SOMATICALLY GENERATED MOUSE MYELOMA VARIANTS SYNTHESIZING IgA HALF-MOLECULES*

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The monomer of the IgA class of antibodies resembles other immunoglobulins in that it consists of two heavy (H)[1] chains and either two kappa or two lambda light (L) chains. This H_2L_2 monomer is often further assembled and secreted as a polymer of two or more monomers associated with a J chain and joined together by disulfide bonds. Many species, including the mouse, express two structurally distinct allotypes of IgA. The covalent structure of one of these is similar to that of other immunoglobulin classes in that its H chains are linked to each other and to its L chains by disulfide bonds (1). The other allotype, which represents all of the IgA expressed by BALB/c mice, is unique among normal immunoglobulins in that it lacks a disulfide bond between its H and L chains (2). This lack of an inter H-L disulfide bond must be due to structural differences in the heavy chain because the same light chains form disulfide bonds with other heavy chain classes (3).

Potter and Kuff (4) isolated BALB/c myeloma tumors that synthesized unusual IgA molecules. The H chains of six such tumors were significantly smaller than normal alpha chains (5). One of them, MOPC 47A (47A), has been completely sequenced, revealing a precise deletion of its third constant region (C_H3) domain (6). In addition, 47A and the other five myeloma proteins were only partially assembled to form H-L half-molecules (7, 8). Perhaps the most unusual aspect of these variant immunoglobulins was that they all contained disulfide bonds between their H and L chains (7, 8). Serologically similar proteins have been detected in small amounts in the intestinal contents of normal BALB/c mice (5).

These variant IgA proteins are interesting not only because of their unusual structure but also because of questions they raise about interdomain effects of deletions

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[1] *Abbreviations used in this paper:* C_H1, C_H2, and C_H3, first, second, and third constant regions, respectively; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; H, heavy; KLH, keyhole limpet hemocyanin; K_a, association constant; L, light; MOPS, 3-(4-morpholino)propanesulfonic acid; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphocholine; RIA, solid-phase radioimmunoassay; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes; TCA, trichloroacetic acid; Tm, tunicamycin.

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in one part of the molecule upon the structure and function of other parts of the molecule. However, it has been difficult to directly address questions about the origin and function of these proteins due to the problem of establishing the geneology of in vivo tumors and because none of the variant immunoglobulins bound known antigens. These problems can be alleviated by using somatic cell genetic systems in which cells producing variant immunoglobulins arise at high frequency in vitro from cloned myeloma and hybridoma cell lines of known antigen-binding specificity (9). In this paper we report that variants with phenotypes and serological characteristics similar to those of the IgA half-molecules (*a*) can rise spontaneously in culture (*b*) can have heavy chains of different sizes, and (*c*) can have the same affinity for hapten as the parental antibody in spite of the abnormal H-L disulfide bond. DNA and RNA studies suggest that these somatic variants arise through premature terminations that result in C-terminal deletions.

Materials and Methods

Cell Culture Condition and Detection of Variants. The S107 cell line and the S_1U_9 variant were grown at 37°C in an atmosphere of 8% CO_2 in Dulbecco's modified Eagle's medium (DME) (Gibco H-21, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS, Flow Laboratories, Rockville, Md.), 5% NCTC 109 (Microbiological Associates, Walkersville, Md.), streptomycin, and penicillin. The W3082 line and the T7 variant were grown in DME supplemented with 10% horse serum (Flow Laboratories). Cells were cloned in soft agarose over rat embryo fibroblast feeders, overlaid with antigen, and variants were identified and recovered as previously described (10–12). Diazophenolphosphocholine was prepared as described by Chesbro and Metzger (13) and reacted with keyhole limpet hemocyanin (KLH) (10). Levan was prepared by differential ethanol precipitation from culture supernates of *Leuconostoc mesenteroids* NRRL B512 (14).

Purification of S107 and S_1U_9 Proteins. Approximately 10^7 cells per mouse were injected intraperitoneally into Pristane-primed BALB/c mice (15). After 7–10 d the ascites fluid was recovered. S107 and S_1U_9 purification was achieved by affinity chromatography on a phosphocholine-Tyr-Gly-Sepharose 4B column. Bound material was eluted with 10 mM phosphocholine (PC) and subsequently dialyzed (16). A sample of purified 47A was generously provided by Dr. Fred Mushinski (National Cancer Institute).

Labeling of Cells. S107, S_1U_9, W3028, and T7 cells were biosynthetically labeled in amino acid-depleted medium as previously described (17) except for the use of DME and 10% FCS. [^{14}C]Lysine, [^{35}S]methionine, and [^{3}H]tyrosine (New England Nuclear, Boston, Mass.) were used at 50, 50, and 100 μCi/ml, respectively. Labeling in the presence of tunicamycin (Tm; generous gift of Dr. Robert Hamill, Eli Lilly and Co., Indianapolis, Ind.) was done similarly except that cells were preincubated for 1 h in growth medium containing 1.5 μg/ml Tm and labeled in medium also containing 1.5 μg/ml Tm. Cell lysates were prepared and immunoprecipitated as described below.

Lactoperoxidase-catalyzed ^{125}I-surface labeling was carried out by a modification of the technique described by Oi et al. (18). Between 0.5 and 2 × 10^7 cells were washed twice in phosphate-buffered saline (PBS), resuspended in 2 ml of PBS, and 0.5 mCi Na ^{125}I (Amersham Corp., Arlington Heights, Ill.) and 200 μg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) were added. The reaction was started by the addition of 0.025 ml of 0.03% hydrogen peroxide (H_2O_2). Equal amounts of H_2O_2 were added at 3, 6, and 9 min. At 10 min the reaction was stopped by washing the cells three times in cold PBS containing 50 μM potassium iodide. The cell pellet was then resuspended gently, lysed by the addition of 0.5 ml of 0.5% Nonidet P-40 (NP-40) in an isotonic buffer (0.01 M Tris, 0.15 M NaCl, and containing 8.5 ml 1 M HCl and 0.305 g $MgCl_2$-$6H_2O$ per liter), placed on ice for 20 min, and centrifuged for 10 min at 400 *g* in order to pellet the nuclei. The supernatant was recovered and saved for immunoprecipitation.

Immunoprecipitation and Gel Electrophoresis. To 0.5 ml of either cell supernatant ([^{14}C]lysine

and [^{3}H]tyrosine labeling) or lysate (^{125}I labeling) was added 0.1 ml of a 25% suspension of rabbit anti-mouse IgA coupled to Sepharose 4B (19). The mixture was incubated overnight on a rotating mixer at 4°C. The Sepharose was then washed twice in cold PBS, once in 0.5% NP-40 (in the same buffer as above except that it was 0.3 M NaCl), and finally in a buffer containing 10 mM phosphate buffer, pH 7.2, and 10 mM NaCl. Samples were eluted from the Sepharose by boiling for 1 min in 2% sodium dodecyl sulfate (SDS). Samples to be reduced and alkylated were made 25 mM in dithiothreitol, incubated for 2 h at 37°C, and then made 50 mM with recrystallized iodoacetamide, and incubated for another 30 min at 37°C. Before electrophoresis, glyerol and phenol red were added to all samples at a final concentration of 10 and 0.02%, respectively.

Slab gel SDS-polyacrylamide gel electrophoresis (PAGE), using either the phosphate or discontinous Tris-glycine buffer system, was done as described by Maizel (20). Depending on the size resolution desired, final polyacrylamide concentrations of 5%, 7.5%, or 10% were used.

Serology. Monoclonal anti-IgA constant region and anti-T15 variable region antibodies were kindly provided by Angela Giusti (Albert Einstein College of Medicine). Rabbit anti-mouse IgA Fc was prepared by repeatedly injecting rabbits with 1 mg of IgA Fc fragment prepared by papain digestion of the myeloma protein W3129 in complete Freund's adjuvant. Double diffusion (Ouchterlony) analysis was done as described (21). Inhibition of hemagglutination assays were carried out by a modification of the method of Evans et al. (22). Affinity-purified S107 protien was attached to sheep erythrocytes (SRBC, 3 mg protein/0.5 ml packed cells in 10 ml PBS) by treatment for 30–60 min with glutaraldehyde (Sigma Chemical Co.) at a final concentration of 0.25%. The titer of the various hemagglutinating antibodies was determined by reacting them with S107-SRBC in PBS containing 1% bovine serum albumin (BSA). A fourfold excess of antibody was then incubated with dilutions of S107 or variant protein and S107-SRBC to determine the ability of these proteins to inhibit hemagglutination.

Anti-PC binding site antiserum was prepared according to Claflin and Davie (23). Solid-phase radioimmunoassay (RIA) using this serum was performed as has been described (10). The only modification was that the incubation with labeled S107 and inhibitor was carried out for 3 h rather than overnight. RIA with monoclonal antibodies were done similarly except that the wells were coated with 0.05 ml of the respective affinity-purified monoclonal antibody (3 μg/ml) instead of with the antibinding site antiserum.

Equilibrium Dialysis. Equilibrium dialysis was done by the steady-state, flow dialysis method of Colowick and Womack (24). The flow rate used was 3.0 ml/min. The upper chamber of 1.5 ml contained 5.5 mg of S_1U_9 protein and 3.75×10^{-9} mol of [^{14}C]PC (55 mCi/mmol, New England Nuclear). In order to drive off increasing amounts of the [^{14}C]PC, successive 0.015-ml aliquots of 6.25×10^{-4} M PC solution were added.

Carboxypeptidase A and B Digestions. C-terminal analysis was done by a modification of the procedure described by Kehry et al. (25). Phenylmethylsulfonylfluoride-treated carboxypeptidase A (Sigma Chemical Co.) was prepared by method ii as described by Ambler (26). Phenylmethylsulfonylfluoride-treated carboxypeptidase B (Worthington Biochemical Corp., Freehold, N. J.) was thawed and diluted 1:10 in 0.1 M ammonium bicarbonate just before use. Digestions were done in 0.1 M ammonium bicarbonate, 0.1% SDS at a molar enzyme to substrate ratio of 1:40. Each 0.25-ml sample contained 130,000–250,000 cpm of reduced and alkylated (as for SDS-PAGE) [^{3}H]tyrosine-labeled parent or variant protein and 0.25 mg of carrier rabbit IgG (Pentex Biochemical, Kankakee, Ill.). Incubations were done at 37°C for 2.5, 5, 15, 40, and 90 min. Substrate controls that received enzyme boiled in 0.1 M acetic acid for 15 min were incubated for 90 min. All reactions were stopped by the addition of equal volumes of 40% trichloroacetic acid (TCA), placed on ice for 20 min, centrifuged at 400 *g* for 10 min, and then an aliquot of each supernatant was counted. Substrate controls were subtracted from each time point. Mole tyrosine released per mole protein was calculated by multiplying the ratio of released supernatant cpm to initial TCA precipitable cpm by the number of tyrosine residues present. Assuming an equimolar ratio of H and L, S107 protein contains 30 tyrosine residues (27–29 and P. Tucker, personal communication). Because the complete sequence of W3082 antibody has not been determined, it was also assumed to contain 30 tyrosine residues. S_1U_9 and T7 protein were estimated to contain 27 and 26 residues, respectively, based on the size of their deletions and the location of tyrosine residues.

DNA Isolation and Gel Analysis. High molecular weight DNA was isolated from the myeloma cells essentially as described by Wigler et al. (30) except that lysates were treated with RNase (50 μg/ml, 30 min at 37°C) before pronase treatment. DNA was digested with restriction enzymes as directed by the supplier (Bio-Rad Laboratories, Richmond, Calif., or New England Biolabs, Beverly, Mass.). 10 μg of DNA was applied per lane of a Tris-borate (31) agarose gel. Molecular hybridizations were done by standard procedures (30) except that 10% dextran sulfate was added to the hybridization. The ^{32}P nick-translated DNA probe (32) was prepared using the 1.6 kilobase (kb) Eco R1-Sma 1 fragment isolated from a recombinant plasmid containing the 4.4 kb Eco R1 fragment of the alpha gene. The plasmid was the generous gift of Dr. S. P. Kwan (Albert Einstein College of Medicine).

RNA Isolation and Gel Analysis. Myeloma cells were washed in PBS (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.6), resuspended in 20 mM Tris, 2 mM Ca^{++}, 2 mM Mg^{++}, pH 8.3, and lysed with 0.5% NP-40. Nuclei were removed by centrifugation, SDS, NaCl, and EDTA added to 0.5%, 0.25 M, and 10 mM, respectively, and the cytoplasmic lysates extracted three times with 90% phenol. The RNA was then precipitated with ethanol. Total cytoplasmic RNA was fractionated by electrophoresis on agrose gels containing 2.2 M formaldehyde, 20 mM 3-(4-morpholino)propanesulfonic acids (MOPS), 5 mM sodium acetate, and 5 μg/ml ethidium bromide. Samples were prepared by resuspending RNA in 50% formamide (ultrapure from Bethesda Research Laboratories, Rockville, Md.), 2.2 M formaldehyde and MOPS/sodium acetate/EDTA, as above, heated at 60°C for 5 min, quickly chilled on ice, and applied to the agarose gel after the addition of Ficoll-bromophenol blue. Electrophoresis was carried out at 100 V in the cold with buffer circulation for ~18 h. RNA markers were visualized by ultraviolet light. RNA was blotted to nitrocellulose as described by Thomas (33).

In Vitro Translation. Ethanol-precipitated RNA was resuspended in 400 mM NaCl, 10 mM Tris 1 mM EDTA, 0.2% SDS, pH7.5, and bound to oligo-dT-cellulose (Collaborative Research Inc., Waltham, Mass.). The column was washed with 100 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.2% SDS, pH 7.5, and the bound poly A+ mRNA eluted with 10 mM Tris, 1 mM EDTA, and 0.2% SDS, pH 7.5. NaCl was added to 0.3 M and the RNA precipitated with 2 vol of ETOH at −20°C.

In vitro translation was done using a rabbit reticulocyte lysate and [^{35}S]methionine (1,370 Ci/mmol, Amersham Corp.). A mix of 80% lysate and 20% labeled amino acid was prepared. mRNA (400 ng) was added in 1–10 μl of the assay mix, and the reaction mixture incubated 90 s at 30°C. It was then diluted with 200 μl of PBS, and 1 μl of rabbit anti-mouse alpha and kappa were added. Immune complexes were precipitated with Staph A essentially as described by Kessler (34).

Results

Isolation of Variants. In an effort to identify variant mouse myeloma cells producing antibodies that no longer bound antigen, the S107 and W3082 cultured myeloma cell lines were cloned in soft agarose and overlaid with their respective antigens (10, 12). Both S107 and W3082 produce IgA, kappa antibodies (35, 36). S107 reacts with the hapten PC and was overlaid with PC attached to the protein carrier KLH, whereas W3082 was overlaid with levan. Most of the clones secreted polymers of IgA that reacted with the antigen in the agarose to form an antigen-antibody precipitate visible around the myeloma colonies. Approximately 1% of the clones were not surrounded by such a precipitate and a number of these were recovered from the agar, expanded to mass culture, and examined in more detail. Some of these spontaneous variants produced superficially normal IgA antibodies that bound to antigen less well than the parental myeloma proteins, whereas others had simply lost their ability to synthesize or secrete detectable amounts of IgA. However, one of the S107 variants, called S_1U_9, and one of the W3082 variants, called T7, secreted structurally altered immunoglobulins. Because both of these spontaneous somatic variants resembled the variant

tumors originally described by Potter and Kuff (4), the variants and their parental myelomas were examined in more detail.

Polypeptide Size and Covalence Structure of the Variants. Fig. 1 compares the electrophoretic migration on SDS-PAGE of the immunoglobulins secreted by the W3082 and S107 parents, and their respective T7 and S_1U_9 variants. As can be seen in lanes a and d, in the absence of reducing agents, SDS dissociates the parental proteins into H chain polymers (H_2 and H_n), L chain dimers, and free L chains. This demonstrates that the IgA produced by W3082 and S107 resemble normal BALB/c IgA in lacking inter-H-L disulfide bonds. Reduction converts the H_2 and H_n polymers into free H chains, thus showing the presence of inter-H chain disulfide bonds (Fig. 1, compare lanes a and d with e and h). The variants display a strikingly different pattern. In the absence of reducing agents, each of the variant proteins exhibits a major band whose mobility is intermediate between that of parental H_2 and L_2 (Fig. 1, lanes b and c). When the variant proteins are reduced in the presence of SDS, two major bands are generated, one of which comigrates with parental L chains while the other migrates more rapidly than the parental H chains (Fig. 1, lanes f and g). This electrophoretic behavior, similar to that described for the in vivo generated half-molecules such as 47A (5), indicates that both the S_1U_9 and T7 immunoglobulins contain a small H chain. Furthermore, it suggests that although the variant H chains do not form inter-H chain disulfide bonds, most of them do form an aberrant H-L chain bond. This was confirmed by recovery experiments (data not shown). It should be mentioned that the H chains of T7 and 47A comigrate exactly on both phosphate and Tris-glycine SDS gels (data not shown).

To determine whether the size differences among the S_1U_9, T7, and parental H chains were due to primary amino acid changes or to glycosylation differences, the various proteins were radioactively labeled in the presence or absence of Tm, which inhibits glycosylation by preventing the addition of the core sugars (37). Fig. 2, lanes a, c, f, and h show the proteins labeled without the drug, whereas lanes b, d, e, and

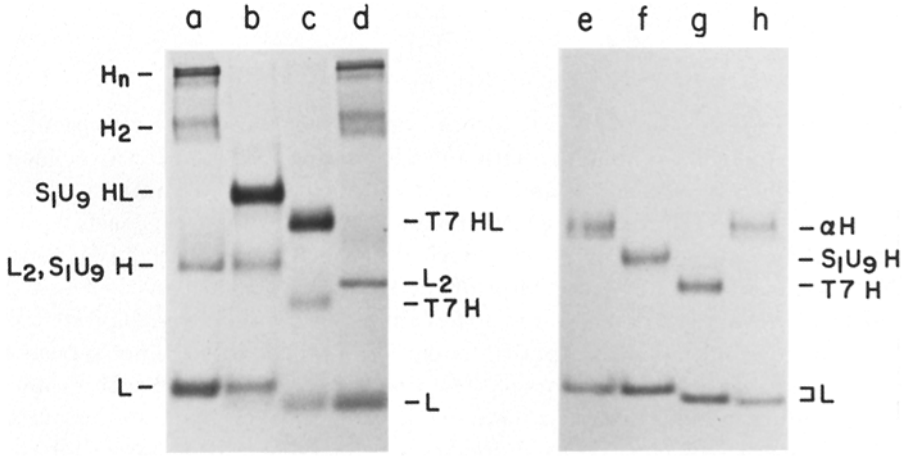

FIG. 1. SDS-PAGE of immune precipitated secretions of S107, S_1U_9, W3082, and T7. Cells were labeled with [^{14}C]lysine, secretions immune precipitated, and run on a 7.5% phosphate gel as described in Materials and Methods. Samples in lanes e–h were reduced and alkylated, while samples in lanes a–d were untreated. Samples are as follows: a and e, S107; b and f, S_1U_9; c and g, T7; d and h, W3082.

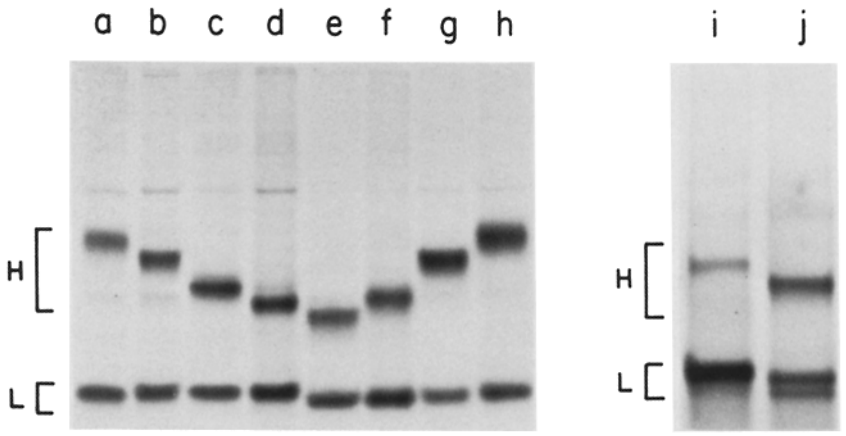

FIG. 2. Size comparison of S_1U_9 and T7 proteins with Tm-treated cells and cell-free synthesis. Cells were either treated with 1.5 μg/ml Tm or left untreated, labeled with [^{35}S]methionine, lysed with 0.5% NP-40, and immune precipitated as described in Materials and Methods. Cell-free synthesis in a rabbit reticulocyte system was also carried out as described. SDS-PAGE was done on a 7.5% phosphate gel. Samples b, d, e, and g were from Tm-treated cells, while a, c, f, and h were from untreated cells. Samples are as follows: a and b, S107; c and d, S_1U_9; e and f, T7; g and h, W3082; i, in vitro synthesized S_1U_9; j, in vitro synthesized T7.

g show the proteins labeled in the presence of the drug. The difference in mobilities of the unglycosylated S_1U_9 and T7 H chains (lanes d and e) shows that their size difference is not due to carbohydrate differences. Similarly, comparison of lanes d and e with b and g shows that the size difference between the variants and their respective parents is also not due solely to an alteration in carbohydrate structure. In vitro synthesis of S_1U_9 and T7 protein utilizing poly A^+ mRNA and a rabbit reticulocyte system (lanes i and j) confirms the polypeptide deletions. Comparison with the in vitro products of the parental cell lines (data not shown) demonstrates that the deletions are not due to in vivo proteolysis.

Using the mobilities of the Tm samples to estimate the apparent molecular weights of the carbohydrate-free polypeptides, the approximate weights of parental, S_1U_9, and T7 H chains were 53,000, 42,000, and 40,000, respectively. The deletions of S_1U_9 and T7 are thus of ~95 and 115 amino acid residues, respectively. For making the molecular weight estimates, phosphate rather than Tris-glycine gels were used because they have been reported to be less susceptible to artifactual errors (38). Taken together these studies indicate that variants phenotypically similar to the 47A group of tumors can arise spontaneously in culture from myelomas producing normal IgA molecules.

Constant Region Serology. Since the 47A protein is known to lack its C_H3 domain (6), its serology was compared with S_1U_9, T7, and their respective parents. Double diffusion in agar (Ouchterlony technique) was used to compare the reactivity of all these proteins in all possible combinations with a rabbit antiserum specific for the Fc region of BALB/c IgA. There was complete identity among S_1U_9, T7, 47A, and between the two parental molecules (data not shown). In all cases the parental molecules spurred over each of the three variants, indicating that the parental proteins contain Fc-region determinants not present in the three variant molecules. This was confirmed by absorbing antiserum with S_1U_9 attached to Sepharose and showing that

the absorbed antiserum did not react in agar diffusion with T7 or 47A, but continued to react strongly with S107 and W3082.

The serological similarity of S_1U_9, T7, and 47A was further supported by their reactivities with two rat anti-IgA constant region antibodies. Table I shows the reactivity of the two parental proteins, their respective variants, and 47A with monoclonals R21 and R99 as measured by the inhibition of hemagglutination. All five proteins reacted similarly with R99. In contrast, the three variant molecules did not react with R21, whereas the parental molecules did. Parenthetically, this suggests that R21 recognizes antigenic determinants on the C_H3 domain of the IgA molecule.

Carboxypeptidase Digestion. The electrophoretic behavior and serological characteristics of the variants taken together with the known structure of 47A indicated that S_1U_9 and T7 no longer contained some or all of the C_H3 domain. To determine whether this was due to an internal or C-terminal deletion, an attempt was made to compare the C-terminal sequence of S107 with S_1U_9 and of W3082 with T7. Since it has been reported that MOPC 511 (another BALB/c IgA myeloma protein) has a C-terminal tyrosine (29), the parental and variant proteins were labeled with [^{3}H]tyrosine, digested with carboxypeptidases A and B, and the release of TCA soluble counts was measured. Fig. 3 shows the expected rapid release of tyrosine from S107 and W3082, whereas with S_1U_9 and T7 the release is only at background levels. This suggests that the variants do not have the normal C-terminal sequence. However, it is also possible that the C-terminal amino acid is protected in the variants due to a conformational change. In order to gain further sequence information, carboxypeptidase digestion of unlabeled, purified H chain followed by amino acid analysis was attempted. However, at all time points examined the yield was too low and the background too high to provide meaningful results.

Membrane Association. It is generally believed, but not formally proven, that a C-terminal hydrophobic segment in membrane immunoglobulin serves to anchor it in the plasma membrane. Since S107 contains membrane immunoglobulin (39), the presumptive C-terminal deletion of the variant S_1U_9 made it interesting to determine whether the variant possessed membrane immunoglobulin. S107 and S_1U_9 cells were both treated with cycloheximide for 3 h to void the cells and membranes of "in transit"-secreted immunoglobulin and then surface-labeled by the ^{125}I/lactoperoxidase method. The membranes were then solubilized, immunoprecipitated, and analyzed

TABLE I

Constant Region Serology: Hemagglutination Inhibition (HAI) with Two Monoclonal Antibodies

Inhibiting protein	HAI ($\log_2$ titer)	
	Hemagglutinating antibody	
	R99	*R21*
W3082	8.0*	8.5
T7	8.5	0
S107	6.5	8.0
S_1U_9	8.5	0
47A	9.5	0

* Value represents average of determinations done in duplicate. In all cases the duplicates were no more than one well apart.

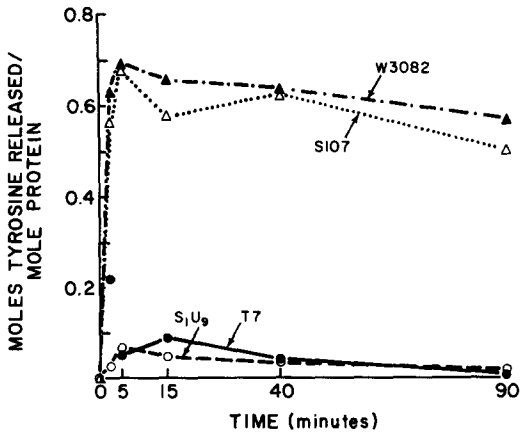

FIG. 3. Carboxypeptidase digestions of S107, S_1U_9, W3082, and T7 proteins. S107, S_1U_9, W3082, and T7 proteins were labeled with [^{3}H]tyrosine and digested with a mixture of carboxypeptidases A and B for 2.5, 5, 15, 40, and 90 min as described in Materials and Methods. The amount of TCA-soluble radioactivity was measured for each time point. Control samples were incubated for 90 min with carboxypeptidase that had been boiled for 15 min in 0.1 M acetic acid.

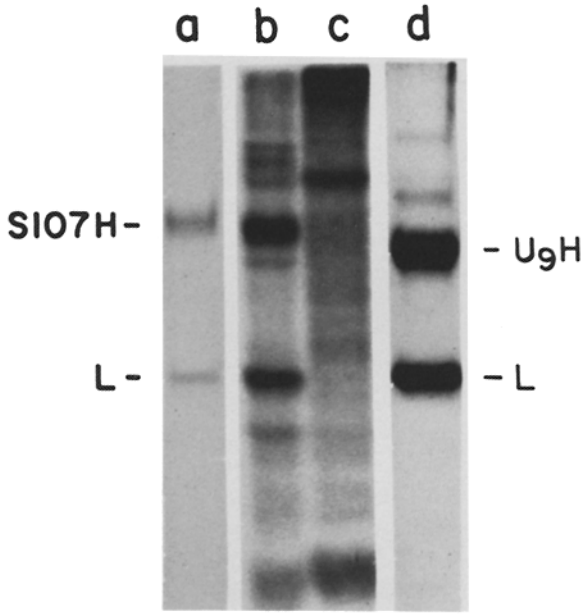

FIG. 4. Surface labeling of S107 and S_1U_9 cells. S107 and S_1U_9 cells were surface labeled by the ^{125}I-lactoperoxidase method as described in Materials and Methods. Membranes were solublized in NP-40, immune precipitated, and electrophoresed on a 7.5% phosphate gel. Samples are as follows: a, control [^{14}C]lysine-labeled secreted S107 protein; b, ^{125}I surface-labeled S107; c, ^{125}I surface-labeled S_1U_9; d, control [^{14}C]lysine-labeled secreted S_1U_9 protein. Equal numbers of TCA precipitable counts were added to lanes b and c.

by SDS-PAGE. Although Fig. 4, lane b, clearly shows bands corresponding to S107 H and L, there are no bands in lane c corresponding to S_1U_9 H and L. For comparison, lane d shows the positions of secreted S_1U_9 H and L. The greater intensity of contaminating bands in lane c relative to lane b is due to the fact that equal numbers

of counts were loaded in both lanes, although lane c contained no H or L. When the cells were not pretreated with cycloheximide, clear bands corresponding to S_1U_9 H and L were seen, indicating that it is possible to iodine label the in transit-secreted immunoglobulin passing through the plasma membrane (data not shown). A similar experiment with T7 was not possible because attempts at surface labeling W3082 and T7 yielded little, if any, labeling of membrane immunoglobulin, presumably because of its absence or the inacessibility of its tyrosine residues.

Analysis of DNA and mRNA. It is possible to distinguish between DNA deletions, processing defects leading to mRNA deletions, and premature terminations by directly examining the sizes of the constant region genes and mRNA coding for the IgA H chains. When the parental and variant DNA were compared by restriction analysis no differences were detected. Fig. 5 shows genomic Southern blots of Eco R1 and Msp I fragments of the parental and variant cell lines probed with an Eco R1-Sma I fragment containing half of the C_H2 and all of the C_H3 constant region domains. In all four cell lines, the probe hybridizes to a single 4.4 kb Eco R1 fragment (lanes a–d) and a single 2.3 kb Msp I fragment (lanes e–h).

Fig. 6 shows a Northern blot of the cytoplasmic mRNA probed with the same nick-translated Eco R1-Sma I fragment. The sizes of the IgA heavy chain mRNA were identical in all four cell lines. It should be noted that using this same system one of us was able to clearly detect a size difference in the mRNA that coded for an IgA molecule with a partially deleted C_H1 domain (Sherie Morrison, unpublished results). These results taken together with the carboxypeptidase experiment and the electrophoretic behavior of the variants indicate that the short heavy chains do not result either from the deletion of the gene or from a splicing defect.

Variable Region Serology. It was important to determine whether the variants contain the same variable region as the parental proteins. This could be done serologically with S107 and S_1U_9 because S107 bears the well-defined T-15 idiotype, the predominant idiotype expressed in BALB/c mice immunized with PC (40, 41). The idiotype of S_1U_9 was examined by comparing its reactivity to that of S107 with a panel of seven monoclonal anti-T-15 antibodies, which have been shown to react with at least six independent idiotopes (Angela Giusti and Catherine Desaymard, unpublished results). S107 and S_1U_9 inhibited the hemagglutination of S107-coated SRBC by each of the monoclonals to the same relative degree. The reactivity of one of these monoclonals (R 101) with both S107 and S_1U_9 was also the same when measured by the more sensitive technique of RIA (data not shown).

Rabbit antibodies specific for the hapten-binding site of S107 were prepared by the method of Claflin and Davie (23). This antibody was attached to the wells of a polyvinyl microtiter plate, and the ability of S107 and S_1U_9 proteins to inhibit the binding of [^{35}S]methionine-labeled S107 was compared. Within the error of the method, the two inhibition curves are identical (Fig. 7). These results indicated that S107 and S_1U_9 contain the same variable region and suggest that the formation of the inter H-L chain disulfide bond does not cause major changes in the conformation of the hapten-binding site nor in at least some other sites of the variable region.

Affinity of S107 and S_1U_9 for Hapten. Although S107 protein is easily purified by affinity chromatography, initial attempts at purification of S_1U_9 protein yielded very little bound material. Analysis of the protein content of the pre-PC (wash) fractions revealed two peaks of 280 nm absorbing material. The second peak (peak II) was

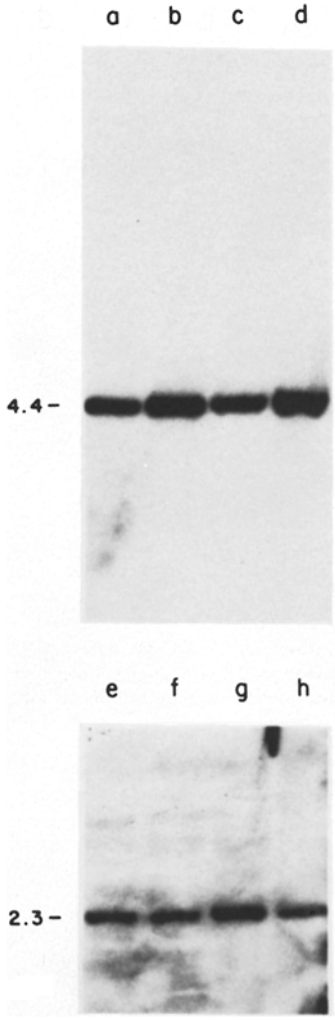

FIG. 5. Southern blot hybridization of S107, S_1U_9, W3082, and T7 genomic DNA. DNA was prepared, restricted, electrophoresed, and hybridized as described in Materials and Methods. The ^{32}P nick-translated probe used was the 1.6 kb Eco R1-Sma I alpha gene fragment that contains half of C_H2 and all of C_H3. The DNA in lanes a–d and e–h was restricted with Eco R1 and Msp I, respectively. Samples are as follows: a and e, S107; b and f, S_1U_9; e and g, T7; c and d, W3082.

enriched for serologically detectable S_1U_9 protein. SDS-PAGE showed that the peak II material was only partially purified. However, when the peak II material was passed over the PC-column a second time, it eluted as before but did not contain any contaminating proteins by SDS-PAGE.

In order to further evaluate this apparent difference in binding between S_1U_9 and S107, the affinity of the parent and variant for PC was measured using the steady-state method of equilibrium dialysis (24). Fig. 8 shows the results for S_1U_9 plotted in the form of a Scatchard analysis. The plot, drawn according to the least squares analysis (with a correlation coefficient of −0.991), yields an association constant (K_a) of $2.23 \times 10^5\ M^{-1}$. The number of binding sites per molecule is calculated to be 1.09.

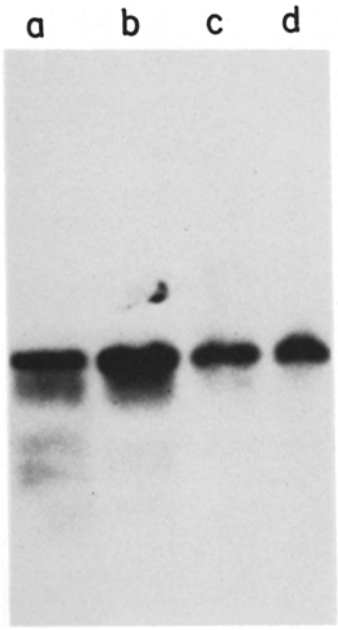

FIG. 6. Northern blot hybridization of S107, S_1U_9, W3082, and T7 cytoplasmic RNA. Cytoplasmic RNA was prepared, electrophoresed, and hybridized as described in Materials and Methods. The probe used was the same as that for Fig. 5. Samples are as follows: a, S107; b, S_1U_9; c, T7; d, W3082.

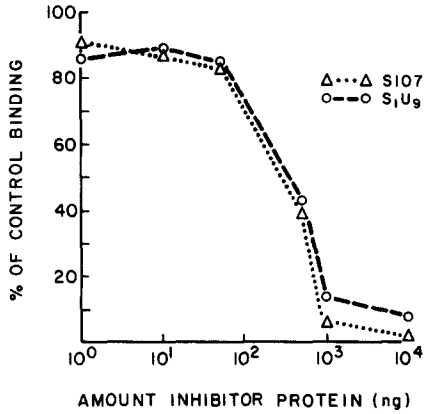

FIG. 7. RIA comparison of the reactivity of S107 and S_1U_9 protein with a rabbit anti-PC binding site-specific antibody. Binding site-specific antibody was prepared and used in an RIA as described in Materials and Methods. PC-KLH was attached to polyvinyl plates and a constant amount of [^{35}S]S107 was added to each well. Increasing amounts of purified S107 or S_1U_9 protein were added as cold inhibitors.

A Sips plot of the same data (not shown) gives a homogeneity index of 0.992 where 1.0 would indicate a totally homogeneous population. Using the same methodology we obtained a K_a of 2.20×10^5 M^{-1} for the parental S107 molecule. The difference between the parent and variant K_a is certainly within the error of the method, and we conclude that they have the same affinity for the hapten. Because S_1U_9 protein has

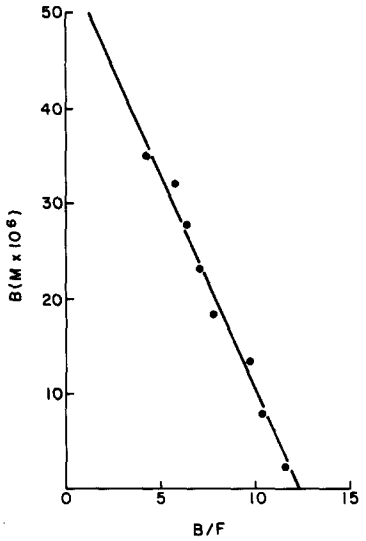

FIG. 8. Equilibrium dialysis of purified S_1U_9 protein. Equilibrium dialysis was done by the steady-state, flow dialysis method described in Materials and Methods. The data are plotted in the form of a Scatchard analysis.

unaltered affinity for the hapten PC as compared with S107 protein, the difference in their binding to PC-sepharose columns most likely reflects a difference in avidity—partially reduced S107 is bivalent, whereas S_1U_9 is monovalent. With TEPC 15 protein (which has a sequence identical to that of S107 protein), a similar binding difference between the bivalent H_2L_2 monomer and monovalent Fab fragment has been observed (42).

It is more difficult to compare the affinities of W3082 and its T7 variant for levan. T7 was neither bound to, nor retarded in elution from, a levan-Sepharose column. However, when the T7 molecules were made polyvalent by the sequential addition of rabbit anti-IgA and sheep anti-rabbit immunoglobulin, binding of some of the molecules was detected (data not shown). This suggests that T7 retains at least some capacity to bind antigen.

Discussion

The results presented above demonstrate that cultured mouse myeloma cells producing normal IgA antibody can spontaneously give rise to variants that produce IgA half-molecules containing H chains lacking all or part of their C_H3 domain. These variant proteins closely resemble those produced by a series of BALB/c tumors first described in 1964 by Potter and Kuff (4). It is not possible at this time to estimate the exact rate at which such variants arise. The two variants described in this paper were identified during experiments designed to detect other phenotypes that arise with frequencies of 0.1–1%. Since only one half-molecule producer apiece has been isolated from S107 and W3082 so far, their frequency could be quite low. A large number of somatic variants with constant region deletions or premature terminations have been isolated from a variety of IgG- and IgM-producing mouse myeloma and hybridoma cell lines and their frequency varies enormously (9, 43–45).

Both S_1U_9 and T7 have the unusual covalent structure of 47A and the other half-

molecule-producing tumors. Based on a comparison of the amino acid sequences of 47A and MOPC 511 (511), a PC-binding IgA myeloma, Robinson and Appella (29) concluded that the formation of inter-H-L disulfide bonds and absence of inter-H chain disulfide bonds were due solely to the deletion of the C_H3 domain. Assuming that this is also true of S_1U_9, it would mean that even a partial deletion of the C-terminal domain can have a long-range affect on the folding and covalent structure of the hinge and C_H1 domain. Since x-ray crystallography has shown, at least for a human Fc fragment (46), that there is essentially no C_H2-C_H2 contact though there is significant C_H3-C_H3 contact, the half-molecule phenotype may be due to a loss of C_H3-C_H3 stabilizing interactions that might be necessary in an IgA molecule to form inter-H chain disulfide bonds. The resulting lack of interchain bonds might make cysteines available to participate in the formation of an abnormal H-L bond. Such a finding indicates that the domains can affect each other, as has been postulated in the initiation of effector functions such as complement fixation and Fc receptor binding (47). On the other hand, the formation of the disulfide bond between the H and L chains did not affect the serology or affinity of the S_1U_9 variable region.

Since the proteins of S107 and its variant S_1U_9 both bind the hapten PC with the same affinity and both bear the T-15 idiotype, they almost certainly have the same H chain variable region. As T7 still reacts with levan, it is probable that W3082 and its variant also express the same variable region. The identity of constant genes between parents and variants presents a potentially more difficult question. Although unlikely, it is possible that the H chain deletions are not due to somatic mutation but rather to a class switch to a rarely expressed alpha subclass or even to a pseudogene. Arguing against such a subclass switch or germ-line pseudogene is the difference in size between the S_1U_9 and T7 H chains and the restriction map identity of the parents and variants. Furthermore, although S107 and W3082 both contain at least three alpha genes (our unpublished results), none is in the germ-line configuration. Thus, a switch would have to involve rearrangement to an already rearranged gene, an event which has not been observed.

On the basis of the 47A and 511 sequence comparison and the finding that the mRNA coding for 47A was of normal size, it was proposed that a single base has been deleted from the codon for valine 336, resulting in the generation of a termination codon (29, 48). From the apparent molecular weights of the Tm-treated samples, the S_1U_9 H chain lacks ~11,000 daltons or 95 amino acids, whereas while the T7 H chain has lost 13,000 daltons or 115 amino acids. Both S_1U_9 and T7 lack C_H3 antigenic determinants and do not appear to have the parental C-terminal tyrosine. The size difference between the variant H chains is not the result of proteolysis or glycosylation differences because similarly short H chains were synthesized in a cell-free system and by Tm-treated cells. The cytoplasmic mRNA from both variants were the same size as parental messenger. These findings suggest that both variants also arise through a premature termination, either due to a nonsense or frameshift mutation. However, detailed sequence analysis will be required to prove such a mechanism. It is worth noting that two of the four IgG variants described by Adetugbo and his colleagues (43, 49) arose through premature terminations and that the M311 variant of MPC 11 probably has a similar origin (50).

The appearance of a number of IgA-producing mouse myeloma tumors with similar phenotypes might suggest a hot spot (29). It is interesting that 47A and T7 have the

same mobility on SDS gels. However, S_1U_9 has a smaller deletion, indicating that mutations can occur within the C_H3 domain as well as at the C_H2-C_H3 boundary. Furthermore, rather than reflecting a localized mutagenic process, the occurrence of relative hot spots for deletion may simply be the result of areas of nucleotide sequence that are easily altered into termination signals, or may reflect unrealized biases in the selection techniques utilized or restrictions arising from the need to maintain cell viability.

Summary

Whereas mouse myelomas that secrete IgA half-molecules have been shown to arise in vivo, their origin has not been definitely established. We show that somatic variants secreting phenotypically similar molecules can arise directly from the normal IgA-secreting myelomas S107 and W3082. In addition to being improperly assembled, the variant proteins have distinct carboxy-terminal deletions and an aberrant heavy-light chain disulfide bond. For at least one of the variants, variable region serology and affinity for hapten are both unaffected by these changes. Southern and Northern blot analyses indicate normal size DNA restriction fragments and mRNA, suggesting premature termination as the mechanism of deletion. These results are discussed in relation to possible mutational hot spots and long-range interdomain interactions.

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