arginine (Appella & Ein, 1967; Quattrocchi *et al.*, 1967). The analogous peptide was prepared from IgG1/ λ (Byn), and found to have the same *N*-terminal sequence through the first five residues.

Discussion

The results given above show that pepsin in the presence of 8M-urea will digest immunoglobulins of all four γ -chain subclasses. In IgG2/ κ and IgG4/ κ proteins, both the Fb'_2 and $upFc$ fragments produced were exactly equivalent to those produced when IgG1/ κ proteins are digested under the same conditions. Fragment $upFc$ of IgG3/ κ proteins was also equivalent to fragment $upFc$ from proteins of the other subclasses.

For Fb'_2 fragments from IgG3/ κ proteins, one major difference was observed. Although the cleavage sites were in the analogous positions, the mol.wt. of peptide II was 21 000, in contrast with 11 000 for peptide II from the other subclasses.

This observation can readily be explained, since peptide II contains the hinge region, which has been shown to be extended in γ_3 chains by the presence of extra residues (Michaelsen & Natvig, 1972). The mol.wt. of γ_3 chains is about 10 000 higher than that of γ_1 , γ_2 and γ_4 chains (Saluk & Clem, 1971; Turner *et al.*, 1970). The presence of this extended hinge region, which has been shown to consist of a series of duplications of sections of the sequence (Adlersberg *et al.*, 1975), also accounts for the slower mobility of fragment Fb'_2 from IgG3 subclass proteins in starch-gel electrophoresis and its earlier elution from the gel-filtration column.

Peptide III from IgG3(Pla) was found to have the same *N*-terminal sequence as peptide III from the other subclasses (Table 1), but the *N*-terminal sequence of the analogous peptide from IgG3(Pav) was Asp-Val-Trp-Gly-Gln-Gly-Thr-Thr-Val-Thr-. Comparison of this sequence with other published sequences (Cunningham *et al.*, 1969; Press & Hogg, 1969; Capra & Kehoe, 1974) showed that cleavage of γ_3 chain (Pav) had occurred *N*-terminal to an aspartic acid residue in a position homologous with that of proline-105 in γ_1 chain (Eu), instead of *N*-terminal to residue valine-113. [Heavy-chain and κ -chain residue numbering throughout is according to protein Eu (Edelman *et al.*, 1969); λ -chain numbering is according to Bence-Jones protein New (Langer *et al.*, 1968).] The reason for the different cleavage site in IgG3(Pav) is not known, but it seems probable that this difference is a property of IgG3(Pav) rather than being typical of γ_3 chains in general. The possibility of a connexion between the cryoglobulin property of IgG3(Pav) and its apparently abnormal cleavage site cannot be excluded entirely, but IgG(Zie) was also a cryoglobulin, and yet was cleaved at positions homologous with those of immunoglobulins without cryoglobulin properties.

Fig 3. Comparison of urea/pepsin cleavage sites in κ , λ and γ_1 chains

Sequences for κ and γ_1 chains are from protein Eu (Edelman *et al.*, 1969), and for λ chain from Bence-Jones protein New (Langer *et al.*, 1968). ↓ Cleavage site; (↓) limited cleavage site; —, gap introduced to maximize homology; underlined residues show position of homology; V|C indicates switch from variable to constant region.

Determination of the *N*-terminal sequence of a peptide produced by the reduction and alkylation of fragment Fb'₂ from two proteins with λ light chains showed that λ chains are cleaved by pepsin in urea at a different site from κ chains. Comparison of sequences (Fig. 3) shows that there is a high degree of sequence homology between κ and λ chains in the region between residues 111 and 121 (κ -chain numbering), but since no peptides were identified from the λ chains except that with *N*-terminal leucine-182, our results do not exclude the possibility of λ -chain cleavage at any other site, e.g. between residues threonine-120 and leucine-121, the position corresponding to the cleavage site in κ chains. No cleavage of κ chains occurs in the position equivalent to the λ -chain site, despite the similarity in sequence in this region also. Between the V_H and C_H1 domains, the γ -chain sequence shows considerable homology with the sequence between the V_L and C_L domains (Fig. 3), but the *N*-terminal cleavage of peptide III occurs at a position which is not equivalent to the κ -chain site. It is possible that the substitution of a proline residue in the γ chain for the isoleucine residue at the C-terminal side of the κ -chain cleavage site may block the action of the pepsin. Cleavage in the γ chain occurs at a position 14 residues closer to the *N*-terminus, between two hydrophobic residues, leucine-112 and valine-113.

Although a detailed study of the specificity of pepsin in urea has not been done, inspection of cleavage sites identified by the characterization of

fragments Fb'₂ and upFc has led to several noteworthy observations. It appears that, in 8M-urea, pepsin activity is directed towards hydrophobic regions of the polypeptide chain. Of the nine major and three minor cleavage sites identified, 11 have a hydrophobic residue adjacent on the *N*-terminal side, six of which are leucine residues. Similarly, nine of the 12 sites have a hydrophobic residue adjacent on the C-terminal side. Of these 20 hydrophobic residues, only six are aromatic. The relative resistance of the constant regions, where numerous pairs of hydrophobic residues occur without being cleaved, in contrast with the extreme susceptibility of the variable regions, suggests that sufficient tertiary structure may be retained in the IgG molecule, even in the presence of 8M-urea, to influence the activity of the pepsin. The marked difference in susceptibility between the variable and constant regions also indicates that, although the intact disulphide bonds may possibly affect the availability of residues for enzymic attack, these bonds cannot be wholly responsible for the resistance to digestion, since all disulphide bonds, including both the V_H and V_L intrachain loops, are intact. These observations are in agreement with those of Stevenson & Stevenson (1967), who showed that, in some cases, antigen-antibody complexes were only partially disrupted by exposure to 8M-urea for 24h, and that sufficient specific conformation was retained in 8M-urea for hapten-binding activity to be retained.

Several of the urea/pepsin cleavage sites are within

intrachain disulphide loops rather than between them, thus producing peptides which are markedly different from those produced by fragmentation with papain (Porter, 1959) or pepsin in aqueous buffers (Nisonoff *et al.*, 1960; Utsumi & Karush, 1965). These peptides may be used to complete the sequence determinations of γ_2 , γ_3 and γ_4 chains, and also to localize the sites responsible for the effector functions of the antibody molecule.

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