IgG_{2a}-PRODUCING VARIANTS OF AN IgG_{2b}-PRODUCING MOUSE MYELOMA CELL LINE*

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The expression of immunoglobulin genes is usually unstable in mouse myeloma cells (1, 2). Some cell lines spontaneously lose the ability to produce immunoglobulin heavy chain at a rate of 1×10^{-3}/cell/generation (3). One cell line, P3 (MOPC 21), has yielded spontaneous variants which synthesize altered heavy chains. Adetugbo and co-workers have examined 7,000 P3 clones and have identified four such variants, three of which have deletions, and one which has a point mutation (4).

After mutagenesis of the MPC 11 mouse myeloma cell line, as many as 2–6% of the surviving cells are variants in heavy chain production, of which approximately two-thirds have ceased synthesis of heavy chains while continuing light chain production (5). The remaining third of these MPC 11 variants synthesize altered heavy chains and are divided about evenly between two major types: (*a*) those synthesizing heavy chains shorter than the parent (5); and (*b*) those synthesizing heavy chains which lack the γ2b serologic determinants of the parental heavy chain but which express serological determinants of the γ2a subclass (6).

Identification of the original primary variants which produced a γ2a heavy chain was based on serology, assembly patterns, and peptide maps (6). Recently, chemical characterization and partial amino acid sequence determination of one γ2a variant protein has shown that the Fc portion of its heavy chain is distinct from that of MPC 11, the parental IgG_{2b} immunoglobulin, and is identical to that of MOPC 173 (7), an $IgG_{2a}(\kappa)$ mouse myeloma protein of known sequence (8). These γ2a variant heavy chains are identical in size to the parent with the exception of one, which is larger. The latter variant gives rise to secondary variants producing γ2a heavy chains of normal size (9). Additional secondary variants producing γ2a heavy chains of normal size have been derived from variants synthesizing short heavy chains (9, this paper).

Previous instances of the production of a new subclass in immunoglobulin-producing cells have been reported. For example, Hausman and Bosma have described two cases in which myeloma tumors ceased production of the original

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heavy or light chain and began synthesis of a new chain differing in subclass or type, and binding of idiotype (10). However, both these changes appeared during adaptation to continuous in vitro culture by alternate passage between culture and animal, and the origin of the variant cell was thus in doubt. In addition, Kunkel et al., Natvig and Kunkel, and Werner and Steinberg have observed the presence in human serum of "hybrid" immunoglobulins which contain antigenic determinants of two heavy chain subclasses (11–13). A similar observation has been made in mice (14).

We do not know what genetic or biochemical mechanisms are responsible for the instability of immunoglobulin expression in mouse myeloma cells or if this instability is related to any of the normal events in the regulation of antibody production. Since the γ2a variants arise frequently, either directly from the parental cell line or as secondary variants from variants producing short heavy chains, and are expressing either part or all of a gene that was silent in the parent, a more detailed study of these variants should provide some insight into the structure of the immunoglobulin genes and the genetic control of immunoglobulin expression. In this paper, we examine the relationship of the γ2a variants to each other and to their parent.

Materials and Methods

Cell Lines and Tumors. All cell lines used in this study are summarized in Table I. The cell line, 45.6.2.4, was derived from the BALB/c mouse myeloma tumor, MPC 11, which synthesizes an $IgG_{2b}(\kappa)$ immunoglobulin (15). Primary (6) and secondary (9) variants synthesizing $IgG_{2a}(\kappa)$ immunoglobulins were obtained from MPC 11 by using a cloning technique (3). Additional variants synthesizing $IgG_{2a}(\kappa)$ have been isolated during the course of these studies. ICR 4.68.66 and ICR 4.68.110 are secondary variants derived from the short heavy chain producing variant, ICR 4.68. U2 arose spontaneously. The parental cell line and all variants retained tumorigenicity, both as solid tumors and ascites. Cell lines were grown in suspension culture in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 20% heat inactivated horse serum (Grand Island Biological Co., or Flow Laboratories, Inc., Rockville, Md.), nonessential amino acids, L-glutamine (3.33 mM), penicillin (10,000 U/liter), and streptomycin sulfate (50 mg/liter), all purchased from Grand Island Biological Co. MOPC 173, a gift from Dr. Melvin J. Bosma, Institute for Cancer Research, Philadelphia, Pa. and LPC 1, a gift of Dr. Michael Potter, NIH, Bethesda, Md., both IgG_{2a} (κ), were maintained in BALB/c mice.

Preparation of Antisera. Rabbits were immunized by numerous intradermal injections in the flanks and footpads with a total of 100–250 μg protein emulsified by sonication in 1 ml of 50% complete Freud's adjuvant (Difco Laboratories, Detroit, Mich.). After 3 wk, rabbits were boosted with an intravenous injection (1 ml) of 100 μg protein coated onto 300 μg alumina particles. Beginning 1 wk after boosting, rabbits were bled twice a week until the antibody titer fell. The antisera were prepared against immunoglobulin from MPC 11 (IgG_{2b}), LPC 1 (IgG_{2a}), and ICR 9.9.2.1 (secondary variant, IgG_{2a}), and the Fc fragments of immunoglobulin from MPC 11 and LPC 1. Commercial antisera directed against the different classes and subclasses of mouse immunoglobulins were obtained from Meloy Laboratories, Inc., Springfield, Va. Their specificity was confirmed by using a panel of myeloma proteins.

Purification of Immunoglobulin. Cells were injected intraperitoneally into pristane (2,6,10,14-tetramethyl-pentadecane, Aldrich Chemical Co., Milwaukee, Wis.) primed BALB/c mice (16). 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 cellulose acetate microzone electrophoresis (Beckman Microzone Electrophoresis System, Bulletin 7086, Beckman Instruments, Inc., Fullerton, Calif.).

The myeloma protein was precipitated with ammonium sulfate as described (17). Further purification was carried out by using ion exchange chromatography on a column of DEAE-cellulose (DE-52, Whatman, Inc., Clifton, N.J.). The buffers used for the linear gradient were a

modification of Potter's system (17). The starting buffer was 0.005 M Tris-phosphate pH 8.6, and the final buffer was a 4:1 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. The purity of the proteins was verified by microzone electrophoresis, immunoelectrophoresis, and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE)[1] (18).

Radioimmunoassay

PREPARATION OF IMMUNOADSORBENT COLUMN. Sera from normal mice, mice bearing MOPC 141 tumor, and mice bearing MOPC 195 tumor (both IgG_{2b}, κ) were pooled and mixed with an equal volume of phosphate-buffered saline (0.15 M NaCl, 0.02 M sodium phosphate, pH 7). The serum pool was brought to 40% saturation by the addition of saturated ammonium sulfate and stirred at 4°C for 30 min. The precipitated proteins were dialyzed against 0.25 mM phosphoric acid-40 mM Tris and applied to a column of DEAE cellulose (DE-52, Whatman), equilibrated in the same buffer. The IgG fraction was eluted with 12.5 mM phosphoric acid-250 mM Tris and coupled to Sepharose 4B (19). The resulting immunoadsorbent contained 3.72 mg of IgG/ml Sepharose.

PREPARATION OF ANTI-IDIOTYPIC ANTISERA. Rabbit antisera against immunoglobulin from MPC 11 (R132) or ICR 9.9.2.1 (BB 24) were brought to 0.5 M in NaCl and chromatographed in this solution on the immunoadsorbant column to which normal IgG and IgG_{2b}, κ-myeloma proteins had been attached. The eluted (unbound) fractions were tested by Ouchterlony analysis against the immunizing antigen and normal mouse serum, MOPC 195, MOPC 141, and MOPC 173. Those fractions from R132 that were reactive only against the immunizing antigen MPC 11, and those fractions from BB 24 which were reactive only against the immunizing antigen ICR 9.9.2.1, were pooled, and concentrated, when necessary, by dialysis against Sephadex G-200 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.).

PREPARATION OF RADIOLABELED MPC 11 IMMUNOGLOBULIN. MPC 11 cells (10^6/ml) were grown for 24 h in Spinner medium (20) containing 1/10 the normal concentration of methionine, 10% heat inactivated horse serum, and 100 μCi of ^{35}S-methionine/ml (New England Nuclear, Boston, Mass.). 200 μg/ml of unlabeled methionine were added to the supernate to replace nonspecifically absorbed [^{35}S]methionine. The supernate was then dialyzed against phosphate-buffered saline. We used the radiolabeled MPC 11 preparation without further purification since we found that it behaved identically to chromatographically purified radiolabeled protein in the radioimmunoassay.

RADIOIMMUNOASSAY. The solid phase radioimmunoassay of Bosma et al. (21) was followed except that bound radioactive proteins were released from the polystyrene tubes by sonication for 10 min in 1 ml of 1% SDS and transferred for counting to scintillation vials containing 10 ml Aquasol (New England Nuclear).

Electrophoresis in Agarose. The Johansson method (22), as modified by Dr. Chester Alper (personal communication) was used. The electrophoresis buffer (pH 8.6) was composed of 35.05 g sodium barbital and 5.5 g barbital in 4 liters. The fixing, staining, and destaining solutions were methanol:water:acetic acid (5:5:1, vol/vol/vol) with 0.02% Coomassie Brilliant Blue R-250 (Consolidated Laboratories Inc., Chicago Heights, Ill.) added to the staining solution.

Assembly of Immunoglobulin. Logarithmically growing cells (3×10^6) were washed twice in Spinner medium minus valine, threonine, leucine, and containing 2% heat-inactivated horse serum. The cells were preincubated in 1 ml medium for 5 min in a 37°C water bath. 5 μCi each of [^{14}C]L-valine, -threonine, and -leucine were added (New England Nuclear) and the cells were incubated further for 15 min. The incorporation of radioisotopes was stopped by immediate cooling of the cells in an ice bath and the addition of 1 ml cold medium (4°C). The cells were collected and lysed as previously described (5). Radiolabeled immunoglobulins were immunologically precipitated by an indirect technique (5) and were analyzed by 5% SDS-PAGE. Gels were dried and subjected to radioautography on Kodak XR-5 film (Eastman Kodak Co., Rochester, N.Y.). Radioautographs were scanned (log mode) with a reflectance fluorescence transmission (RFT) scanning microdensitometer equipped with an automatic General Computing Integrator (Transidyne General Corp., Ann Arbor, Mich.).

Preparation of Radiolabeled Secreted Immunoglobulin. Logarithmically growing cells were

[1] *Abbreviation used in this paper*: SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

washed twice in Spinner medium containing 1/40 the normal amount 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 were placed in a Petri dish. Radiolabeled L-valine, -threonine, and -leucine were added at 15 μCi ^{14}C each, or at 25 μCi ^{3}H each. The dishes were incubated at 37°C in 5% CO_2 for 48 h, at which time the cells were removed by centrifugation, the immunoglobulin was precipitated from the supernate by the sandwich technique, and heavy and light chains were prepared by using SDS-PAGE (5).

A large quantity of radiolabeled ICR 9.9.2.1 immunoglobulin was prepared from 40 ml of cell suspension in a spinner culture, incubated with 250 μCi each of [^{3}H]L-valine, -threonine, and -leucine. The supernate was dialyzed against phosphate-buffered saline to remove labeled amino acids after which the protein was precipitated with 50% saturated ammonium sulfate. The precipitate was dissolved in phosphate-buffered saline, dialyzed against distilled water, and freeze-dried. The radioactivity in the precipitate was found exclusively in immunoglobulin (15). The protein was subjected to complete reduction and alkylation (23), and radiolabeled heavy and light chains were separated by gel filtration on a column of Sephadex G-100 (2.2 × 180 cm), equilibrated with 4.5 M urea, 1 M propionic acid (24). Elution was monitored by radioactivity. The pooled peaks were freed of salts by passage through a column of Sephadex G-25, coarse (3 × 60 cm), equilibrated with 0.05 M formic acid.

Papain Digestion. Radiolabeled Fab and Fc of ICR 11.8 and M224 were isolated after papain digestion as described by Guyer et al. (25).

Ion Exchange Chromatography of Peptides. A mixture of [^{3}H] and [^{14}C]labeled proteins was subjected to digestion by sequential additions of trypsin and chymotrypsin. The freeze-dried enzymatic digest (5) was dissolved in 1.5 ml of 0.3 M pyridine-HCl, 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 (0.2 × 23 cm), packed with a Dowex-50 sulfonated polystyrene resin (SPHERIX, type XX907, Phoenix Precision Instrument Co., Philadelphia, Pa.) (15), 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 Co., Inc., Gardiner, N.Y.), by 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 (26). 200 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 H_2O and 10 ml Aquasol were added to each vial. The samples were counted in a Beckman scintillation counter (LS-230 or LS-233, Beckman Instruments, Inc., Fullerton, Calif.), with the isosets adjusted so that the spill from the ^{3}H to the ^{14}C channel was <0.001%, and the spill from the ^{14}C to the ^{3}H channel was 7 ± 1%. The data were corrected for background and spill, and plotted as cpm or percent of total counts vs. fraction number, by using a computer program developed for us by Mr. David Medford.

Results

Ouchterlony Analysis of Parent and Variant Immunoglobulins. Cytoplasms and secretions from cultured cells, and immunoglobulins purified from sera or ascites of tumor bearing mice were tested by double diffusion in agarose (5). All of the variants listed in Table I were γ2a positive and γ2b negative when tested with subclass specific (Meloy Laboratories Inc., Springfield, Va.) antisera. These γ2a immunoglobulins showed "complete identity" with each other using a variety of antisera produced against MPC 11 (IgG_{2b}, κ), LPC 1 (IgG_{2a}, κ), the γ2a variant protein – ICR 9.9.2.1, and the Fc fragments of MPC 11 and LPC 1. All the variants shared the same antigenic determinants with the parent and lacked some antigenic determinants which were present on MPC 11 (Fig. 1a). When tested with an antisera raised against the Fc fragment of LPC 1 (IgG_{2a}, κ), the variants shared the same determinants, some of which were absent from the parent (Fig. 1b). This analysis showed that at least part of a different constant region gene is expressed in these variants and distinguished the γ2a variant proteins as a group from the parent γ2b protein. Since many of the

TABLE I
The γ2a Producing Cell Lines Derived from MPC 11

Parent	Mutagen	*Primary variant	*Secondary variant	Heavy chain		
				Size	Sero-type	Secre-tion
45.6.2.4				55,000	γ2b	+
45.6.2.4	–	U2		55,000	γ2a	+
[a]45.6.3.1	Melphalan	‡M224		55,000	γ2a	+
[b]45.6.3.1	Melphalan	M319.2		55,000	γ2a	+
[c]45.6.2.4	ICR 191	‡ICR 11.8		55,000	γ2a	+
[d]45.6.2.4	ICR 191	ICR 16		55,000	γ2a	+
[e]45.6.2.4	ICR 191	ICR 9		75,000	γ2a	–
		§				
		ICR 9.9		75,000	γ2a	–
			→ ICR 9.9.2.1	55,000	γ2a	+
			→ ICR 9.9.1.6.7	55,000	γ2a	+
45.6	ICR 191	ICR 4.68		50,000	–	–
			→ ICR 4.68.66	55,000	γ2a	+
			→ ICR 4.68.110	55,000	γ2a	+
[f]45.6.3.2	ICR 191	ICR 11.19		50,000	–	–
			→ ICR 11.19.2	55,000	γ2a	+
			→ [g]ICR 11.19.3	55,000	γ2a	+

* In the text, we use an abbreviated form of the cell lines starting with the letter M, or ICR.

‡ M and ICR indicate the variant was obtained after mutagenesis with Melphalan or ICR 191, respectively.

§ An arrow indicates that the subclone was obtained without further mutagenesis. a, b, c, d, and e were listed in Preud'homme et al. (6) as: 45.6.3.2.2 M224; 45.6.3.2.2 M319; 45.6.3.1 ICR 11; 45.6.3.1 ICR 16; 45.6 ICR 9; respectively. f was listed as 45.6.3.2 ICR 11 in Birshtein et al. (5). g was listed as 45.6.3.2 ICR 11.19.S3 in Koskimies and Birshtein (9).

antigenic determinants detected by these antisera are in the Fc region, the serology of the variable region was examined directly.

Idiotypic Analysis of Parent and Variant Immunoglobulins by Radioimmunoassay. Rabbit antiserum to MPC 11 (R 132) was absorbed on an immunoadsorbent column, as described in Materials and Methods. This anti-idiotypic antiserum bound to MPC 11, and the binding was not affected by normal mouse serum, two other $IgG_{2b}(\kappa)$ proteins (MOPC 141 and MOPC 195) (Fig. 2), or the $IgG_{2a}(\kappa)$ immunoglobulin, MOPC 173 (Fig. 3). In contrast, proteins purified from six γ2a-producing variants were identical to each other and to the parental MPC 11 protein in their ability to inhibit this binding to the radiolabeled MPC 11 protein (Fig. 3). A second rabbit antiserum (BB24) was raised against the γ2a variant protein, ICR 9.9.2.1, and was also made anti-idiotypic by appropriate absorption (Materials and Methods). In a similar radioimmunoassay, but

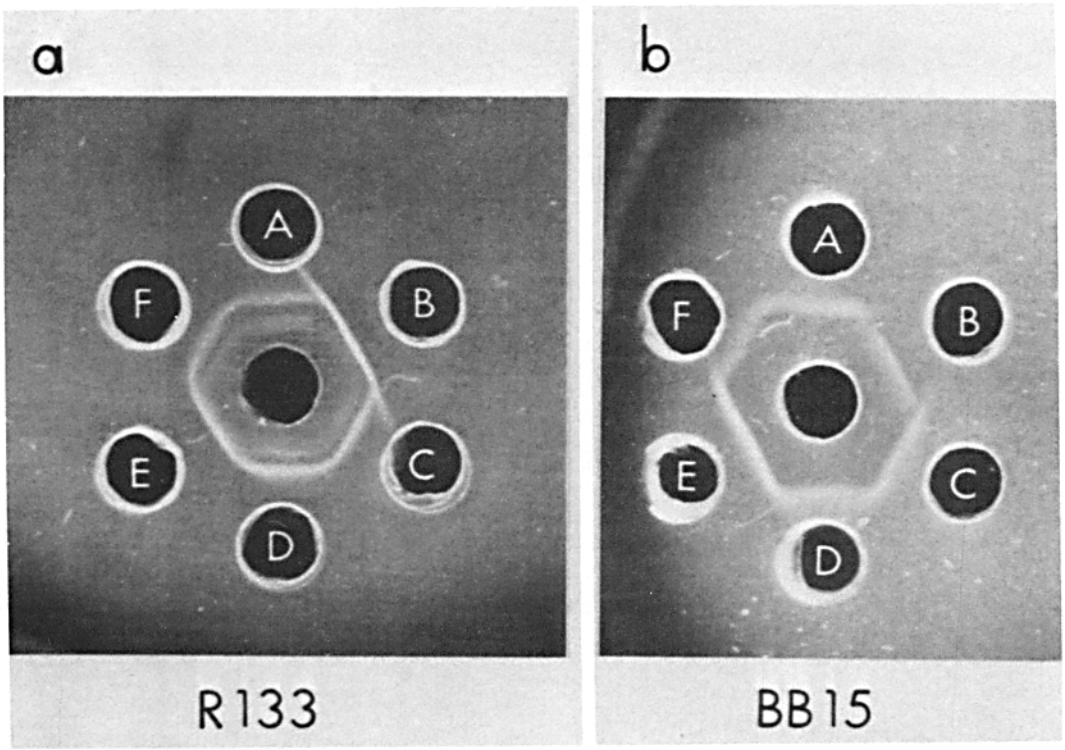

FIG. 1. Ouchterlony analysis of parent and IgG_{2a} variant proteins. A, ICR 9.9.2.1. B, MPC 11 (parent). C, ICR 11.8, D, M224. E, ICR 11.19.2. F, ICR 16. a, R 133 is anti-MPC 11. b, BB 15 is anti-LPC 1-Fc.

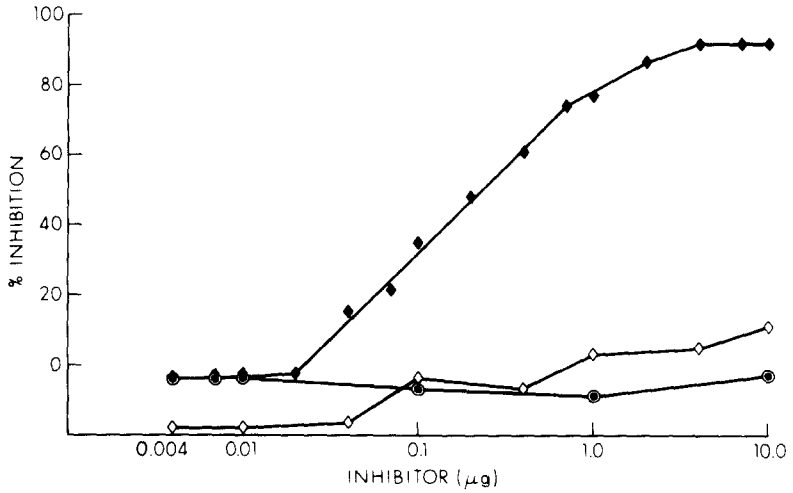

FIG. 2. Inhibition of the binding of [^{35}S]Met-MPC 11 to anti-idiotypic antisera (R 132) directed against MPC 11 by ◆ MPC 11, ◇ MOPC 141, ◉ MOPC 195.

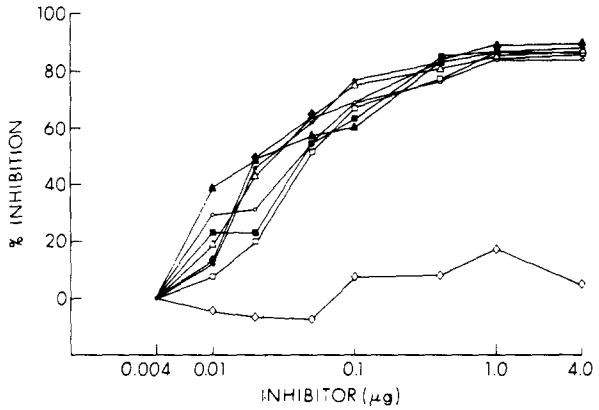

FIG. 3. Inhibition of binding of [^{35}S]Met-MPC 11 to anti-idiotypic antisera directed against MPC 11 by purified IgG_{2a} variant proteins. ◆ MPC 11, □ M224, ■ ICR 16, ○ ICR 11.8, △ ICR 9.9.2.1, ▲ ICR 4.68.66, ● ICR 11.19.3, ◇ MOPC 173.

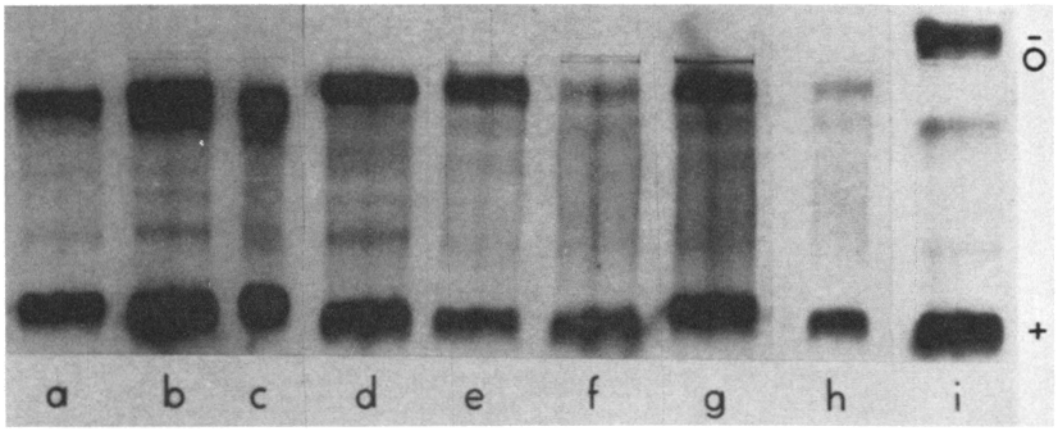

FIG. 4. Agarose gel electrophoresis of ascites. a, ICR 4.68.66. b, ICR 11.19.2. c, ICR 11.19.3. d, MPC 11. e, ICR 11.8. f, ICR 16. g, M224. h, ICR 9.9.2.1. i, MOPC 173. O-origin. The major protein band near the anode is albumin.

using a smaller set of proteins, MOPC 173 did not inhibit the binding of MPC 11 to the second antiserum while the parent, MPC 11, and two variants, ICR 9.9.2.1 and ICR 11.8, inhibited equally well (data not shown). These results showed that the γ2a variant proteins contained MPC 11 variable region antigenic determinant(s).

Electrophoretic Migrations of Variant Proteins in Agarose Gels. To try to discriminate between the variants, we examined ascites from the different γ2a producing variants, from MPC 11, and from MOPC 173 by electrophoresis on agarose plates. The γ2a paraproteins from the variants clearly fell into two groups (Fig. 4). The first group consisted of the secondary variants derived independently from two primary variants (ICR 4.68 and ICR 11.19) which produced heavy chains with a mol wt of 50,000 (Table I). These four proteins, three of which are shown in Fig. 4, panels a–c, were more negatively charged

than the MPC 11 parental protein (panel d). The second group of six variants, four of which are shown, migrated similarly to MPC 11 (Fig. 4, panels e–h). This group consisted of four primary variant proteins with heavy chains of mol wt 55,000 (three of which are shown in panels e–g) and the two secondary γ2a producing variants derived from ICR 9, the primary variant which synthesizes a γ2a heavy chain of mol wt 75,000 (one shown in panel h). For reference, the protein from MOPC 173 ($IgG_{2a}[\kappa]$), which has a cathodal mobility, is shown in panel i. These differences in electrophoretic mobility indicated that the γ2a variants were not identical and suggested that they might fall into subgroups based on their origins. This possibility was examined by comparative peptide map analysis.

Peptide Analysis. For a systematic analysis, the heavy chains of each γ2a variant were compared to ICR 9.9.2.1, a secondary γ2a producing variant whose primary structure we have studied (7).[2] ^{14}C-variant heavy chains were mixed with ^{3}H-heavy chains from ICR 9.9.2.1 and digested with trypsin and chymotrypsin; the resulting peptides were resolved by ion-exchange chromatography. The peptide maps of two primary variants, ICR 16 and ICR 11.8, each vs. ICR 9.9.2.1, are shown in Figs. 5 a and b. Examination of these two maps showed that the profiles of ICR 9.9.2.1 were not identical. In nine independent analyses, the number of distinct peaks generated from ICR 9.9.2.1 ranged from 41 to 52.[3] We have therefore analyzed each map independently. For example, the first line of Table II summarizes the data for the map of ICR 16 vs. ICR 9.9.2.1. ICR 16 had 48 peptide peaks: 36, or 75%, were shared with ICR 9.9.2.1; the extra 12, or 25%, were not found in ICR 9.9.2.1. In this map, ICR 9.9.2.1 had 51 peptide peaks, of which the 36 shared with ICR 16 constituted 71%. 15 (29%) of the ICR 9.9.2.1 peaks were not found in ICR 16. A similar analysis of the other maps, summarized in Table II, showed that ICR 9.9.2.1 differed from each variant by 10% (ICR 9.9.1.6.7, line h) to 29% (ICR 16). These differences were significantly smaller than the 50% difference found when ICR 9.9.2.1 was compared to the γ2b parent, 45.6.2.4 (Table II, line i). These results, like our serological data, enabled us to distinguish the γ2a variants, as a group, from the parent.

The sensitivity of the peptide analysis allowed us to discriminate between the variants and to subdivide them according to their structural similarities. The variant that was most similar to ICR 9.9.2.1 was ICR 9.9.1.6.7 (Table III, line a). Since both were secondary variants derived from the same primary variant (ICR 9), we thought that a comparison of other variants which arose by a single route might also show close similarities. Accordingly, ICR 4.68.66 was compared

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[2] T. Francus and B. K. Birshtein. Manuscript in preparation.

[3] So that a single map might yield as much information as possible, we have done the following: (*a*) we label with valine, threonine, and leucine, all of which are abundant in immunoglobulins, so that a significant percentage of heavy chains might be accounted for, and (*b*) we use both trypsin and chymotrypsin to solubilize as much of the protein as possible and to facilitate chromatography.

The lack of exact reproducibility of peptide profiles from map to map undoubtedly reflects the general problem of resolving numerous peptides and may additionally reflect the varying yields of chymotryptic cleavages. However, use of double-labeled cleavage and maps gives an internal control to this method since a single variant protein, independently labeled, gives identical ^{3}H and ^{14}C profiles under these conditions.

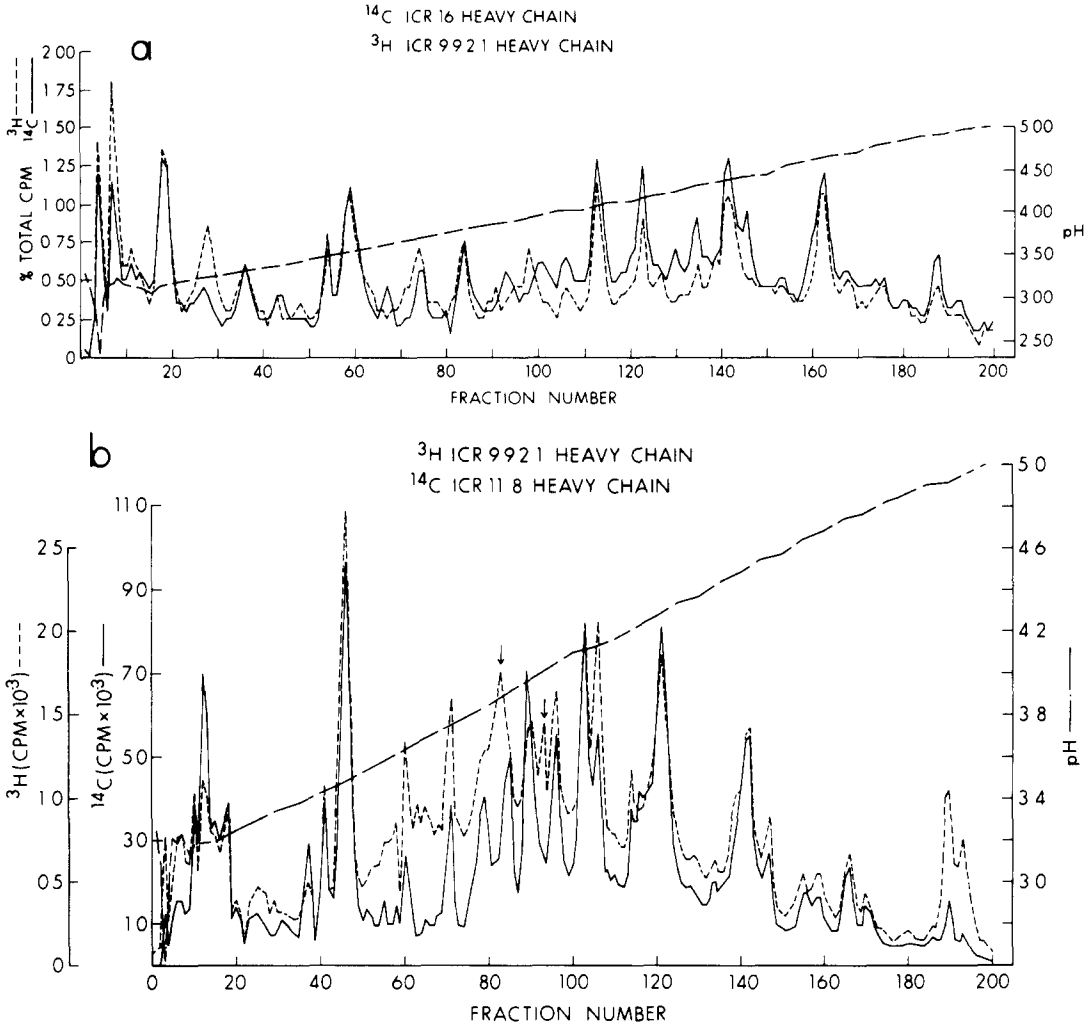

FIG. 5. Tryptic-chymotryptic peptide map of variant γ2a heavy chains. a, [^{3}H]ICR 9.9.2.1 vs. [^{14}C]ICR 16. b, [^{3}H]ICR 9.9.2.1 vs. [^{14}C]ICR 11.8. Details of preparation of sample and chromatography of peptides are described in Materials and Methods.

with ICR 4.68.110, both of which arose from the same short heavy chain (50,000 mol wt) producing primary variant and to ICR 11.19.3, which arose from a different short heavy chain (50,000 mol wt) producing primary variant. The results in Table III (lines b and c) pointed out the similarities in all three proteins and underscored a closer structural relationship in the pair derived from the same primary variant (ICR 4.68.66 and ICR 4.68.110). Similarly, we found that M224 and M319.2, both primary variants obtained after Melphalan mutagenesis were almost indistinguishable. (Table III, line d). Another close structural relationship between the variants M224 and ICR 11.8, both primary variants derived after mutagenesis with two different mutagens, was suggested upon a close examination of their maps each vs. ICR 9.9.2.1 (Table II, lines d

TABLE II

Compilation of Heavy Chain Peptide Map Analyses of Parent and γ2a Variant Proteins vs. ICR 9.9.2.1

Protein source	Total	Peptide peaks			Total	Peptide peaks	
		Shared no.	Extra no.			Shared no.	Extra no.
a. ICR 16	48	36 (75%)	12 (25%)	vs. ICR 9.9.2.1	51	36 (71%)	15 (29%)
b. ICR 11.19.2	36	31 (86%)	5 (14%)	" "	42	31 (74%)	11 (26%)
c. ICR 4.68.66	41	32 (78%)	9 (22%)	" "	41	32 (78%)	9 (22%)
d. M224	39	36 (92%)	3 (8%)	" "	46	36 (78%)	10 (22%)
e. M319.2	38	32 (84%)	6 (16%)	" "	40	32 (80%)	8 (20%)
f. ICR 11.8	43	37 (86%)	6 (14%)	" "	46	37 (80%)	9 (20%)
g. U2	60	46 (75%)	15 (25%)	" "	52	45 (87%)	7 (13%)
h. ICR 9.9.1.6.7	51	43 (84%)	8 (16%)	" "	48	43 (90%)	5 (10%)
i. 45.6.2.4	34	19 (56%)	15 (44%)	" "	41	19 (46%)	22 (54%)

TABLE III

Compilation of Heavy Chain Peptide Map Analysis of Pairs of Variants Generated via the Same Route

γ2a Variant protein	Total	Peptide peaks			γ2a Variant protein	Total	Peptide peaks	
		Shared no.	Extra no.				Shared no.	Extra no
a. *ICR 9.9.1.6.7	51	43 (84%)	8 (16%)	vs.	ICR 9.9.2.1	48	43 (90%)	5 (10%)
b. ICR 4.68.66	39	36 (92%)	3 (8%)	vs	ICR 4.68.110	40	36 (90%)	4 (10%)
c. ICR 4.68.66	44	36 (82%)	8 (18%)	vs.	ICR 11.19.3	43	36 (84%)	7 (16%)
d. M224	49	49 (100%)	–	vs.	M319.2	52	49 (94%)	3 (6%)
e. M224	47	45 (96%)	2 (4%)	vs.	ICR 11.8	46	45 (98%)	1 (2%)
f. M224 Fab	28	28 (100%)	–	vs.	ICR 11.8 Fab	28	28 (100%)	–
g. M224 Fc	27	27 (100%)	–	vs.	ICR 11.8 Fab	27	27 (100%)	–

* These data are taken from Table II, line h

and f, Fig. 5b). Direct comparison of their heavy chains (Fig. 6), and their Fab and Fc fragments showed them to be strikingly similar if not identical (Table III, lines e-g).

Thus, peptide map analyses have enabled us to subdivide the variants, but the grouping that results does not completely correlate with their origin.

Assembly Characteristics. The major pathway used by any mouse myeloma tumor for the assembly of H_2L_2 from newly synthesized heavy and light chains depends on the subclass of the heavy chain that it makes (27). For example, γ2b producing tumors assemble their immunoglobulin mainly via $H + L \rightarrow HL + HL \rightarrow H_2L_2$, while the major pathway in the γ2a producing tumors is $H + H \rightarrow H_2 + L \rightarrow H_2L + L \rightarrow H_2L_2$ (27). Our studies on the short heavy chain producing variants further supported the idea that the structure of the heavy chain dictates the assembly pattern: variants synthesizing heavy chains of 50,000 mol wt fail to assemble past the HL stage, while those synthesizing heavy chains of 40,000 mol wt secrete both H_2L_2 and HL (5).

We asked whether we could discriminate among the γ2a variants by examining their assembly characteristics since preliminary pulse-chase experiments had shown that they might differ (S. Koskimies and B. K. Birshtein, unpublished data). We labeled the γ2a variants, the parent (MPC 11, γ2b), and LPC 1 (γ2a) cells for 15 min, and examined the radiolabeled immunoglobulin assembly

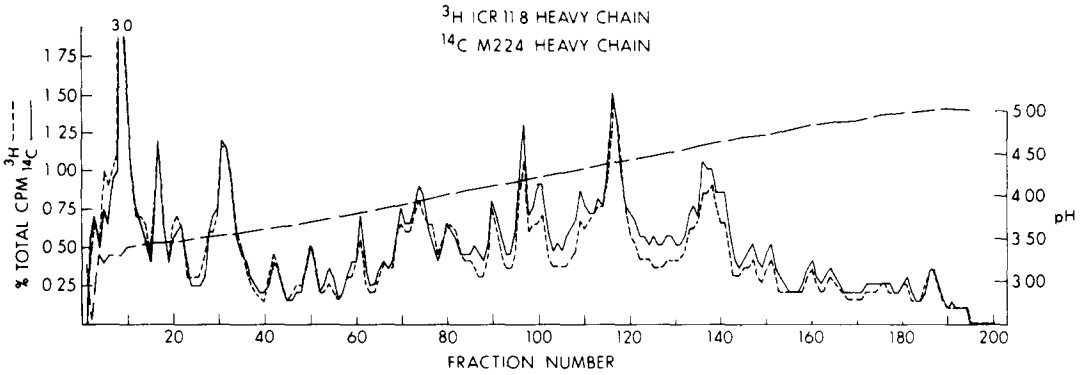

FIG. 6. Tryptic-chymotryptic peptide map of heavy chains of [^{3}H]ICR 11.8 vs. [^{14}C]M224. Details of preparation of sample and chromatography of peptides are described in Materials and Methods.

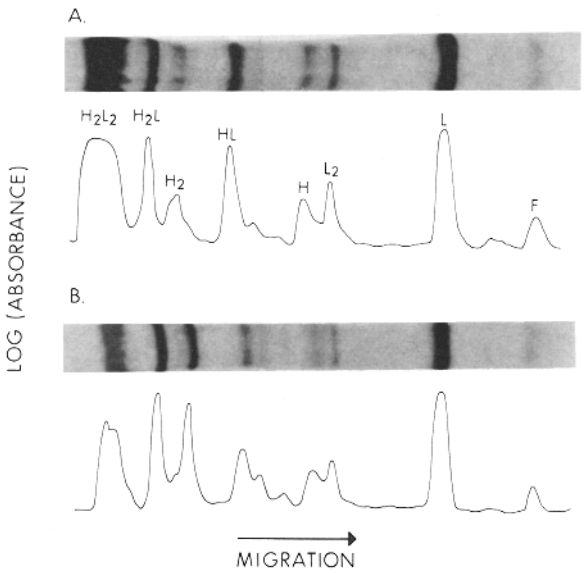

FIG. 7. Assembly patterns of ICR 4.68.66 (A) and ICR 11.8 (B) examined by SDS-PAGE and their densitometry tracing. Details are in Materials and Methods. H, heavy chain; L, light chain; F, constant region of light chain.

components by SDS-PAGE, as described in Materials and Methods. Fig. 7 shows differences in radioautographs and densitometric scans for two variants, ICR 4.68.66(A) and ICR 11.8(B).

The assembly patterns of the γ2a variants have been analyzed both for their rate of assembly to H_2L_2 and for their relative amount of HL (Fig. 8). As noted by Baumal et al. and as shown in Fig. 8 A-panels j and k, the relative amount of HL discriminates between γ2a and γ2b tumors, with γ2a tumors, such as LPC 1 (panel j) having lower relative HL than a γ2b tumor, such as MPC 11 (panel k). All the γ2a variants are lower than MPC 11 in this parameter and are similar to LPC 1, implying that they, like γ2a producing tumors, do not use

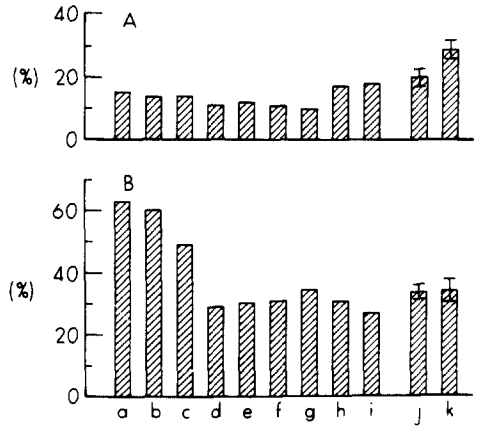

FIG. 8. Assembly patterns of γ2a producing variants compared by normalized HL (A), and normalized H_2L_2 (B). a, ICR 4.68.66. b, ICR 11.19.2. c, ICR 16. d, ICR 11.8. e, M224. f, M319.2. g, ICR 9.9.2.1. h, ICR 9.9.1.6.7. i, U2. j, LPC 1 (γ2a control). k, MPC 11 (parent γ2b). Variants were compared by using integration of densitometric traces. Calculations were normalized to only those assembly components containing heavy chains because of varying amounts of light chains produced by different variants; e.g. normalized H_2L_2 = $H_2L_2/(H_2L_2 + H_2L + H_2 + HL)$; normalized HL = $HL/(H_2L_2 + H_2L + H_2 + HL)$.

the HL intermediate as their major pathway of assembly.

Fig. 8 B shows the histogram of normalized H_2L_2 as a measure of the rate of assembly. γ2a producing variants are shown in panels a–i (ICR 4.68.66, panel a, and ICR 11.8, panel d) and the results of four determinations each of LPC 1 and MPC 11 are shown in panels j and k, respectively. Visually, the γ2a variants differ from each other much more than the error of the procedure. Statistically, with 95% confidence,[4] we can say that the γ2a variants fall into more than one group. Those variants having the fastest rate of assembly are ICR 4.68.66 (panel a), ICR 11.19.2 (panel b), and ICR 16 (panel c). Of these three, ICR 4.68.66 and ICR 11.19.2, both secondary γ2a variants derived from short heavy chain producing variants, were previously grouped together on the basis of charge and peptide maps; while ICR 16 has seemed to be the most distinctive of the γ2a producing variants because of its peptide profile (Fig. 5a) (6).

The remaining six variants (Fig. 8 B, panels d–i) have a similar rate of assembly which does not differ significantly from either MPC 11 (γ2b) or LPC 1

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[4] $\overline{H_2L_2}$ = $H_2L_2/(H_2L_2 + H_2L + H_2 + HL)$ = fraction H_2L_2 is of total assembly components containing heavy chains; $\overline{HL}$ = $HL/(H_2L_2 + H_2L + H_2 + HL)$ = fraction HL is of total assembly components containing heavy chains; $S_1^2{}_{\overline{H_2L_2}}$, $S_1^2{}_{\overline{HL}}$–the variance of $\overline{H_2L_2}$ and $\overline{HL}$, respectively, as calculated for the percent assembled H_2L_2 and HL from four independent labeling experiments of MPC 11. The subscript "1" indicates control. $S_2^2{}_{\overline{H_2L_2}}$, $S_2^2{}_{\overline{HL}}$–the variance of $\overline{H_2L_2}$ and $\overline{HL}$, respectively, in the nine variants studied for the percent assembled H_2L_2 and HL. Subscript "2" indicates variants. $F_{\overline{H_2L_2}} = S_2^2{}_{\overline{H_2L_2}}/S_1^2{}_{\overline{H_2L_2}} = 194.58/12.86 = 15.13$; $F_{\overline{HL}} = S_2^2{}_{\overline{HL}}/S_1^2{}_{\overline{HL}} = 8.6/0.7 = 12.28$; critical value for $F_{.05}$ (8, 3) = 8.84. Since both $F_{\overline{H_2L_2}}$ and $F_{\overline{HL}}$ are > 8.84, we can say at the 95%+ confidence level that the variants fall into more than one assembly group. That is, under the null hypothesis that the variants fall into one group and assuming the experimental errors are the same for the variants and the controls, the true variance of the control $\sigma_1^2{}_{\overline{H_2L_2}}$ will equal the true variance of the variants $\sigma_2^2{}_{\overline{H_2L_2}}$. Similarly, $\sigma_1^2{}_{\overline{HL}}$ will equal $\sigma_2^2{}_{\overline{HL}}$. The sample F values of 15.13 and 12.28 allow us to reject the null hypothesis that the variants fall into one group.

(γ2a). It is noteworthy that ICR 11.8, M224, and M319.2 (panels d, e and f, respectively), the three variants that seem indistinguishable by peptide maps, assemble similarly.

Discussion

We have isolated from the γ2b producing cell line, MPC 11, a dozen variants synthesizing immunoglobulins having a γ2a serotype. The expression of the γ2a structural gene was confirmed when we showed that the Fc region of one variant was identical to the Fc of the IgG_{2a} immunoglobulin MOPC 173, and different from that of the IgG_{2b} immunoglobulin, MPC 11 (7).[2] In this paper, we have shown, by a radioimmunoassay, that the idiotypes of the variants are identical to those of the parent. The simplest explanation for the generation of variants synthesizing heavy chains which have the parental idiotype and a constant region of a previously silent gene is a translocation of the sort suggested by Gally and Edelman (28). This hypothesis would predict that all the γ2a variants would be identical to each other. However, we showed that two γ2a variants (M224 and ICR 16) differed extensively in their peptide profiles (6). This finding suggested that other genetic mechanisms must be involved. Any genetic mechanism proposed must take into account the high incidence of variants and the multiple routes by which they arose. Primary γ2a producing variants generated by either ICR 191 or Melphalan occurred at an incidence of 0.6–2% (6) and secondary variants arose without further mutagenesis at the same frequency (9).

Our focus here was to define differences and similarities among the γ2a variants that might allow us to distinguish and group them and understand the genetic mechanisms of their origins. The major outcome of these studies has been the observation that these many γ2a variant proteins differ from each other. However, our examination of the variants by charge, peptide maps and assembly characteristics has enabled us to subdivide them. Three of the primary variants—M224, M319.2 and ICR 11.8—are very similar by all our assays and, in fact, are almost indistinguishable by peptide maps. It should be emphasized that two of these variants arose after Melphalan mutagenesis and the other after ICR-191 treatment. This group might thus result from a commonly operative mechanism in an immunoglobulin producing cell. Translocation is one such mechanism, but only detailed structural studies will enable us to test this hypothesis.

A second group is comprised of the secondary variants which arose from ICR 4.68 and ICR 11.19, which are primary variants synthesizing short heavy chains of mol wt 50,000. These secondary variants—ICR 4.68.66, ICR 4.68.110, ICR 11.19.2 and ICR 11.19.3—were similar in charge and in assembly pattern. Their peptide maps showed extensive similarities; however, they are not identical.

The rest of the variants do not fall into obvious groups. ICR 16 and ICR 9, both primary variants obtained after ICR 191 treatment, differ markedly from the other γ2a variants, including ICR 11.8, which arose after identical treatment. ICR 16's structure is probably the most different of the γ2a variants, as reflected by its peptide maps and its assembly pattern. ICR 9 is unique by virtue of its 75,000 mol wt heavy chain. The secondary variants derived from ICR 9—ICR 9.9.2.1 and ICR 9.9.1.6.7—could potentially comprise a group since their peptide maps show similarities; but their assembly patterns are rather different.

The spontaneously arising variant, U2, differs in peptide map and assembly pattern and also falls outside of our groupings. Our finding that several γ2a variants differ thus precludes strict translocation as a sole mechanism for their generation.

We considered the possibility that the variants represent the expression of different γ2a constant region genes which are ordinarily silent similar to the observation by Bosma and Bosma of the "wrong" allotype in a congenic mouse (29). However, our findings would seem to require more γ2a constant region genes that one might propose on the basis of nucleic acid hybridization data for light chain genes. In addition, Dr. Melvin Bosma and Ms. Carol DeWitt (unpublished data) showed that all our variants carried the BALB/c γ2a allotype.

It is possible that all our variants could arise by a common mechanism, such as a series of recombination events between γ2b and γ2a constant region genes and that preferred sites of recombination ("hot spots") could explain the high frequency of some types of variants. By this mechanism, one would expect the variant heavy chains to be hybrid molecules containing varying lengths of γ2b and γ2a constant region sequences. Such hybrid molecules have been reported (11-14). Thus far, we have not serologically detected any γ2b determinants in these variants; nor have we found any γ2b specific residues in our sequence studies of the Fc of one variant, ICR 9.9.2.1. Recently, however, we have identified such residues in another γ2a variant protein, ICR 11.19.3 (M. L. Greenberg, R. Campbell, and B. K. Birshtein, unpublished data). Whether our variants reflect the type of DNA sequence arrangements recently seen in genes coding for proteins as diverse as adenovirus, globin, and immunoglobulin (30, 31) remains, of course, a tantalizing question.

Knowledge of the primary structure of the variants should help us to discriminate among these mechanisms and our grouping of the variants helps us to select particular ones to study in depth. In addition, studies at the nucleic acid level to look for gene rearrangements, such as those shown by Hozumi and Tonegawa (32), have also begun.

Summary

12 variant cell lines producing an $IgG_{2a}(\kappa)$ immunoglobulin derived via different routes from the $IgG_{2b}(\kappa)$ synthesizing MPC 11 were studied. These variants all have the parental MPC 11 idiotype as shown by a radioimmunoassay. A comparison of the variants by charge, peptide maps, and assembly patterns has shown that most of them differ from one another, and some can be grouped.

One group consists of three primary variants generated with two mutagenic agents: these three have almost indistinguishable peptide maps. Two other primary variants which arose in a similar fashion differ markedly from each other and from this group. A second group is comprised of the four secondary variants which arose from two short heavy chain producing primary variants. Other secondary variants and the one spontaneously arising variant cannot be grouped. Possible genetic mechanisms such as translocation, expression of previously silent genes and recombination are discussed.

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