Amino Acid Sequence of a Murine Immunoglobulin Fragment That Possesses Complement Fixing Activity†

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ABSTRACT: The amino acid sequence of 62-residue cyanogen bromide fragment located in the Fc region of a murine IgG2a immunoglobulin has been determined using a combination of automated and manual Edman degradation techniques. This fragment, which comprises the major portion of the IgG C_H2 domain, had previously been shown capable of fixing a significant quantity of guinea pig complement when bound to polystyrene latex particles. The sequence was compared with those available for the corresponding region of other immunoglobulins from man, the rabbit, and the guinea pig. The various interspecies sequence comparisons showed that sequence identities for the part of the mammalian IgG heavy chain corresponding to this fragment totaled approximately 60%. This constant region identity value was considerably lower than interspecies sequence homologies for certain segments of immunoglobulin heavy chain *variable* regions. The results indicate that complement fixing molecules can differ by as much as 40% in the amino acid sequence of the C_H2 domain. The absence of a clear relationship between the amino acid sequence and the complement binding property implies that such binding is most dependent on some conformational feature of this region of the immunoglobulin molecule.

The functional duality of immunoglobulin molecules is now well established. A primary function, antigen binding, is carried out by variable regions of both light and heavy polypeptide chains, while the biological, or effector, properties are mediated by heavy chain constant regions (Edelman and Gall, 1969). The exact intramolecular site, or sites, responsible for these various properties have not been precisely localized, however, although the Fc region alone has been shown capable of mediating a number of effector functions (Cohen and Milstein, 1967). According to the "domain hypothesis" for immunoglobulins (Edelman *et al.*, 1969), different and discrete regions of these molecules are responsible for their various properties, including the effector functions.

Previous work with cyanogen bromide fragments of a murine myeloma protein had demonstrated that an isolated 62 amino acid section of the heavy chain was capable of fixing a significant amount of guinea pig complement when attached to polystyrene particles (Kehoe *et al.*, 1969). We report here the complete amino acid sequence of this fragment and compare this sequence with that of the corresponding region of other immunoglobulin molecules. The sequence determination unequivocally localizes this fragment in the amino terminal segment of the Fc region of this molecule and provides for a futher assessment of the extent of the sequence homologies characteristic of the C_H2 domain of mammalian IgG molecules.

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Materials and Methods

The preparation of the IgG2a protein produced by the murine myeloma MOPC 173 has already been described, as have the methods for the purification of its constituent cyanogen bromide fragments (Bourgois and Fougereau, 1970a,b). Working quantities of fragment H-5 were prepared for the present study by scaling up the gel filtration method previously described.

Enzyme Digests. Trypsin, subtilisin, and chymotrypsin digests were prepared from fragment H-5 in 0.5 M NH_4HCO_3 buffer (pH 8.0). Enzyme–substrate ratios were 1:100 and digestion was allowed to proceed for 1 (trypsin, subtilisin) or 16 (chymotrypsin) hr. Digests were lyophilized directly.

Peptide Purification. Enzymatic digests were purified by gel filtration (Sephadex G-50 or G-25 in 0.05 M NH_4HCO_3) or on Dowex 50-X2 columns (0.9 × 55 cm) at 50° using a linear gradient from 0.1 N, pH 3.1 pyridine–formic acid buffer to 2.0 N, pH 5.0 pyridine–formic acid. Aliquots of the Dowex effluent were analyzed using an automatic system (A. Bourgois, M. Fougereau, and J. Rocca-Serra, submitted for publication). Additional peptide purifications were performed by high-voltage paper electrophoresis at pH 3.5 or 6.5 which also permitted net charge determinations (Milstein, 1966; Offord, 1966).

Amino Acid Analysis. Samples were hydrolyzed *in vacuo* for 20 hr at 120° in 6.0 N HCl and analyzed on a Beckman Model 121 amino acid analyzer.

Sequencing Methods. A. Automated Sequencer. The use of the Beckman Model 890 sequencer has been previously described (Capra and Kunkel, 1970).

B. Manual Method. Manual Edman degradations using phenyl isothiocyanate were carried out according to standard methods (Gray, 1967). Phenylthiohydantoins were identified by gas chromatography, thin-layer chromatography (Edman and Sjoquist, 1956), or regeneration of the amino acid from the phenylthiohydantoin by hydrolysis, and identification on the amino acid analyzer (Van Orden and Carpenter, 1964). Dansyl-Edman analyses were carried out as previously described (Bourgois and Fougereau, 1970a).

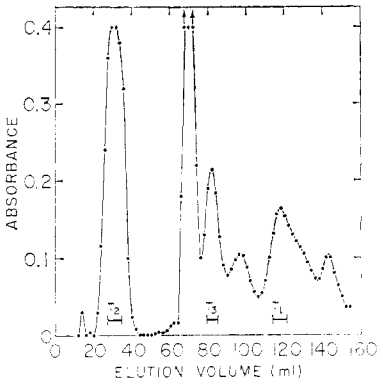

FIGURE 1: Chromatography of soluble tryptic peptides of fragment H-5; 10 μmol of the enzymatic digest of fragment H-5 were loaded on a Dowex 50-X2 column (0.9 × 55 mm) equilibrated at 50°. A linear gradient (250 ml in each reservoir) of 0.1 N, pH 3.1 pyridine–formic acid starting buffer and 2.0 N, pH 5.0 terminal buffer was begun immediately. Aliquots (~5%) of the effluent were monitored at 570 mm after alkaline hydrolysis and reaction with ninhydrin.

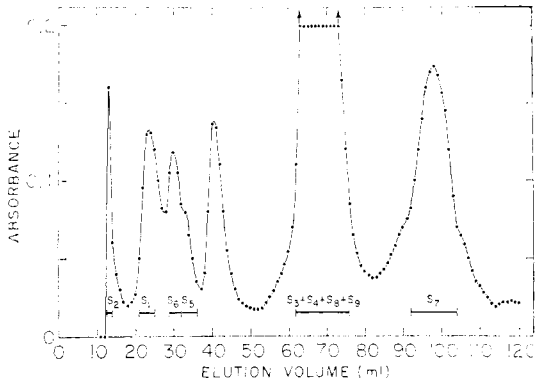

FIGURE 2: Chromatography of 5 μmol of subtilisin digest of fragment H-5. Same chromatography conditions as in Figure 1.

C. End Group Analysis. Amino terminal residues were determined by the dansyl technique (Woods and Wang, 1967). Carboxy terminal residues were determined by carboxypeptidase digestions as described by Ozols and Strittmatter (1968).

Detection of Carbohydrates. Amino sugars were detected on the amino acid analyzer subsequent to a 6-hr acid hydrolysis (6.0 N HCl).

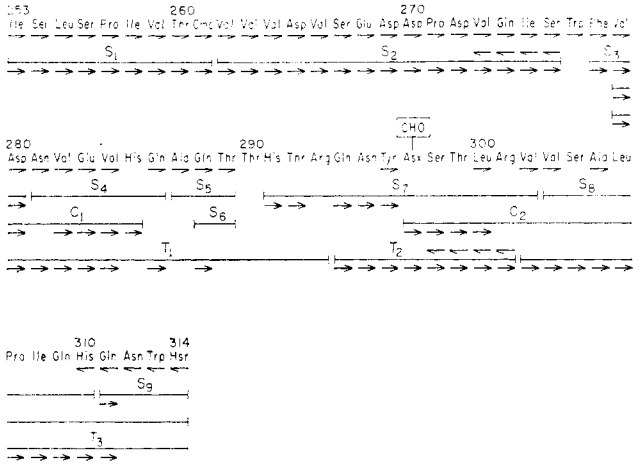

FIGURE 3: Sequence analysis of fragment H-5. The sequence was established using automated Edman degradations (—), manual Edman degradations of peptides (→), and carboxypeptidase digestion (⇀). The numbers indicate IgG residue positions as given by Edelman *et al.* (1969).

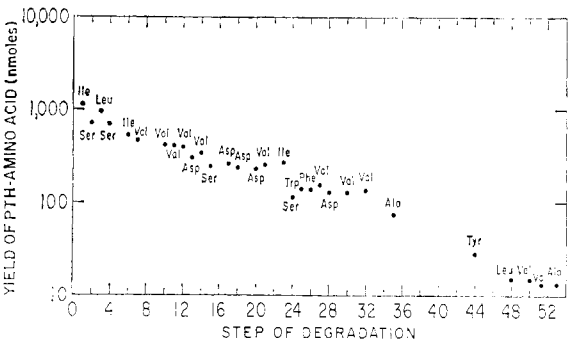

FIGURE 4: Semilogarithmic plot of sequencer yields obtained during direct analysis of intact fragment H-5. The yield for each position shown was obtained either by comparing the sample gas chromatographic patterns with those of standard amounts of known amino acid phenylthiohydantoins, or by amino acid analysis of the hydrolyzed phenylthiohydantoins.

Results

Fragment Preparation. The purity of cyanogen bromide fragment H-5 was initially estimated by amino acid analysis (Bourgois and Fougereau, 1970a), especially by the absence of lysine (Table I). Purity was confirmed by finding a unique amino terminal isoleucine residue and by the demonstration of a single amino acid sequence when the fragment was subjected to analysis in the automated sequencer.

Enzymatic Digests. Elution profiles of trypsin and subtilisin derived peptides of fragment H-5 are shown in Figures 1 and 2. The compositions of peptides derived from these separations are shown in Table I.

Two useful chymotryptic peptides, termed C-1 and C-2, were also isolated by Dowex ion-exchange chromatography. The compositions of these peptides are also given in Table I.

Studies on Intact Fragment H-5. Sequence Analysis. As shown in Figure 3, direct analysis on the automated sequencer of intact fragment H-5 allowed the determination of the sequence of the first 37 amino acid residues (positions 253–291 of the Eu numbering system of Edelman *et al.* (1969), with the exception of residue 285. Five additional residues, 296, 300, 302, 303, and 305, were also identified using the sequencer for a 53-step analysis, as illustrated in Figure 3. Concordant results were obtained on three different sequencer runs. Figure 4 shows yields obtained during an automated sequencer run on intact fragment H-5.

Carboxy Terminal Sequence. Carboxypeptidase A digestion of intact fragment H-5 for various intervals up to 24 hr gave the results indicated in Figure 5. Since other studies showed that residues 311 and 312 actually exist in the amide form (*vide infra*), the establishment of the sequence of the five carboxy terminal residues was possible (Figure 3). Thus 45 of the 62 residues of fragment H-5 were assignable on the basis of studies of the intact fragment.

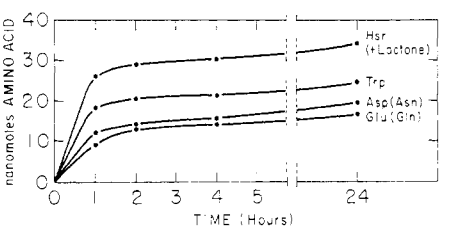

FIGURE 5: Time course of release of amino acids from intact fragment H-5 by carboxypeptidase A. Digestions were performed as described in the text and aliquots removed for direct amino acid analysis at the intervals indicated.

TABLE I: Amino Acid Composition of MOPC 173 Cyanogen Bromide Fragment H-5 and Its Constituent Tryptic, Chymotryptic, and Subtilisic Peptides.[a]

Amino Acid	H-5	T-1	T-2	T-3	C-1	C-2	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9
Lysine															
Histidine	3.2 (3)	1.9 (2)		0.9 (1)	0.8 (1)	0.6 (1)				1 (1)			0.9 (1)	1.0 (1)	
Arginine	2.1 (2)	1.0 (1)	0.9 (1)			0.7 (1)							1.9 (2)		
CM-cysteine	0.8 (1)						0.8 (1)								
Aspartic acid	9.1 (9)	2.0 (2)	1.9 (2)	1.0 (1)	2.1 (2)	1.8 (2)		4.1 (4)	1.2 (1)	1.2 (1)			1.8 (2)		1.2 (1)
Threonine	4.9 (5)	3.0 (3)	0.9 (1)			1.4 (1)	1.0 (1)				0.9 (1)	0.8 (1)	1.7 (2)		
Serine	5.8 (6)		1.1 (1)	1.1 (1)		2.3 (2)	2.0 (2)	1.7 (2)					1.0 (1)	0.8 (1)	
Glutamic acid	8.2 (8)	2.8 (3)	1.1 (1)	2.1 (2)	1.1 (1)	2.4 (2)		2.1 (2)		1.8 (2)	1.1 (1)	1.2 (1)	1.0 (1)	1.0 (1)	0.7 (1)
Proline	3.0 (3)			1.0 (1)		0.7 (1)	1.1 (1)	1.1 (1)						1.1 (1)	
Glycine															
Alanine	2.0 (2)	1.1 (1)		0.9 (1)		1.0 (1)					1.0 (1)			1.0 (1)	
Valine	10.8 (11)	3.1 (3)		2.0 (2)	2.9 (3)	1.9 (2)	1.0 (1)	4.9 (5)	1.0 (1)	1.8 (2)			1.2 (1)	1.0 (1)	
Homoserine	0.9 (1)			0.9 (1)		0.8 (1)									1.0 (1)
Isoleucine	4.0 (4)			1.0 (1)		1.1 (1)	2.0 (2)	1.0 (1)						1.1 (1)	
Leucine	3.0 (3)		1.0 (1)	0.9 (1)		1.8 (2)	1.1 (1)						1.0 (1)	1.0 (1)	
Tyrosine	0.9 (1)		1.0 (1)						0.8 (1)				1.0 (1)		
Phenylalanine	1.0 (1)														
Tryptophan	2.3 (2)			(1)[b]		N.D. (1)									(1)[b]
No. of residues		15	9	13	7	18	9	15	3	6	3	2	12	8	4
% yield		40	60	50	20	35	80	90	20	20	90	10	10	10	20
Position in sequence		279–293	294–301	302–314	279–285	297–314	253–261	262–276	278–280	281–286	287–289	288–289	291–302	303–310	311–314
$(CH_2)O_n$			+										+		

[a] Numbers in parentheses represent nearest integer values. [b] Deduced by fluorescence. N.D., not determined.

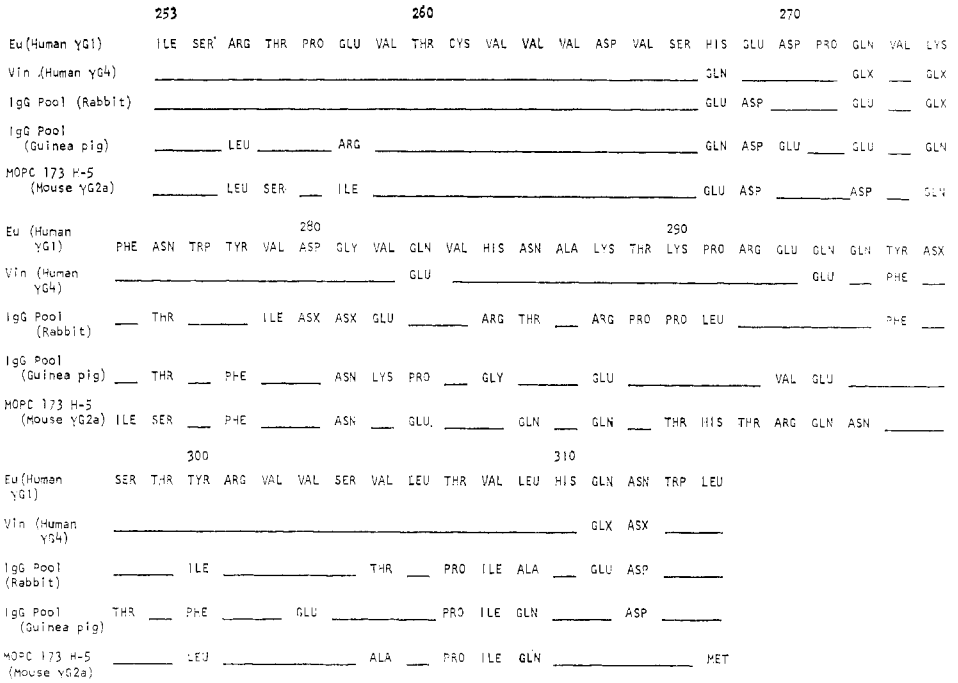

FIGURE 6: Sequence comparisons for various immunoglobulins in the region covered by MOPC 173 fragment H-5. Other sequences are from: Edelman *et al.* (1969), Eu; Pink *et al.* (1970), Vin; Hill *et al.* (1966) and Appella *et al.* (1971), rabbit IgG; Tracey, D. E., and Cebra, J. J. (1973), submitted for publication, guinea pig IgG.

Studies of Tryptic Peptides of Fragment H-5. The sequence analyses performed on tryptic peptides are indicated in Figure 3. The localization of the carbohydrate moiety of fragment H-5 was carried out on peptide T-2, the only tryptic peptide which contained carbohydrate (Table I). After three steps of Edman degradation, carbohydrate was still present on the residual peptide. Also, digestion with carboxypeptidases A and B released the four carboxy terminal residues, leaving only the Asx at position 297 as the point of attachment for the carbohydrate, in correspondence with other immunoglobulin heavy chains (Howell *et al.*, 1967; Hunt and Dayhoff, 1970). The presence of homoserine and its lactone identified peptide T-3 as the carboxy terminal tryptic peptide of fragment H-5.

Studies of Subtilisin Peptides of Fragment H-5. The manual sequence analyses performed on the subtilisin peptides are shown in Figure 3.

Studies of Selected Chymotryptic Peptides. The manual sequence analysis of peptides C-1 and C-2 is shown in Figure 3.

Localization of Constituent Peptides within Fragment H-5. Peptide T-1 was localized by its correspondence with the results of the sequencer analysis performed on intact fragment H-5, as shown in Figure 3. T-3 contained the homoserine and was thus carboxy terminal. Since the intact fragment contains only two arginines and no lysine, peptide T-2 must be localized between T-1 and T-3. In addition, a significant overlap was obtained for peptide T-2 with peptides S-7 and C-2, as well as by sequence analysis of the intact fragment (Figure 3).

The subtilisin peptides S-1 through S-9 account for the composition of the entire fragment with the exception of one tryptophan and one threonine residue. The sequencer analysis of intact H-5 showed that the missing tryptophan residue was localizable to position 277 (Figure 3). The threonine at position 290 was presumably liberated during the subtilisin digestion then lost during the peptide isolation procedures. Peptides S-1, S-2, S-3, and S-4 were ordered by comparison with the results of the direct sequencer analysis of intact fragment H-5 (Figure 3). Peptides S-4, S-5, S-6, and S-7 were overlapped by peptide T-1, while S-7 and S-8 were overlapped by peptides T-3 and C-2. Peptide S-9, like peptide T-3, was carboxy terminal because of its content of homoserine.

Assignment of Aspartic Acid, Asparagine, Glutamic Acid, and Glutamine Residues. These residues were identified by thin-layer chromatography following automated sequencer analysis with the exception of the residues in positions 274, 294, 295, 297, 309, 311, and 312, which were deduced as follows: carboxypeptidase digestion of peptide S-2 released the glutamine at position 274, as shown in Figure 3. The low difference in mobility at pH 6.5 between intact peptide T-2 and that of the same peptide following two steps of Edman degradation indicated that positions 294 and 295 were glutamine and asparagine, respectively. The neutrality of peptide T-3 indicated that residues 309, 311, and 312 were glutamine, glutamine, and asparagine, respectively. A precise determination of the charge on residue 297 was not possible because of the attachment of acidic carbohydrate at this point. This asparaginyl residue is a common site of carbohydrate attachment in IgG immunoglobulins (Howell *et al.*, 1967; Hunt and Dayhoff, 1970).

Sequence of Fragment H-5. The composite of these results, summarized in Figure 3, allowed the proposal of a complete sequence for fragment H-5. This sequence is displayed in Figure 6, with available corresponding sequences from man, the rabbit, and the guinea pig.

Discussion

The determination of the amino acid sequence of MOPC 173 cyanogen bromide fragment H-5 unequivocally places it at residues 253–314 (Eu numbering, Edelman *et al.*, 1969) of the IgG heavy chain, within the amino terminal half of the Fc region. No part of the IgG hinge region (Smyth and Utsumi, 1967) is included in this fragment. The validity of the sequence is supported by the isolation of constituent enzymatically derived peptides which show appropriate overlaps and whose compositions account for the whole fragment (Table I,

Figure 3). This region falls within the C_H2 domain of the IgG molecule as defined by Edelman *et al.* (1969).

The determination of this sequence required the combined use of automated and manual Edman degradation procedures and illustrated the complementary nature of these two approaches to protein sequencing. The automated sequencer was particularly valuable in reducing the total amount of fragment H-5 that had to be used (the complete sequence of 62 residues was established using approximately 30 μmol (or 200 mg) of fragment). Manual sequencing of selected tryptic, subtilisic, and chymotryptic peptides prepared from intact fragment H-5, together with a number of carboxypeptidase analyses, were required to extend the sequence to the carboxy terminal homoserine residue and to fill in those residues not identified by the automated approach; 150 mg of the 200 mg of fragment H-5 used was consumed in the enzymatic digestions and peptide isolations were carried out prior to the manual degradations.

The completed sequence of fragment H-5 allows some specific analyses of interspecies sequence variations for this part of the IgG heavy chain. The results of various comparisons are shown in Table II. As previously determined by Pink *et al.* (1970), the intraspecies identity for the two human proteins (IgG1 Eu and IgG4 Vin) that have been sequenced in this region exceeds 90%. However, the interspecies comparisons, no matter how they are made among man, rabbit, mouse, and guinea pig, show very close to 60% identities for the region covered by fragment H-5. Since the constant region of the immunoglobulin molecule is believed to be under strong selection pressure to retain structure so that the various effector functions will in turn be retained (Edelman *et al.*, 1969), the value of only 60% interspecies homology in this part of two complement fixing molecules (MOPC 173 and the human IgG1 Eu) seems somewhat lower than might be expected *a priori*. Also, consideration of this value of 60% with the greater than 90% homology of the human IgG1 (classical complement pathway positive) and IgG4 (classical complement pathway negative—Ishizaka *et al.*, 1962) proteins in this region could indicate that, if a complement component does bind to the protein part of immunoglobulins (*i.e.*, not to a carbohydrate moiety), only a few amino acids may be directly involved.

The recent availability of sequence data on the heavy chain of human IgM proteins (Watanabe *et al.*, 1973; Putman *et al.*, 1973) made possible a search for regions of sequence homology between fragment H-5 and the Fc region of the IgM molecule, which has been shown capable of complement fixation (Plaut *et al.*, 1972). Except for some very weak homology with certain sections of the IgM C_H3 domain, which were limited to a maximum of eight corresponding residues in a 62 amino acid stretch (comparing, for example, the H-5 sequence with that section of the IgM heavy chain beginning at residue 380 as described by Putnam *et al.* (1972)), no significant sequence correspondence between the IgG heavy chain and that of the IgM proteins could be detected. The expression of the complement fixing property would thus not seem, by this comparison alone, to be dependent on a particular amino acid sequence stretch.

Four independent studies, involving three distinct species, have now implicated the amino terminal section of the Fc region of IgG in a complement fixing function. Utsumi (1969) showed that ablation of this region of purified rabbit Fc fragment resulted in the loss of its complement fixing capacity. Our previous studies with fragment H-5 demonstrated that this fragment alone possessed a significant amount of complement fixing ability and thus provided the first direct evidence for the participation of the C_H2 domain in this biological function of immunoglobulins. Subsequently, Connell and Porter (1971) reported that a fragment of a rabbit antibody preparation, which they termed Facb, and which includes the entire light chain and all of the heavy chain except the carboxy terminal 120 residues (*i.e.*, approximately heavy chain residues 1–326), retains complement fixing activity. Finally a recent study by Ellerson *et al.* (1972) has corroborated the C_H2 assignment for complement fixation in experiments carried out utilizing intact heavy chain constant region domains prepared from a human IgG1 myeloma protein.

TABLE II: Homology Comparisons among Various IgG Proteins for the Region Covered by MOPC 173 Fragment H-5.

Proteins Compared	No. of Identities in Residues 253–314 of Heavy Chain	% Identities
Mouse γG2a : human γG1	37/62	60
Mouse γG2a : human γG4	38/62	61
Rabbit γG pool : human γG1	40/62	65
Rabbit γG pool : human γG4	39/62	63
Mouse γG2a : rabbit γG pool	32/62	53
Mouse γG2a : guinea pig γG pool	38/62	61
Guinea pig γG pool : human γG1	39/62	63
Guinea pig γG pool : human γG4	43/62	69
Guinea pig γG pool : rabbit γG pool	39/62	63
Human γG1 : human γG4	57/62	92

One aim of the present study was thus to identify, within the region of the IgG heavy chain represented by MOPC 173 fragment H-5, any specific amino acid residues that might participate directly in the binding of complement components. In particular, we looked for positions where a given residue occurs in proteins such as the mouse IgG2a or human IgG1 subclasses, that are known to be capable of fixing complement by the classical pathway beginning with C-1 (Muller-Eberhard, 1971), and a different residue exists for proteins negative in such systems, such as human IgG4 proteins. As shown in Figure 6, no such correlation could be made.

The carbohydrate moiety characteristic of most immunoglobulin heavy chains was localized for the MOPC 173 protein at asparagine-297, a common site of attachment for such carbohydrate (Howell *et al.*, 1967). Howell *et al.* had in fact suggested, even before specific structure–function correlates had been proposed for complement fixation, that the glycopeptide of this region might be involved in certain of the biological properties of immunoglobulins. While it is conceivable that carbohydrate attached to fragment H-5 could participate in the complement binding function, no direct evidence for such a carbohydrate role has yet been obtainable for any immunoglobulin.

Expression of the latent capacity of any immunoglobulin to activate the complement cascade requires either union with specific antigen or some artificially induced distortion of the immunoglobulin molecule by chemical means (such as bisdiazotized benzidine aggregation), heat aggregation or attachment to polystyrene latex particles (Ishizaka *et al.*, 1962; Utsumi, 1969). Such processes presumably mimic, at least in part, a conformational change that occurs in an antibody molecule following its interaction with antigen. Although precise data are limited, all indications to date suggest that

immunoglobulin conformational changes following union with antigen are not extensive (Green, 1969; Metzger, 1970). This could imply that the activation process for complement fixation, whatever its exact nature, is probably a subtle one (Thompson and Hoffman (1971) have presented evidence for the allosteric nature of the activation process.) Since the two heavy chains of an IgG molecule are bound by covalent disulfide linkages in the hinge region, and since the carboxy-terminal half of the Fc region (the C_H3 domain) has been shown to be the area where the strongest noncovalent bonds exist between the two heavy chains (Charlwood and Utsumi, 1969), the region of fragment H-5 (within the C_H2 domain) may be able to undergo a conformational alteration relatively more easily than other portions of the heavy chain. The accumulated data imply at present that some general conformational feature of the IgG C_H2 domain (rather than particular amino acid residues within the region), which is characteristic of certain IgG proteins from different species, is capable, subsequent to its revelation by the process of antigen–antibody union, to activate the complement system.

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