An IgG_{2a}-Producing Variant of an IgG_{2b}-Producing Mouse Myeloma Cell Line. Structural Studies on the Fc Region of Parent and Variant Heavy Chains†

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ABSTRACT: The IgG_{2b}-producing MPC 11 mouse myeloma cell line has yielded a number of variants which synthesize heavy chains characteristic of a different immunoglobulin subclass, IgG_{2a}, as previously shown by serology, peptide maps, and assembly profiles. We have studied the Fc regions of the IgG_{2a} protein synthesized by one variant, ICR 9.9.2.1, and of the IgG_{2b} protein synthesized by MPC 11 and compared them to MOPC 173, an IgG_{2a} protein of known sequence. We analyzed the Fc regions of the three immunoglobulins by several analytical techniques, such as immunoelectrophoresis of papain digests, $NaDodSO_4$-polyacrylamide gel electrophoresis analyses of Fc CNBr fragments, and comparative ion-exchange chromatography of radiolabeled tryptic and chymotryptic Fc peptides. In addition, Fc CNBr fragments of the variant γ2a and MPC 11 γ2b molecules were isolated and subjected to amino acid analysis and partial sequence determination. From these data, we concluded that the Fc fragment of ICR 9.9.2.1 is most probably identical to that of MOPC 173 and different from the parental γ2b Fc fragment. A number of residue positions which discriminate between γ2b and γ2a sequences are described. In two of three segments sequenced, γ2b and γ2a molecules share more identical residues than either shares with another mouse subclass, γ1.

We have isolated a number of variants from the IgG_{2b}-producing MPC 11 mouse myeloma cell line which synthesize immunoglobulins of the IgG_{2a} subclass. Some variants were isolated from the parent cell line either directly (Francus et al., 1978) or after mutagenesis with either ICR-191 or Melphalan (primary variants) (Preud'homme et al., 1975), while others arose spontaneously from primary variants (secondary variants) (Koskimies and Birshtein, 1976). Our isolation of a number of these variants undoubtedly reflects the high instability of immunoglobulin gene expression in mouse myeloma cells (Coffino and Scharff, 1971; Milstein et al., 1974).

The loss of γ2b serological markers and the expression of the γ2a gene product were originally detected by serology using subclass-specific antisera (Preud'homme et al., 1975). Peptide maps comparing two variant proteins with LPC 1 (IgG_{2a}, κ) showed the presence of γ2a-specific peptides (Preud'homme et al., 1975), and immunoglobulin assembly profiles of the variants showed relatively low amounts of HL, a characteristic feature of γ2a-producing tumors (Preud'homme et al., 1975; Francus et al., 1978). We have recently found that these variant proteins retain the original idiotype of the parent but differ from each other in charge, peptide maps, and assembly kinetics (Francus et al., 1978).

Because this group of γ2a-producing variants expresses a gene originally silent in the parent, we decided to examine the relationships of the variant proteins to each other, to MOPC 173 [an IgG_{2a} immunoglobulin of known sequence (Fougereau et al., 1976)], and to the parent γ2b MPC 11 heavy chain by a comparison of primary structures. Since only limited data were available on a γ2b heavy chain (dePréval et al., 1970), we have isolated and characterized the CNBr fragments of the parent γ2b heavy chain, focusing initially on the Fc region, which comprises two-thirds of the constant region.

We have also examined in detail the Fc region of a 55 000 molecular weight γ2a heavy chain synthesized by ICR 9.9.2.1, a secondary variant. This variant is of particular interest, since it was derived by recloning ICR 9, a primary variant synthesizing a γ2a heavy chain of 75 000 molecular weight (Koskimies and Birshtein, 1976). While especially interesting because of its extra length, the primary variant protein is difficult to study, since it is not secreted from the cell and thus it cannot be obtained from the sera or ascites of tumor-bearing mice. In contrast, the secondary variant molecule, ICR 9.9.2.1, is secreted and can be isolated in quantities suitable for primary structural analysis. This variant has also served as a model in developing analytical techniques with which we can examine other interesting variant molecules, such as ICR 9, that are available only in radiolabeled quantities.

Materials and Methods

Cell Lines and Tumors. The cell line 45.6.2.4 was derived from the BALB/c mouse myeloma tumor, MPC 11, which synthesizes an IgG_{2b}, κ immunoglobulin (Laskov and Scharff, 1970). ICR 9.9.2.1, an IgG_{2a}, κ-producing secondary variant of MPC 11, was derived on recloning of the primary variant which synthesizes a γ2a immunoglobulin having a heavy chain of 75 000 molecular weight (Koskimies and Birshtein, 1976). Both MPC 11 and ICR 9.9.2.1 synthesize heavy chains of 55 000 molecular weight. Cell lines were maintained in suspension culture in Dulbecco's modified Eagle's medium, supplemented with 20% heat-inactivated horse serum, nonessential amino acids, L-glutamine, penicillin, and streptomycin sulfate, all purchased from Grand Island Biological Co. MOPC 173 (IgG_{2a}, κ), a gift from Dr. Melvin J. Bosma, and LPC 1 (IgG_{2a}, κ), a gift from Dr. Michael Potter, were carried in BALB/c mice.

Purification of Immunoglobulin. Myeloma cells were injected intraperitoneally into pristane (2,6,10,14-tetrameth-

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ylpentadecane, Aldrich Chemical Co., Milwaukee, Wis.) primed BALB/c mice (Potter et al., 1972). The ascites from the tumor-bearing mice were collected, centrifuged to remove cells and debris, treated at 56 °C for 30 min, and stored frozen. The presence of paraprotein was verified by microzone electrophoresis (Beckman Microzone Electrophoresis System, Bulletin 7086).

The myeloma protein was precipitated with 50% saturated ammonium sulfate as described (Potter, 1967). Further purification was carried out using ion-exchange chromatography on a column of DEAE-cellulose (Whatman DE-52). The buffers used for the linear gradient were a modification of Potter's system (1967). The starting buffer was 0.005 M Tris–phosphate, pH 8.6, and the final buffer was a 3:1 mixture of 0.005 M Tris–phosphate, pH 8.6, and 0.5 M Tris–phosphate, pH 5.1. The purified protein was dialyzed against distilled water and freeze-dried. A typical preparation used 150 mL of ascites obtained from 20 mice and yielded ~1 g of purified protein.

Papain Cleavage and Isolation of Fab and Fc Fragments. The method of Guyer et al. (1976) for papain digestion was followed with some modifications. The activity of mercuripapain was taken to be that specified by the manufacturer (Worthington Biochemical Corp., Freehold, N.J.). Before each preparation, we did a pilot experiment to test the enzyme's efficacy and to ascertain the appropriate time of digestion. Cleavage of immunoglobulin was followed by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (Maizel, 1971) and by immunoelectrophoresis. For most preparations, the optimum time of cleavage was 10 min. Any uncleaved immunoglobulin was separated and Fab and Fc fragments were isolated as described (Guyer et al., 1976). Radiolabeled immunoglobulin was treated identically, except that the Sephadex gel-filtration step (Guyer et al., 1976) was omitted.

Separation of Heavy and Light Chains. Mildly reduced and radioalkylated IgG was filtered through a column (2.2 × 180 cm) of Sephadex G-100, equilibrated in 4.5 M urea–1 M propionic acid (Spring and Nisonoff, 1974).

Preparation of Radiolabeled Secreted Immunoglobulin. Logarithmically growing cells were washed twice in Spinner medium (Eagle, 1959) containing $^1/_{40}$ the normal amounts of valine, threonine, and leucine, and supplemented with 10% heat-inactivated horse serum. The cells were resuspended in the same medium at a final concentration of 5×10^5 cells/mL, and 5 mL was placed in a Petri dish. Radiolabeled L-valine, L-threonine, and L-leucine were added at 15 μCi of ^{14}C each or at 25 μCi of ^3H each. The dishes were incubated at 37 °C in 5% CO_2 for 48 h, at which time the cells were removed by centrifugation. Radiolabeled immunoglobulin was precipitated from the supernatant with 50% $(NH_4)_2SO_4$ and dissolved in and dialyzed against 0.01 M potassium phosphate, pH 8, prior to papain digestion.

Ion-Exchange Chromatography of Peptides. A mixture of ^3H- and ^{14}C-labeled Fc fragments was subjected to digestion by sequential additions of trypsin and chymotrypsin (Birshtein et al., 1974). The freeze-dried enzymatic digest was dissolved in 1.5 mL of 0.3 M pyridine hydrochloride, pH 1.7, and the pH was adjusted to <pH 2 with glacial acetic acid. The peptides were applied to a heated (56 °C), water-jacketed column (0.9 × 23 cm), packed with a Dowex-50 sulfonated polystyrene resin (SPHERIX, type XX907, Phoenix Precision Instrument Co., Philadelphia, Pa.) (Laskov and Scharff, 1970), which was equilibrated with 0.05 M pyridine–acetic acid, pH 3.13. The peptides were eluted with a gradient generated in a varigrad (Virtis Instrument Co.), using 110 mL each of the following pyridine–acetic acid buffers: (a) 0.05 M, pH 3.13; (b) 0.10 M, pH 3.54; (c) 0.20 M, pH 4.02; (d) 0.5 M, pH 4.5; and (e) 2.0 M, pH 5.0 (Brown et al., 1974). Two hundred fractions of 2.5 mL each were collected into glass scintillation vials, the pH was recorded, the buffer was evaporated in an oven, and 0.5 mL of H_2O and 10 mL of Aquasol (New England Nuclear Corp., Boston, Mass.) were added to each vial. The samples were counted in a Beckman scintillation counter (Model LS-230 or LS-233, Beckman Instruments, Inc., Palo Alto, Calif.), with the isosets adjusted so that the spill from the ^3H to the ^{14}C channel was <0.001%, and the spill from the ^{14}C to the ^3H channel was 7 ± 1%. The data were corrected for background and spill.

Amino Acid Analysis and Sequence Determination. Purified samples of peptides were hydrolyzed in distilled HCl (6 N) at 110 °C for 22 h. The amino acid compositions were determined either with a Beckman 121 analyzer or Durrum D500 analyzer. Automatic sequence analysis (Edman and Begg, 1967) was carried out on a Beckman 890C sequencer, using a DMAA double-cleavage program (10/29/74) and giving a repetitive yield of 90–94%. The amino acid phenylthiohydantoins were identified either by gas chromatography (Beckman GC-65) (Pisano and Bronzert, 1969) or by thin-layer chromatography (Kulbe, 1974; Summer et al., 1973), where solvent I contained toluene/*n*-pentane/acetic acid [60:30:22 (v/v)] plus 27 mg of BBOT (5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene/100 mL (Dr. J. D. Capra, personal communication). In some cases, we identified the amino acid on the amino acid analyzer after regeneration of the amino acid by hydrolysis of the phenylthiohydantoin with HI (Smithies et al., 1971). In our hands, we could identify serine only by its conversion to alanine after HI hydrolysis. In certain cases, manual Edman degradation of peptides was done (Turner and Cebra, 1971). Residues were identified by at least two methods or sequencer runs.

Other Methods. The following procedures were used as described (Birshtein et al., 1970): mild and complete reduction and radioalkylation of disulfide bonds, cyanogen bromide cleavage of the proteins, deionizing of urea, and removal of thiol groups from dialysis tubing. Homoserine lactone was converted to homoserine by the method of Ambler (1965). Trypsin and thermolysin were used as described (Turner and Cebra, 1971).

Nomenclature. Cyanogen bromide fragments of the Fc of the variant ICR 9.9.2.1 receive the number of the corresponding fragment from MOPC 173 (Bourgois and Fougereau, 1970), except that an asterisk is added to show its origin. The residue positions of MPC 11 and ICR 9.9.2.1 are numbered according to MOPC 173 (Fougereau et al., 1976).

Results

Comparison of Papain Digests from MPC 11, ICR 9.9.2.1, and MOPC 173

Immunoelectrophoretic Analysis of Papain Digests. Papain digests of the three immunoglobulins were compared by immunoelectrophoresis. The digest of ICR 9.9.2.1 (Figure 1c,d) showed two arcs. Using specific antisera, we identified the arc near the origin as the Fab fragment and the arc with an anodal mobility as the Fc fragment (data not shown). The Fc of ICR 9.9.2.1 has a similar mobility to that of MOPC 173 (Figure 1e,f) while both differ from MPC 11 (Figure 1a,b). The mobilities of the Fab and Fc fragments of MPC 11 are similar, though distinguishable.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis of CNBr-Treated Fc Fragments. Purified Fc fragments from ICR 9.9.2.1, MOPC 173, and MPC 11 were subjected to

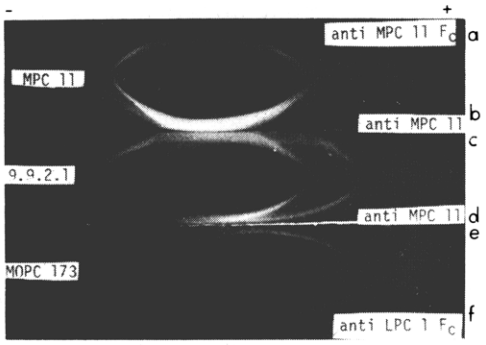


FIGURE 1: Immunoelectrophoresis of papain digests of MPC 11, ICR 9.9.2.1, and MOPC 173. Rabbit anti-MPC 11 was not made specific for the γ2b heavy chain. It reacts well with both γ2b and γ2a chains.

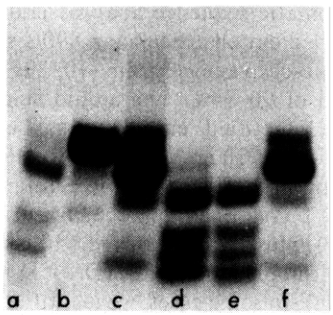


FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5%) of CNBr fragments of the Fc of MPC 11 (a reduced and b unreduced), ICR 9.9.2.1 (c unreduced and d reduced), and MOPC 173 (e reduced and f unreduced).

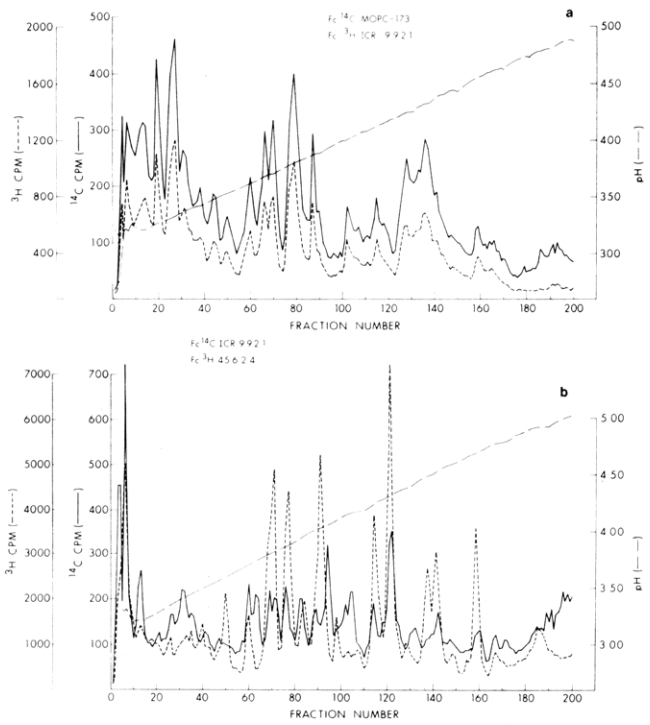
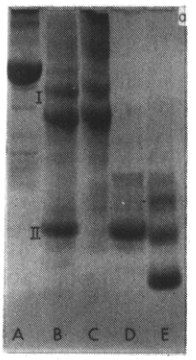


FIGURE 3: Tryptic–chymotryptic peptide maps of Fc fragments of (a) ICR 9.9.2.1 vs. MOPC 173 and (b) ICR 9.9.2.1 vs. 45.6.2.4 (MPC 11). Details of the procedure are under Materials and Methods.



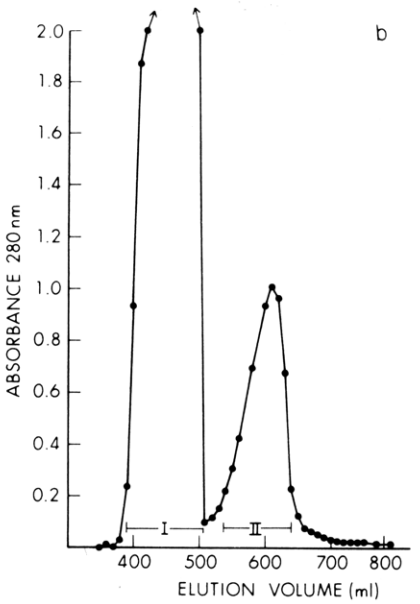


FIGURE 4: CNBr fragments of MPC 11 immunoglobulin. (a) $NaDodSO_4$–polyacrylamide gel electrophoresis (5%) of: (A) H_2L_2; (B) CNBr digestion of H_2L_2; (C) pool I from Figure 4b; (D) pool II from Figure 4b; (E) molecular weight markers, 14 300, 28 600, 42 900, 57 200, are shown. Markers were purchased from BDH through Gallard-Schlessinger, Carle-Place, N.Y., and consist of a series of oligomers produced by cross-linking a monomeric 14 300 molecular weight protein. (b) Elution profile of CNBr fragments of MPC 11 immunoglobulin on a column (3.6 × 180 cm) of Sephadex G-100, equilibrated in 8 M urea, 0.1 M formic acid. Fraction volume was 5 mL.

CNBr cleavage and examined by $NaDodSO_4$–polyacrylamide gel electrophoresis before and after complete reduction. Figure 2 shows that ICR 9.9.2.1 (Figure 2c,d) and MOPC 173 (Figure 2e,f) had identical fragment patterns which differed from MPC 11 (Figure 2a,b).

Comparative Peptide Maps of Fc Fragments. Radiolabeled immunoglobulins from ICR 9.9.2.1, MOPC 173, and MPC 11 were digested with papain and their Fc fragments were purified. Comparative peptide maps showed that the elution profiles of the Fc fragments of ICR 9.9.2.1 and MOPC 173 were very similar (Figure 3a) and differed from that of 45.6.2.4, the MPC 11 parent cell line (Figure 3b).

These results suggested that the Fc of ICR 9.9.2.1 was identical to the Fc of MOPC 173 and differed from MPC 11. To confirm these observations, we undertook primary sequence analysis of the Fc regions.

Primary Sequence of the Fc from MPC 11 and ICR 9.9.2.1

Isolation of CNBr Fragments of MPC 11. The $IgG_{2b}(\kappa)$ immunoglobulin was subjected to CNBr cleavage. The cleavage products by $NaDodSO_4$–polyacrylamide gel electrophoresis showed three major bands—a doublet (I) having a molecular weight of ~100 000 and a single band (II) of ~29 000 molecular weight (Figure 4a, panel B). These fragments, I and II, were separated by gel filtration on a column of Sephadex G-100, equilibrated in 8 M urea, 0.1 M formic

TABLE I: Amino Acid Compositions of Fragments and Peptides of the Fc of MPC 11.[g]

	II.1	II.2	II.3	T2	T3	T4	Th2	Th3	Th4	II.1 + II.2 + II.3	pool II[d]
SCM	1.6[a]	0.83[a]	0.41 (1)[b]					0.82		2.8	+[e]
Asp	10.9	8.2	3.0 (3)				1.2	1.3		22.1	22.3
Thr	4.8	6.4	2.8 (3)			0.88	0.92			14.0	13.6
Ser	8.8	5.1	4.1 (5)		0.96	1.0	0.98	1.0		18.0	14.8
Hse	0.98	0.86								1.8	1.9[f]
Glu	7.9	8.4	2.2 (2)	1.02			0.94		1.0	18.5	21.1
Pro	8.6	3.6	1.0 (1)		0.92					13.2	12.2
Gly	5.8	0.91	2.2 (2)	1.05	1.12				1.1	8.9	9.2
Ala	3.0	1.4								4.4	6.4
Val	7.1	9.5	0.70 (1)						1.0	17.3	18.4
Ile	5.3	4.6	0.89 (1)							10.8	9.8
Leu	7.7	2.7	1.9 (2)	0.94						12.3	13.8
Tyr	3.7	1.1	1.6 (2)							6.4	6.6
Phe	2.8	1.0	0.85 (1)							4.6	6.1
His	0.98	2.8	1.2 (1)	0.84					1.0	5.0	4.7
Lys	8.8	1.6	5.6 (6)	0.98		1.1	0.94			16.0	15.6
Arg	3.1	2.1	1.9 (2)						0.91	7.1	7.4
Trp		+[c]	(1)								

[a] This is a single representative amino acid analysis. The compositions of II.1 and II.2 were normalized to 92 and 62 residues, respectively. [b] The numbers in parentheses are the actual numbers of amino acid residues from the primary sequence. [c] The presence of tryptophan was confirmed by automated sequential degradation. [d] This is the average of two analyses. [e] Detected as cystine. [f] Measured as methionine. [g] Abbreviations used: T, tryptic peptides from II.3; Th, thermolytic peptides from II.3.

acid (Figures 4b and 4a, panels C and D). Subsequent studies have shown that pool I contains the N-terminal half ("Fab") of the molecule. In this paper, we describe our studies of pool II, which we show contains fragments comprising almost a complete papain-generated Fc fragment.

After complete reduction and alkylation, both pool II and CNBr-treated Fc gave the same three major fragment bands on $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel pattern for the Fc fragment is shown in Figure 5a, panel B. The three fragments—II.1, II.2, and II.3—were separated from pool II by filtration on a column of Sephadex G-75, equilibrated in 8 M urea, 0.1 M formic acid (Figures 5b and 5a, panels C, D, and E). The observation that pool II gave rise to three smaller fragments, all of which were radiolabeled after reduction and alkylation with iodo[^{14}C]acetic acid (Figure 5b), indicated that the fragments were originally joined by intrachain disulfide bridges. From the general structure of the immunoglobulin molecule, one would expect the middle fragment to contain two half-cystine residues. The higher specific radioactivity of fragment II.1 is consistent with its being this middle fragment. The presence of two half cystines in II.1 was confirmed by the isolation of two distinct radioalkylated tryptic peptides (data not shown).

Amino acid compositions of pool II, II.1, II.2, and II.3 are shown in Table I. Fragment II.3 lacks homoserine and therefore is the C-terminal fragment of the γ2b heavy chain.

Sequence Studies of Intact Fc and CNBr Fragments of MPC 11. The papain-generated Fc of MPC 11 was subjected to 26 cycles of automated sequential degradation. The sequence of this segment is shown in Figure 6. The three Fc CNBr fragments were also subjected to automated sequential degradation. Figure 6 shows the results and includes additional sequence information on II.3, obtained from tryptic and thermolytic digestions. Amino acid analyses of peptides are shown in Table I. As shown in Figure 6, residues 20–26 of the Fc (following the methionine at position 19) correspond to the first seven residues of II.2 and establish it as the N-terminal CNBr fragment of pool II. With II.3 placed as the C-terminal fragment by its lack of homoserine, the CNBr fragments isolated from pool II were aligned: N-II.2, II.1, II.3-C.

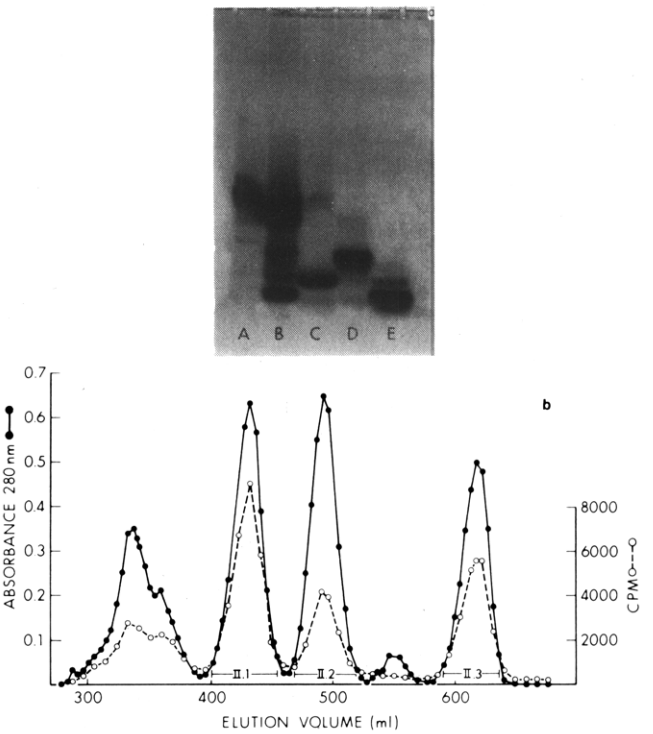


FIGURE 5: CNBr fragments of MPC 11 pool II. (a) $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5%) of: (A) Fc after CNBr cleavage; (B) Fc after CNBr cleavage and complete reduction; (C) II.1 from Figure 5b; (D) II.2 from Figure 5b; (E) II.3 from Figure 5b. (b) Elution profiles of CNBr fragments of MPC 11 pool II after complete reduction and radioalkylation on a column (2.8 × 180 cm) of Sephadex G-75, equilibrated in 8 M urea, 0.1 M formic acid. Fraction volume was 5 mL.

Figure 7 gives the comparison of the partial sequence of the Fc of MPC 11 (γ2b) with published sequences of MOPC 173 (γ2a) (Fougereau et al., 1976) and MOPC 21 (γ1) (Milstein et al., 1974). The MPC 11 sequences have been placed by homology. Of 102 positively identified positions, we have found 27 residue positions which discriminate between γ2b and γ2a sequences.

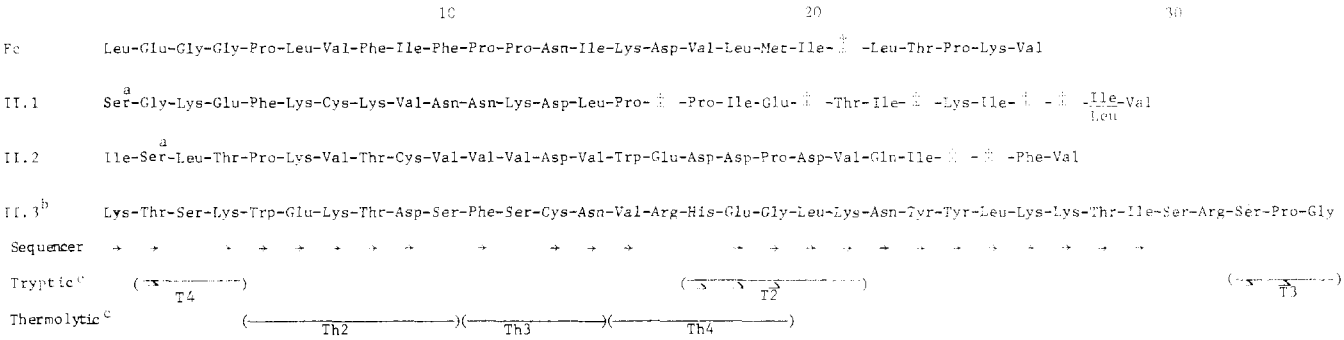


FIGURE 6: Partial sequence of MPC 11. N-terminal sequences of the intact Fc fragment and of fragments II.1 and II.2 were obtained by automated sequential degradation. The complete sequence of II.3 was derived from automated sequential degradation (→) and isolation and manual Edman degradation (—) of tryptic (T-) and thermolytic (Th-) fragments. Footnotes used: (a) The serine at this position was detected by a single back-hydrolysis. (b) Serine and arginine have been determined for positions 30 and 31 on the basis of the following data. The amino acid composition of II.3 gave a total of 34 residues. The difference between the total composition and the firmly established residues is Ser_1Arg_1. The isolation of the C-terminal tryptic peptide (T3) requires the base to be at position 31, and the Ser is thus placed at 30. (c) Tryptic and thermolytic digests were resolved by gel filtration on a column (2.0 × 245 cm) of Sephadex G-25, equilibrated in 0.05 M NH_4OH. (‡) The residue at this position could not be identified.

FIGURE 7: Comparison of partial primary sequence of MPC 11 (γ2b) with published sequences of MOPC 173 (γ2a) (Fougereau et al., 1976) and MOPC 21 (γ1) (Milstein et al., 1974). Identical residues have been enclosed. Footnotes used: (a) Residues 236–261 are from intact Fc. Residues 255 to 281 are from II.2. (b) We subjected the intact Fc fragment of MOPC 173 to automated sequential degradation, and our determination of N-247 and N-252 in MOPC 173 as Pro and Val, respectively (instead of N-247 Val and N-252 Pro), was confirmed by Dr. J. Rocca-Serra (personal communication). See Figure 10. (c) The serine at this position was detected by a single back-hydrolysis. (d) This residue position is counted as different although we were unable to confirm the presence of the amide in our automated sequential degradation of intact Fc from MOPC 173. See Figure 10. (e) This residue position is counted as different although automated sequential degradation of *H-10 from ICR 9.9.2.1 failed to show the presence of the amide. See Figure 10. (‡) The residue at this position could not be identified.

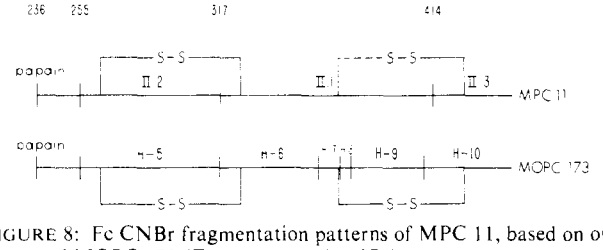


FIGURE 8: Fc CNBr fragmentation patterns of MPC 11, based on our data, and MOPC 173 (Fougereau et al., 1976).

Primary Sequence of the Fc Fragment of ICR 9.9.2.1 Compared with MOPC 173 and MPC 11. With the knowledge of these discriminating residues, we asked which sequence ICR 9.9.2.1 followed. CNBr fragments were isolated by gel filtration both from the Fc fragment and the heavy chain (Figure 9) of ICR 9.9.2.1. The elution profile of the Fc CNBr fragments (data not shown) was identical to that found by Bourgois and Fougereau (1970) for MOPC 173. Fragments *H-5, *H-6-7, *H-9, and *H-10 (see Figure 8) were isolated by subsequent recycling of pools on columns of Sephadex G-50 or G-75, equilibrated in 0.05 M formic acid (data not shown).

The bulk of our sequence studies, however, has been done on CNBr fragments isolated from intact heavy chain. ICR 9.9.2.1 was mildly reduced and radioalkylated with iodo[^{3}H]-acetic acid to label half-cystine residues involved in interchain disulfide bridges. Heavy chains were then isolated and cleaved with CNBr. The CNBr digest was completely reduced and radioalkylated with iodo[^{14}C]acetic acid to label the half-cystine residues involved in intrachain disulfide bridges. The digest was then filtered on a column of Sephadex G-75, equilibrated with 4.5 M urea, 1 M propionic acid (Figure 9). Pools I and II contained uncleaved heavy chain and aggregates and were not purified further. Pool III had the bulk of the tritium label and therefore contained the mouse Fd CNBr fragment(s) which has interchain disulfide bridges. However, automated sequential degradation of this pool gave a single sequence identical to H-5 of the MOPC 173 heavy chain (Figure 10). We concluded that Fd fragment(s) in this pool were inaccessible to automated sequential degradation.

Pools V–VII were each recycled on a column of Sephadex

TABLE II: Fc CNBr Fragments of ICR 9.9.2.1.

	*H-5	*H-6-7	*H-9	*H-10
SCM	1.4 (1)[a]	1.0 (2)[a]		1.2 (1)[a]
Asp	8.2 (9)	5.3 (3)	6.3 (7)[a]	3.2 (3)
Thr	4.2 (5)	4.0 (4)	2.8 (3)	2.6 (2)
Ser	4.4 (6)	3.1 (3)	2.2 (2)	3.5 (7)
Hse	0.84 (1)	0.93 (2)	0.82 (1)	
Glu	7.4 (8)	6.4 (6)	4.2 (4)	3.8 (3)
Pro	4.0 (3)	5.5 (7)	2.0 (2)	1.0 (1)
Gly	1.9 (0)	2.0 (2)	2.3 (2)	1.6 (2)
Ala	2.5 (2)	1.9 (2)	0.23 (0)	0.70 (0)
Val	8.8 (11)	6.3 (5)	1.9 (2)	4.8 (5)
Ile	3.6 (4)	2.1 (2)	0.86 (1)	0.61 (0)
Leu	3.8 (3)	2.9 (3)	2.2 (2)	2.7 (2)
Tyr	1.2 (1)	1.3 (1)	2.6 (3)	2.2 (2)
Phe	1.5 (1)	1.1 (1)	0.76 (1)	0.98 (1)
His	2.2 (3)	1.2 (0)		3.4 (3)
Lys	2.2 (0)	5.6 (8)	2.2 (2)	3.8 (4)
Arg	2.1 (2)	2.1 (2)		1.4 (3)
Trp	(2)[b]		(1)[b]	(1)[b]

[a] The numbers in parentheses are the actual number of amino acids as determined by Fougereau et al. (1976). Compositions were normalized according to the lengths of the corresponding fragments of MOPC 173. [b] Detected by N-terminal sequence analysis (Figure 10).

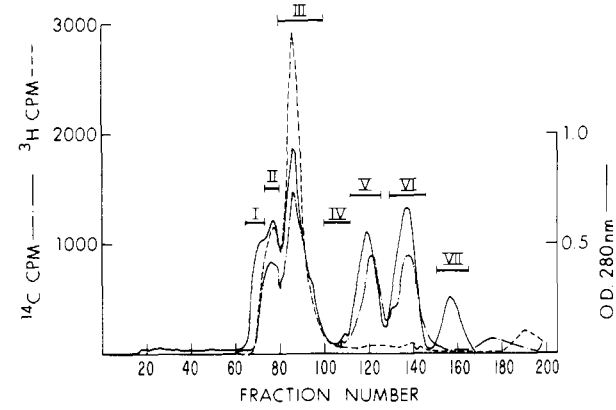


FIGURE 9: Elution profile of CNBr fragments of the heavy chain of ICR 9.9.2.1 after complete reduction and radioalkylation on a column (2.2 × 180 cm) of Sephadex G-75, equilibrated in 4.5 M urea, 1 M propionic acid. Fraction volume was 3 mL.

G-50, equilibrated in 0.05 M formic acid, and yielded fragments which, based on their amino acid compositions (Table II), were identified as H-6-7, H-10, and H-9, respectively, of MOPC 173 (Figure 8). The sequence data obtained by automated sequential degradation of intact Fc, *H-5, *H-6-7, *H-9, and *H-10 are shown in Figure 10 with the corresponding sequence data from MOPC 173, LPC 1, and MPC 11. At each of the 106 positively identified positions, which include 16 of the 27 which distinguish γ2b from γ2a, the variant follows the γ2a sequence. In vitro deamidation could account for observed differences at residues N-251, -396, and -431.

Discussion

We embarked on our structural studies of γ2a variant proteins with two goals in mind: (a) the determination of the primary structure of one variant IgG_{2a} protein and of the parental IgG_{2b} molecule to enable us to examine their relationships to each other and to MOPC 173, an IgG_{2a} of known sequence (Fougereau et al., 1976); (b) the development of analytical techniques to screen the numerous variants with relative ease.

Papain digestion has been an exceedingly useful tool in our structural studies, since we have found that the enzyme cleaves both IgG_{2a} and IgG_{2b} at the same position (at N235/236), giving rise to comparable Fc fragments. The Fc fragments from different variants can therefore be directly compared by immunoelectrophoresis, by the pattern of their CNBr fragments on $NaDodSO_4$-polyacrylamide gel electrophoresis, and by peptide maps. These analytical techniques initially showed that the Fc fragments of ICR 9.9.2.1 and MOPC 173 were identical and differed from the Fc fragment of MPC 11. Subsequently, our sequence data have confirmed these observations.

Automated sequential degradation of intact Fc showed that ICR 9.9.2.1 followed the γ2a sequence. This method is a good initial step to examine a variant, since three of the residues distinguishing γ2a and γ2b sequences lie within the N-terminal 13 residues. By examining CNBr fragments as well, we have analyzed 127 of the 212 amino acid residues of the Fc of IRC 9.9.2.1 and have positively identified 106 of them. All 106 residues, which include 16 which discriminate between γ2b and γ2a sequences, are identical to MOPC 173, except for three which might reflect in vitro deamidation.

Using the analytical techniques of immunoelectrophoresis, peptide mapping, and $NaDodSO_4$-polyacrylamide gel electrophoresis of CNBr fragments, we have found that another γ2a variant protein, ICR 11.19.3, differed in its Fc from ICR 9.9.2.1 (Greenberg et al., 1978). Our ability to distinguish the Fc CNBr fragments of MPC 11 by $NaDodSO_4$-polyacrylamide gel electrophoresis should enable us to focus on relevant segments in order to define the molecular structure. These same methods should allow us to analyze radiolabeled immunoglobulin from the cytoplasm of nonsecreting variants like ICR 9, the primary variant synthesizing a heavy chain of 75 000 molecular weight, which gave rise to the secondary variant, ICR 9.9.2.1, studied in this paper (Koskimies and Birshtein, 1976).

We have isolated and aligned three CNBr fragments from the C-terminal half of the MPC 11 IgG_{2b} protein. Although we cannot rule out the possibility of additional small CNBr fragments until we isolate methionine-containing overlapping peptides, we can account for most, if not all, of the Fc region. This conclusion is based on the size of the pool II fragment (Figure 4a), the amino acid composition of the three CNBr fragments comprising pool II, and $NaDodSO_4$-polyacrylamide gel electrophoresis analysis of these fragments and of the Fc before and after CNBr treatment.

The MPC 11 partial sequence is compared to MOPC 173 (IgG_{2a}) (Fougereau et al., 1976) and MOPC 21 (IgG_1) (Milstein et al., 1974) in Figure 7. In two of the three stretches sequenced, γ2b and γ2a share more identical residues than either of them shares with γ1, an observation that might be related to the ease with which we isolate γ2a-producing variants from MPC 11. In position N-236-281, γ2a and γ2b differ in 7 of 44 (16%) confirmed positions. In this segment, γ1 differs from γ2a in 17 of 46 (37%) and from γ2b in 17 of 44 (39%) confirmed positions. From position N-317-345, γ2a and γ2b differ in 2 of 22 (9%) confirmed positions, while γ1 differs from γ2a in 10 of 29 (34%) residues and from γ2b in 9 of 24 (38%) confirmed positions.

The C-terminal fragment of MPC 11, which extends from N-414-447, shows extensive differences in all three sequences. In this segment, γ2a and γ2b differ in 20 of 34 (59%) confirmed positions. γ1 differs from γ2a in 16 of 34 (47%) and from γ2b in 18 of 34 (53%) positions. Perhaps most serologically defined γ2a and γ2b subclass-specific determinants will

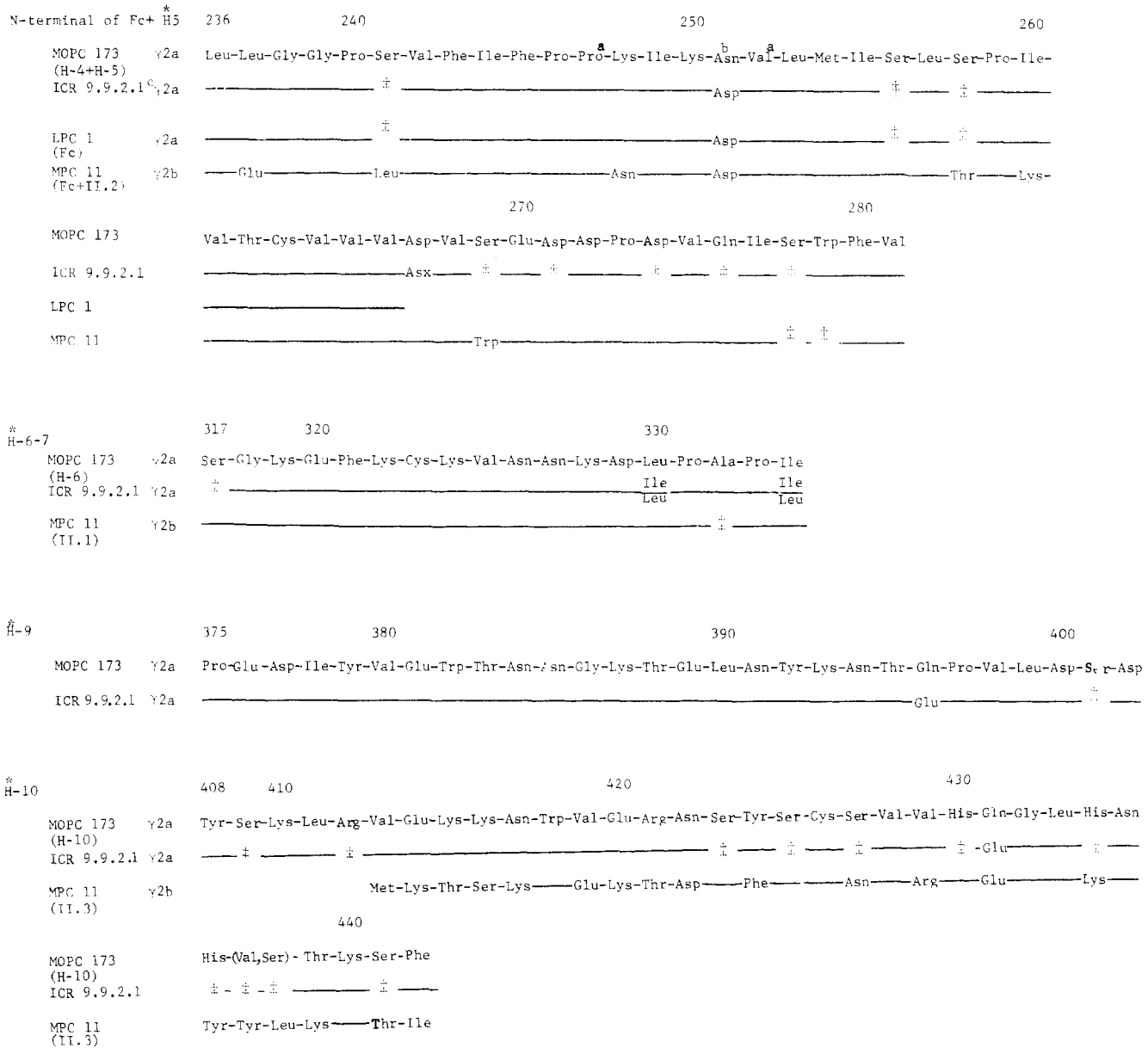


FIGURE 10: Partial primary sequence of ICR 9.9.2.1 obtained by automated sequential degradation of intact Fc and Fc CNBr fragments compared to MOPC 173 (Fougereau et al., 1976), LPC 1, and MPC 11. Footnotes used: (a) We subjected the intact Fc fragments of MOPC 173 and LPC 1 to automated sequential degradation. Our determination of N-247 and N-252 in MOPC 173 as Pro and Val, respectively (instead of N-247 Val and N-252 Pro), was confirmed by Dr. J. Rocca-Serra (personal communication). (b) In our hands, N-251 of MOPC 173 was shown to be Asp. (c) Residues 236–261 are from intact Fc. Residues 255–281 are from *H-5. (‡) The residue at this position could not be identified.

be shown to reside in this C-terminal fragment.

The structural information we have thus far obtained permits us to explore the genetic basis of these variants. One hypothesis for the generation of variants is translocation (Gally and Edelman, 1970) of the parental heavy-chain variable-region gene from the γ2b to the γ2a constant-region gene. If such a mechanism were operative, all γ2a variants should be identical. In fact, the variable regions of the variants are clearly related to the parent MPC 11, since they are idiotypically indistinguishable (Francus et al., 1978). In addition, we have isolated from MPC 11 a variable-region CNBr fragment that begins at position N-21 and should encompass two hypervariable regions (B. K. Birshtein, unpublished observation), and a similar fragment has been isolated from several variants (B. K. Birshtein, M. L. Greenberg, and S. L. Morrison, unpublished data; Francus, 1978). Nonetheless, we have shown that many of these variants differ from each other (Francus et al., 1978), and in one variant we have found γ2b-specific residues in the Fc region (Greenberg et al., 1978). Consequently, we can rule out translocation as the sole mechanism for generating all γ2a-producing variants. For any one variant, such as ICR 9.9.2.1, this mechanism could still apply.

The generation of all our variants could be explained by a single mechanism, that of recombination between γ2b and γ2a genes at different sites. Differences in the variants would then reflect varying ratios of γ2b and γ2a sequences expressed. Such hybrid molecules have been reported for immunoglobulins of mice (Warner et al., 1966) and man (Kunkel et al., 1969; Natvig and Kunkel, 1974; Werner and Steinberg, 1974) and for hemoglobins (Baglioni, 1962). If ICR 9.9.2.1 resulted from such a recombination event, the cross-over must have occurred in CH_1 or the hinge region, since the Fc fragment contained no detectable γ2b sequence.

Recent evidence [reviewed for a number of systems by Robertson (1977) and Williamson (1977)] has shown insertions of untranslated DNA segments within structural genes.

Of particular interest to us are studies on immunoglobulins where an insertion within the variable-region gene has been described [unpublished data of O. Bernard, W. Gilbert, A. Maxam, and S. Tonegawa, as quoted by Brack and Tonegawa (1977)]. Should constant-region genes have similar insertions, our variants might have arisen by improper splicing and joining of γ2b and γ2a segments. Structural analysis of variant proteins combined with studies at the nucleic acid level should help us discriminate among these possible mechanisms.

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