MUTANT MONOCLONAL ANTIBODIES WITH ALTERATIONS IN BIOLOGICAL FUNCTIONS*

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The hybridoma technology (1) has made it possible to generate immunological reagents that are in many ways superior to antisera obtained through conventional immunization (2). Cell lines secreting monoclonal antibodies provide large amounts of well-defined homogeneous reagents that can be replenished indefinitely. Whenever a battery of different monoclonals is available for an antigen, it is possible to select the ones best suited for particular assays (3). Unfortunately, some of the properties of antibodies complicate their use in research and might be a barrier to their fullest use in diagnosis and therapy. For example, antibody binding to complement and to Fc receptors on lymphocytes and phagocytic cells forms the basis of many immunoassays but is a constant problem in immunofluorescence experiments and might seriously hamper attempts to use monoclonals in vivo. The approach to overcoming such problems with conventional antibodies has usually involved proteolytically removing the Fc portion of the molecule. Because monoclonal antibodies are produced by cells in culture, we thought it might be possible to identify mutant subclones producing structurally altered antibodies using techniques previously developed to identify somatic mutants in mouse myeloma cells (4, 5). One purpose in these studies was to determine whether similar techniques could identify mutant monoclonal antibodies with defects in those properties that limit the usefulness of the intact molecule.

In addition to their benefits as serological reagents, monoclonal antibodies offer advantages over myeloma proteins for studying immunoglobulin synthesis and the structural basis of antibody effector functions. Because most myeloma proteins do not bind known antigens, these immunoglobulins or fragments of them have been artificially aggregated to simulate antigen-antibody complexes that fix complement or bind to Fc receptors (6). Now that it is possible to generate monoclonal antibodies of all classes that react with convenient antigens, homogeneous immunoglobulins can be purified by antigen affinity chromatography and activated physiologically by forming immune complexes. Although mutant human (7) and mouse myeloma (8) proteins have been useful in studying antibody functions, structurally altered monoclonal antibodies should provide additional information on the structural basis of effector functions.

In this paper, we show that a hybridoma producing an IgG2b antibody that binds p-azophenylarsonate (Ar)¹ generates somatic mutants producing antibody molecules

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¹ Abbreviations used in this paper: Ar, p-azophenylarsonate; BSA, bovine serum albumin; CNBr, cyanogen bromide; DME, Dulbecco's modified eagle's medium; H-chain, immunoglobulin heavy chain; L-chain, immunoglobulin light chain; KLH, keyhole limpet hemocyanin; Mab, monoclonal antibody; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes.
with deletions in various parts of the Fc region. These mutants arise frequently after mutagenesis. The mutant immunoglobulin molecules exhibit aberrant assembly patterns and have lost the ability to carry out certain effector functions. In addition, we report generating a battery of rat monoclonal antibodies that react with different domains of the IgG2b constant region and are useful in locating the deletions in the mutant monoclonal antibodies.

Materials and Methods

Cell Lines. All myeloma and hybridoma cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum (FCS) in a humidified atmosphere of 5–10% CO2. The M3.11 and B50-10.1 mutant lines from the MPC-11 myeloma have been reported previously (9–12). B50-10.1 was kindly provided by Dr. Sherie Morrison of Columbia University, New York. The methods for producing hybrid cell lines have been reported in detail (13). The X63-Ag8.653 myeloma cell line that synthesizes no immunoglobulin chains (14) was used to produce the hybrids reported here. The Ar13.4 anti-azophenylarsonate, IgG2bK hybridoma was obtained by fusing splenic lymphocytes from a BALB/c mouse immunized with p-azophenylarsonate attached to keyhole limpet hemocyanin (Ar:KLH) by the method of Nisonoff (15). 250 μg of Ar:KLH in complete Freund's adjuvant was injected intraperitoneally, followed by a boost 4 wk later of 250 μg Ar:KLH in saline intravenously. Immunizations and fusions generating hybridomas secreting rat monoclonal anti-IgG2b antibodies have been reported (16). Rat Mab 187, 116, 180, and 168 were derived from a Sprague-Dawley rat; 31, 178 and 196 were derived from a Lewis rat. All cell lines have been subcloned to insure clonality.

Rabbit Antisera. Rabbits were immunized (17) with purified MPC-11 myeloma protein (18). Antisera specific for restricted parts of the γ2b heavy chain were obtained by passing the rabbit antisera over a column to which purified immunoglobulin had been attached by activating Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) with cyanogen bromide (CNBr) (19). Antiserum defined as “specific” for the IgG2b subclass was extensively absorbed with an affinity-purified IgG2a anti-Ar monoclonal antibody. Antiserum specific for the CH3 domain was absorbed with purified M3.11 protein. Both antisera were considered fully absorbed when they could not hemagglutinate sheep erythrocytes (SRBC) coated with the absorbing protein.

Selecting H-Chain Mutants. The method of selecting heavy chain (H-chain) mutants from Ar13.4 was adapted from that used previously to select variants from the MPC-11 cell line (4, 5). Cells were mutagenized for 24 h with 2.5 μg/ml of the acridine half-mustard ICR-191 (20). After 48 h, ~30% of the original number of cells remained viable, as judged by trypan blue exclusion. The cells were then cloned in soft agarose (500–1,000 clones/60-mm petri dish) containing 100 μl of antisera specific for a restricted segment of the constant region of IgG2b. As the clones reached the 8–32 cell stage, a visible antigen-antibody precipitate formed around clones secreting immunoglobulin still reactive with the antiserum. Clones without any precipitate were marked as putative variants. Clones were marked by inserting three pieces of sterile costume glitter into the agarose forming an asymmetric triangle. Under microscopic visualization, marks were placed above the glitter on the dish cover using a fine felt-tipped marker with water-insoluble ink. Unstained clones were also marked on the dish cover and relocated by reorienting the dish cover using the glitter. After unstained clones were identified, agarose containing an unabsorbed antiserum (50 μl/dish) reactive with the entire IgG2b constant region was layered over the clones. This must be done before the clones reach the 100–200 cell stage. Clones that did not react with the first antiserum but did react with the second were likely to be producing mutant immunoglobulins. Although this double overlay scheme does not completely eliminate picking false positive and false negative clones and requires some technical finesse, it has proved superior to attempts at brute force recovery and screening of all clones unreactive with the restricted antiserum.

Proteins. Production of ascites (21) and purification of myeloma proteins (18) have been described. Proteins from the Ar13.4 parent and mutant hybridomas were purified by affinity chromatography from ascites fluid or occasionally from culture supernatants using Sepharose
4B to which a gly-tyr dipeptide (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) had been attached with CNBr and subsequently conjugated with p-azophenylarsonate. Adsorption of antibody to the column was carried out overnight at 4°C in the presence of 0.5 M NaCl and 0.5% Nonidet P-40 (Shell Oil Co., Houston, TX). The Sepharose was washed thoroughly with Tris-saline (0.15 M NaCl; 25 mM Tris, pH 7.5). Antibodies were eluted with 50 mM p-azophenylarsonate in Tris-saline, dialyzed exhaustively to remove hapten, concentrated to ~10 mg/ml using an Amicon PM-10 membrane (Amicon Corp., Lexington, MA), made 10 mM with NaN3, and stored at 4°C. Some mutant proteins are secreted in low amounts and appear to have a short half-life in vivo, making purification of more than a few milligrams laborious.

Radioimmunoassay. Methods for endogenously labeling monoclonal antibodies by growing hybridomas in 35S (or 14C) amino acids have been described (16). After dialysis to remove the unincorporated label, the culture supernatants can be used in radioimmunoassays without further purification. Radioimmunoassays used in these experiments are patterned after those of Pierce and Klinman (22). The wells of round-bottomed polyvinyl chloride microtiter dishes (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) were coated with antigen (either Ar:KLH or purified immunoglobulin) by adsorption overnight at 4°C of 100 µl of a 10 µg/ml solution of antigen diluted in PBS (0.15 M NaCl; 20 mM Na PO4, pH 6.8). Wells were washed thoroughly with PBS and filled with a solution of 1% bovine serum albumin (BSA-Pentex) in PBS for 2 h at 25°C to block unreacted sites. These antigen-coated wells can be used in sandwich assays to detect antibody to the antigen or in inhibition assays to examine the specificity of a monoclonal (see figure legends for details). For either assay, subsequent antibody-binding incubations were carried out overnight at 4°C. Antibodies were diluted with 1% BSA in PBS. After being washed with the PBS-BSA, wells were cut out and counted in a Beckman scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Hemagglutination Assays. Two types of hemagglutination assays were used in these experiments and have been described (16). The first, designated "facilitated hemagglutination," is designed to quantitate roughly the amount of anti-azophenylarsonate antibody (Ar13.4 or mutant) in solution. Because the Ar13.4 antibody and some other monoclonals are weak agglutinins and many mutants secrete HL half-molecules incapable of cross-linking erythrocytes, a rabbit anti-mouse immunoglobulin serum was added to promote cross-linking via the monoclonal antibody bound to Ar:SRBC (see below).

The second assay is an inhibition of hemagglutination assay designed to determine whether the "inhibiting" protein contains determinants recognized by an antibody (Mab or conventional antiserum). If a protein is recognized, it will compete with the antigen on the SRBC and prevent agglutination. This assay is modified from Evans et al. (23). The antigen-SRBC used for titering anti-Ar antibodies was conjugated with p-azophenylarsonate (15) using 20 µmoles p-azophenylarsonate per ml of packed SRBC (Ar:SRBC). The cells were fixed with glutaraldehyde (13) and stored at 4°C in PBS with 10 mM NaN3 for up to several weeks. Antigen-SRBC used in inhibition of hemagglutination assays contained IgG2b fixed onto the SRBC with glutaraldehyde and stored as above. The IgG2b protein was from an anti-SRBC hybridoma that does not agglutinate SRBC, even at very high concentrations. This permitted a large amount of IgG2b to be fixed to the SRBC, improving the sensitivity of the assay. All solutions were diluted into PBS containing 1% (wt/vol) BSA.

Polyacrylamide Gel Analysis of Biosynthetically Labeled Immunoglobulin. Conditions for labeling cells were similar to those previously described (24). 25 µCi of [35S]methionine (New England Nuclear, Boston, MA) was used per 106 viable cells. Incorporation times for intracellular material was 2 h and for secreted material 5 h, unless noted otherwise. Labeled immunoglobulins were precipitated either by indirect immunoprecipitation (25) or by adsorption onto 50 µl of haptenated Sepharose 4B. Because both methods gave identical results with all mutants, the latter method was generally used for convenience. After washing, immune precipitates or Sepharose were boiled in 2% SDS in electrophoresis sample buffer (26) for 2 min. Reduction was carried out with 10 mM dithiothreitol (Sigma Chemical Co., Saint-Louis, MO) for 2 h at 37°C, followed by alkylation for 1 h at 37°C with a twofold molar excess of iodoacetamide (Sigma Chemical Co.) recrystallized from absolute ethanol. See figure legends for details of electrophoresis conditions. When labeling was done in the presence of tunicamycin, cells were
pretreated for 2 h with 2.5 μg/ml of tunicamycin (27) before the isotope was added.

Sodium dodecyl sulfate (SDS) acrylamide gels were formed either in phosphate or Tris-glycine buffers as described by Maizel (26). Gels were fixed and stained, treated with Autofluor (National Diagnostic, Inc., Advanced Applications Institute Inc., Somerville, NJ), and analyzed by autoradiography using Kodak SB-5 film (Eastman Kodak Co., Rochester, NY).

Reagents. Protein A from Staphylococcus aureus (protein A) was obtained from Sigma Chemical Co. Commercial antisera for typing mouse immunoglobulin class were obtained from Meloy Laboratories Inc., Springfield, VA, and for rat immunoglobulins from Miles Laboratories Inc., Research Products Div., Elkhart, IN. KLH was obtained from Sigma Chemical Co. ICR-191 was kindly provided by Dr. Howard Creech of the Institute for Cancer Research in Fox Chase, PA, and tunicamycin was provided by Dr. Robert Hamill, Eli Lilly and Co.

Results

Isolation of Heavy Chain Mutants. The hybridoma Ar13.4 produces IgG2bK antibody that binds the hapten p-azophenylarsonate. Subclones of this hybridoma producing mutant immunoglobulin molecules were identified using the same approach used to obtain constant region mutants from the MPC-11 IgG2bK-producing mouse myeloma cell line. Cells were mutagenized with the acridine half-mustard ICR 191 and cloned in soft agarose containing rabbit antibody that was specific either for the CH3 domain or for the subclass-specific antigenic determinants of the IgG2b constant region. Clones secreting intact immunoglobulin molecules became surrounded by a visible antigen-antibody precipitate. Approximately 5% of the clones were not surrounded by such a precipitate. In preliminary experiments (28), some of these presumptive mutants were recovered from the agarose and further analyzed. They were found either (a) to secrete less immunoglobulin than the parental cells, (b) to have lost the ability to synthesize detectable amounts of heavy chains (H-chains), or (c) to be secreting immunoglobulin molecules that had structural changes resulting in a loss of reactivity with the selecting antiserum. Loss of H-chain production was especially common, presumably due to the segregation of chromosomes that occurs during the early propagation of hybridomas.

To eliminate the need to recover and characterize low producers or H-chain nonproducers, a double antibody overlay assay was used. Clones that were not surrounded by a visible antigen-antibody precipitate with the selecting antibody were marked. The dishes were then overlaid with agarose-containing antibody reactive with the whole IgG2b constant region. Only clones that did not react with the first antiserum and did react with the second were recovered. The medium from these clones was assayed for Ar-binding antibody by facilitated hemagglutination of Ar:SRBC. Positive clones were further examined for their reactivity with the selecting antiserum and with monoclonal anti-IgG2b antibodies by inhibition of hemagglutination. Eight mutants that clearly differed from the parent were further studied serologically and by polyacrylamide gel electrophoresis.

Phenotypes of Mutant Immunoglobulins. Parental and mutant cells were incubated with [35S]methionine, and the secreted immunoglobulins were analyzed by SDS-polyacrylamide gel electrophoresis. As can be seen in Fig. 1 (channel A), most of the antibody secreted by the parental Ar13.4 hybridoma was assembled into covalently linked H2L2 molecules. The bands that migrate slightly faster than H2L2 may represent proteins that have not been completely glycosylated. Mutants ArM17 and ArM19 (Fig. 1, channels B and C) were isolated in a single experiment and could be progeny of the same mutant cell. Mutants ArM16, ArM18, and ArM20 (channels F-
FIG. 1. SDS-polyacrylamide gel electrophoresis of secreted Ar13.4 and mutant immunoglobulins. Parent Ar13.4 (channel A) and mutant immunoglobulins (ArM17:B; ArM19:C; ArM1:D; ArM2:E; ArM16:F; ArM18:G; ArM20:H; and ArM17:I) were biosynthetically labeled by incubating 1.5 × 10^6 cells in 1.5 ml of medium containing 50 Ci [35S]methionine for 24 h. After removing cells by centrifugation, the labeled immunoglobulins were precipitated with haptenated Sepharose. Immunoglobulins were analyzed on a 5% polyacrylamide gel in the phosphate buffer system of Maizel (26).

FIG. 2. SDS-polyacrylamide gel electrophoresis of cytoplasmic and secreted immunoglobulin. Parent Ar13.4 (channels A) and mutant cell lines (ArM17:channels B; ArM1:channels C; ArM2:channels D; ArM7:channels E; ArM16:channels F) were biosynthetically labeled in [35S]-methionine as described. Anti-Ar antibody was precipitated on haptenated Sepharose and eluted by boiling in SDS. C represents cytoplasmic immunoglobulin and S represents secreted protein. Aliquots of each sample were reduced and alkylated and electrophoresed in the Tris-glycine SDS buffer system (26) through a 10% polyacrylamide gel with a 3% stacking gel. Because of variable labeling efficiencies between cell lines, some channels represent longer exposures.

H) were also from a single experiment, but ArM16 and ArM20 differ serologically (see below). ArM17 and ArM19 (channels B and C) have H2L2 molecules that co-migrate with the parent but differ in that small amounts of HL molecules are secreted. The remaining mutants (channels D–I) are dramatically different from the parent. Because the L-chains of all of these mutants have the same mobility as the parent on SDS gels, whereas, their H-chains are smaller than those of the parent (Fig. 2), these changes in the covalent assembly and size of the secreted proteins are almost certainly due to changes in the structure of their heavy chains.

The mutants with large changes in molecular weight secrete predominantly molecules that are incompletely covalently assembled (Fig. 1, channels D–I). The ratio of H2L2 to HL molecules varies over a broad range. Mutants ArM16, ArM18, and ArM20 (channels F, G, and H) secrete barely detectable amounts of covalently assembled H2L2.

The noncovalent assembly of the secreted immunoglobulin of representative mutants was examined by molecular-seiving in nondenaturing buffers (Fig. 3, Table I). The polymers secreted by the cells do not always correlate with the covalent assembly patterns illustrated in Fig. 1. For example, ArM20 does not form significant amounts
FIG. 3. Gel filtration profiles of Ar13.4 and representative mutants. Secreted parent and mutant immunoglobulins biosynthetically labeled as for Fig. 1 were purified by affinity chromatography on haptenated Sepharose 4B. Approximately $3 \times 10^6$ cpm of each purified antibody were mixed with the indicated markers (blue dextran, bovine serum albumin, ovalbumin, carbonic anhydrase, and myoglobin) and chromatographed through a $1.5 \times 170$-cm AcA44 (LKB) column at a linear flow rate of from 3–8 cm/h in Tris-saline. The OD$280$ of each fraction was determined to locate peaks of the marker proteins, and aliquots of each fraction were counted. Because of differences in flow rates, the markers do not chromatograph identically from run to run. The profiles have been aligned with respect to the BSA marker, which elutes between H$_2$L$_2$ and HL molecules.

FIG. 4. The effect of tunicamycin on the migration of ArM1. Parent Ar13.4 (channels A) and ArM1 (channels B) were biosynthetically labeled with [35S]methionine in the presence (+) or absence (−) of tunicamycin. Cytoplasmic immunoglobulin was precipitated with haptenated Sepharose, reduced and alkylated, and analyzed as in Fig. 2.

Fig. 3 shows reduced intracellular and secreted H- and L-chains of representative mutants. The H-chains synthesized by the parent (channel A) and ArM17 (channel B) are very similar. The double band seen in the secreted $\gamma_2b$ heavy chain has been attributed to heterogeneity of glycosylation (29). Multiple distinct H-chain bands are seen in the other mutants. The positions of some of these bands shift strikingly after secretion. With some mutants, the multiple cytoplasmic bands are eliminated by pretreating the cells with tunicamycin to prevent N-linked glycosylation (27). This is illustrated for ArM1 in Fig. 4. The nonglycosylated parental H-chain migrates slightly more rapidly than the glycosylated chain (channel A). The double H-chain bands of ArM1 (channel B) are reduced to a single band in the presence of tunicamycin.

of inter H-chain disulfide bonds (Fig. 1, channel H), yet is secreted as an H$_2$L$_2$ molecule in which the H-chains are held together by noncovalent interactions, whereas most of the secreted ArM2 is in the form of HL half-molecules (Fig. 2). The noncovalent assembly of mutants representing each of the phenotypes is summarized in Table I.

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Table I

|       | H₂L₂ | HL |
|-------|------|----|
| Ar13.4| 100  | 0  |
| ArM17 | 100  | 0  |
| ArM1  | 27   | 73 |
| ArM2  | 18   | 82 |
| ArM16 | 100  | 0  |
| ArM20 | 100  | 0  |
| ArM7  | 18   | 82 |

Biosynthetically labeled immunoglobulins from parent and mutant cell lines were subjected to gel filtration with AcA44, as described for Fig. 2. The percentage of counts in each peak are listed. For mutant ArM2, the counts in the small peak between H₂L₂ and HL (that might correspond to the middle band in channel E, Fig. 1) are disregarded.

Fig. 5. The effect of tunicamycin on migration ArM7. Parent Ar13.4 (channels A and B) and ArM7 (channels C and D) were labeled with (+) and without (−) tunicamycin as in Fig. 4. After labeling, samples were divided, and immunoglobulins were precipitated either by indirect immunoprecipitation (channels A and C) or hapten-Sepharose (channels B and D). Reduced and alkylated samples were electrophoresed and autoradiographed as in Fig. 2.

Fig. 6. Comparison of ArM1 and M3.11. Immunoglobulins (intracellular) were biosynthetically labeled, immunoprecipitated, reduced, and electrophoresed as described for Fig. 2. The channels represent the following: A: Ar13.4 parent; B: MPC-11; C: ArM1; D: M3.11.

For other mutants, tunicamycin treatment did not eliminate the complexity of the electrophoretic pattern. The slowly migrating band in the cytoplasm of ArM2 (Fig. 3, channel D [c]) remained after tunicamycin treatment. With mutant ArM7, tunicamycin changed the pattern of heavy chain bands, but at least two and probably three bands were detected in the presence of the inhibitor (Fig. 5), suggesting that this
mutant synthesizes more than one heavy chain polypeptide. Northern blots of ArM7 RNA also reveal three mRNA bands of similar size that hybridize to a $\gamma_2b$ probe (Sau-Ping Kwan, unpublished results). The multiple heavy chain bands in Fig. 5 were precipitated by both antisera and antigen, indicating that all forms of the H-chains participate in the interaction with antigen. This is true for all of the mutants (data not shown).

Some of the mutants illustrated above are remarkably similar to those that have been reported for mutants of the MPC-11 cell line (4). In Fig. 6, the electrophoretic mobility of the ArM1 heavy chain (channels C) is compared with that of M3.11 (channel D), an extensively studied mutant of MPC-11 (9, 10, 24). The double heavy chain band, its conversion to one band by tunicamycin (Fig. 4), and the noncovalent assembly of M3.11 and ArM1 (Table I and Fig. 3) are similar. There is a difference in the migration of the nonglycosylated form of the heavy chains of these two mutants that may be due to a small difference in their size.

Characterization of Rat Monoclonal Anti-IgG$_{2b}$ Antibodies. To serologically map the defects in the mutants of Ar13.4, we generated a battery of rat monoclonal antibodies that react with the mouse IgG$_{2b}$ immunoglobulin. Rats were immunized with the MPC-11 myeloma protein, and their spleen cells were fused to a nonproducing mouse myeloma cell line. 15 cloned, stable rat hybridomas that made high titer antibody against mouse IgG$_{2b}$ were examined for their reactivity with various mouse myeloma proteins and for their ability to cross-inhibit each other in competitive binding assays. Three of these have been reported previously (16): 187, which reacts with the constant region of mouse K L-chains; 116, which recognizes all of the IgG subclasses equally; and 168, which reacts preferentially with IgG$_{2b}$. 116 and 168 are further characterized below along with additional rat monoclonal antibodies that bind different antigenic determinants. These were identified by their distinctive reactivities with the subclasses of mouse IgG and their inability to inhibit each other’s binding.

The availability of myeloma mutants derived from MPC-11, whose structures had been extensively studied, made it possible to localize the determinants recognized by each anti-IgG$_{2b}$ monoclonal. Two mutants with large deletions, one in C$_{H}$1 (B50-10.1) (11-13) and the other encompassing all of C$_{H}$3 (M3.11) (10), divided the IgG$_{2b}$ constant region into three sections roughly equivalent to the three domains. A determinant could be assigned to one of these segments, depending on whether the antibody bound to either or both of these mutants. The parent Ar13.4 protein was bound to polyvinyl plates, and the ability of the M3.11 and B50-10.1 mutants to inhibit binding of each biosynthetically labeled anti-IgG$_{2b}$ monoclonal was determined (Table II). This approach depends upon the assumption that, when a monoclonal does not react with a deletion mutant, its determinant is contained within the deletion. It is possible, of course, that a deletion (or any mutation) could induce a conformational change in the protein, destroying the tertiary structure recognized by an antibody, even though the determinant’s primary sequence remains. Because the domains of antibody molecules fold relatively independently of one another, we hoped the choice of the B50-10.1 and M3.11 myeloma mutants, whose deletions are restricted to a single domain, would avoid this problem. Another potential source of error is that a monoclonal could bind two similar determinants in the homologous regions of different domains. Although we believe that such complications are unlikely, it should be emphasized that we consider these assignments tentative, pending a chemical
Table II
Reactivity of Rat Anti-IgG2a Monoclonals with MPC-11 Mutants

| Mab   | Percent inhibition of binding by |
|-------|---------------------------------|
|       | B50-10.1 | M3.11 |
|       | (-C\textsubscript{H}1) | (-C\textsubscript{H}3) |
| 116   | 8        | 94    |
| 180   | 98       | 90    |
| 196   | 93       | 9     |
| 178   | 99       | 0     |
| 31    | 91       | 0     |
| 168   | 100      | 0     |

Ar13.4 was adsorbed to polyvinyl chloride microtiter wells, and [\textsuperscript{35}S]biosynthetically labeled anti-IgG\textsubscript{2a} was added in the presence of each mutant immunoglobulin or no inhibitor. Mutant immunoglobulins were concentrated 20-fold by 50% NaSO\textsubscript{4} precipitation from culture supernatants, and controls consisted of unused growth medium concentrated in the same way. Results are the means of triplicate determinations expressed as percent inhibition as control cpm (no inhibitor control) - experimental cpm (with inhibitor)/control cpm X 100. Background counts bound to wells not coated with antigen have been subtracted.

Table III
Protein A Competes with Monoclonal 196 for Binding to Mouse IgG2a

| Mab   | cpm bound in absence of protein A | cpm bound in presence of protein A |
|-------|----------------------------------|-----------------------------------|
| 187   | 26,224                           | 29,567 (113%)                     |
| 196   | 14,272                           | 1,292 (11%)                       |
| 178   | 15,777                           | 11,495 (71%)                      |
| 31    | 10,967                           | 12,297 (112%)                     |
| 168   | 2,973                            | 2,649 (86%)                       |

Ar13.4 (1 \mu g/well) was bound to polyvinyl chloride microtiter wells via Ar:KLH antigen and probed with the indicated \textsuperscript{35}S-labeled monoclonal anti-IgG\textsubscript{2a} antibodies in the presence and absence of protein A (10 \mu g/well; approximately 30-fold molar excess over IgG\textsubscript{2a}). Diluent for this assay was Tris-saline, pH 8.0, with 1% BSA because protein A requires alkaline pH to bind to IgG\textsubscript{2a} (32). Counts represent mean of triplicate determinations.

analysis of additional mutants studied with these antibodies.

B50-10.1 protein does not inhibit the binding of rat monoclonal 116, suggesting that 116 recognizes an antigenic determinant on the C\textsubscript{H}1 domain (Table II). 180's binding is inhibited by both mutants, suggesting that it binds within the structural segment the two mutants share. This segment includes the C\textsubscript{H}2 domain, hinge, and possibly some C-terminal sequences in C\textsubscript{H}1. Although these two mutants also share the same variable region, all of the rat monoclonals were shown to react with constant rather than variable region determinants by methods described previously (16). Rat monoclonals 196, 178, 31, and 168 are all inhibited by B50-10.1 and not by M3.11. It was concluded that they recognize determinants on the C\textsubscript{H}3 domain, although, as discussed above, we cannot rule out reactivity with a determinant on the C\textsubscript{H}2 domain that changed its conformation as a result of the C\textsubscript{H}3 deletion.

We attempted to further define the site of reactivity of these putative anti-C\textsubscript{H}3
Table IV

Reactivity of Rat Monoclonal Anti-IgG2b with Ar13.4 and Mutant Immunoglobulin

| Parent and mutant immunoglobulins from culture supernatants (100 μl) were bound to polyvinyl chloride microtiter wells via Ar:KLH antigen, after which wells were thoroughly washed. 35S-labeled rat monoclonals were added in excess, and the counts bound to each well were determined. Each assay was done in triplicate. The results are expressed for each monoclonal as the percentage of counts bound to the Ar13.4 parent immunoglobulin when it was at a concentration (30 μg/ml) that saturated all Ar:KLH sites. For some monoclonals (ArM17 and ArM7), less antibody is secreted by the mutant, and probably not all antigen sites are saturated. The approximate H-chain molecular weights listed for the mutants were determined for the most rapidly migrating H-chain bands in Fig. 2. The location of the determinants recognized by each Mab is indicated in parentheses.

Monoclonals by determining whether protein A competes with any of them for binding to IgG2b. Protein A has been shown by x-ray crystallography to bind to the Fc portion of human IgG at the CH2-CH3 junction (30). As can be seen in Table III, when IgG2b is pretreated with protein A, only 196 is prevented from binding. This would suggest that the 196 determinant is located in the N-terminal part of CH3 (or C-terminal CH2). Despite the fact that protein A has a molecular weight of 45,000 and induces conformational changes in the Fc region (31), it does not inhibit the binding of 178, 31, and 168.

Localizing the Deletions in the Mutant Monoclonal Antibodies. The rat anti-IgG2b monoclonal antibodies were used to determine roughly what part of the constant region had been deleted from the mutants derived from Ar13.4. Ar:KLH was adsorbed to polyvinyl plates. Culture supernatants from the mutants were then incubated with the antigen-coated plates. After washing, biosynthetically labeled (35S) rat monoclonal antibody was added to each well. Because different rat monoclonal antibodies were labeled to different specific activities, the binding to a mutant for each monoclonal was calculated as the percent of counts that bound to saturating amounts of Ar13.4 parent (Table IV). Because the mutants and parent are polymerized to different degrees and may bind to the antigen-coated plates with different avidities, we consider this a qualitative assay. In each case, however, the reactivity with one or more monoclonals (vertical column, Table IV) reveals that adequate amounts of the mutant protein bound to the plate. ArM1, ArM2, and ArM7 resemble M3.11 (Table II) in that they do not react with any of the four rat monoclonals assigned to the CH3 domain. Based on their molecular weights (Table IV), these mutants have undergone deletions equal to approximately one domain. Because preliminary studies (Sau-Ping Kwan, unpublished observations) indicate the messenger RNA that hybridize to a γ2b probe are approximately normal in size, we would predict that these three mutants resemble M3.11 in having undergone premature terminations and are missing all or
most of their C\(_\text{H}3\) domains (10). ArM16 and ArM20 do not react with 180 and 196, which we have assigned to the C\(_\text{H}2\) and C\(_\text{H}3\) domains, respectively. They do react weakly (5–6 times background) with 178 and strongly with 31, both of which recognize C\(_\text{H}3\) determinants. Although the gel pattern in Fig. 1 suggests that ArM16 and ArM20 are similar, 168 reproducibly distinguishes between them (Table IV). Furthermore, the \(\gamma_b\) mRNA present in these two mutants differ in size (Sau-Ping Kwan, unpublished observations). Because ArM16 and ArM20 are missing approximately a domain equivalent, we would predict that they have undergone an internal deletion involving all or most of C\(_\text{H}2\), due either to deletion at the DNA level or a splicing defect. The loss of reactivity with 196 and greatly decreased reactivity with 178 could be due to loss of part of C\(_\text{H}3\) or conformational changes resulting from the abnormal covalent joining of the hinge or part of the N-terminal C\(_\text{H}2\) to the C\(_\text{H}3\) domain.

ArM17 is grossly abnormal serologically (Table IV), but its size is very similar to the parent, and its assembly pattern (Fig. 1) is only slightly aberrant. Its lesion also appears to include C\(_\text{H}3\) sequences because it lacks the determinants seen by monoclonals 196, 178, and 31. The increased reactivity of 168 with ArM17 is reproducible. Similar phenomena here have been seen in other systems analyzed with monoclonals, where determinants close to sequence changes sometimes show increased reactivity (33). The ArM17 protein does not react in agar diffusion with rabbit antibody specific for the \(\gamma_b\) C\(_\text{H}3\) domain and reacts only weakly with IgG\(_{2a}\) and IgG\(_{2b}\)-specific antisera. Although we have been unable to detect a reaction between ArM17- and IgG\(_{2a}\)-specific antisera, it might represent a subclass switch mutant producing a hybrid IgG\(_{2b}a_2\) molecule (4). In our experience with similar myeloma mutants, these proteins have often proved difficult to classify with standard reagents. ArM17 will require extensive chemical characterization before we can determine the nature of its defect.

It is interesting that 196, the monoclonal whose binding is inhibited by protein A, does not react with any of the mutants. This could be because the conformation of the antigenic determinant recognized by 196 is readily changed by sequence changes in C\(_\text{H}2\) or C\(_\text{H}3\). For example, 196 might recognize an antigenic determinant formed by the interaction of C\(_\text{H}2\) and C\(_\text{H}3\) sequences. It is also possible that it recognizes sequences that are deleted from all of the mutants because they are the major antigenic determinants recognized by the rabbit antisera used to select these mutants.

**Effector Functions of Mutant Immunoglobulins.** We determined which of the effector functions exhibited by IgG\(_{2b}\) were retained by each of the hybridoma mutants for two reasons. First, although controversy exists, several reports have assigned specific functions to individual domains (6). Knowledge of activities of each mutant offers an independent test of the structures we inferred from their reactivity with the rat anti-IgG\(_{2b}\) monoclonals. Second, and more importantly, we might be in a position to further refine our knowledge of immunoglobulin effector functions by determining the structure of mutants with interesting properties. The antigen-binding activity of mutant monoclonal antibodies makes studying their effector functions not only very convenient but possibly more relevant to physiologic antibody functions than studies with chemically aggregated proteolytic fragments. We examined the ability of the Ar13.4 mutants to bind protein A, bind mouse macrophage Fc receptors, and mediate complement-dependent lysis of SRBC.

To study their interaction with protein A, parental and mutant proteins bound to polyvinyl plates via Ar:KLH were probed with either \(^{35}\)S-labeled anti-K light chain
MUTANT MONOCLONAL ANTIBODIES

Interaction of Protein A with Mutant Immunoglobulins

| Immunoglobulin | 187 Bound  | Protein A Bound |
|---------------|------------|-----------------|
|               | cpm        | cpm             |
| Ar13.4        | 9,244      | 8,313           |
| ArM17         | 4,390      | 46              |
| ArM1          | 10,683     | 45              |
| ArM2          | 6,250      | 57              |
| ArM7          | 5,254      | 47              |
| ArM20         | 10,119     | 68              |
| ArM16         | 9,785      | 88              |
| X63/Ag8.653   | 351        | 53              |

Parent and mutant immunoglobulins were bound to microtiter wells, as in Table IV, except using Tris-saline as in Table III. Excess 35S-labeled 187 (anti-k constant region) monoclonal or 125I-labeled protein A was added to each well, after which wells were washed and counted in a Beckman LS-9000. Because 187 is in excess, the amount of 35S counts reflects the relative amounts of anti-Ar antibody bound to each well. It is clear that adequate amounts of each mutant bind.

Protein A was iodinated by the glycouril method (35), using 10 µg of glycouril per 20 µg of protein A and 1 mCi of 125I (Amersham Corp., Arlington Heights, IL).

(Mab 187) to determine the relative amount of immunoglobulin in each well or 125I-labeled protein A (Table V). None of the mutants bound protein A. This result is consistent with the previous results that protein A interfered with the binding of monoclonal 196 to IgG2b and that 196 does not bind to any of these mutants.

To determine whether the mutant proteins would bind macrophage Fc receptors, parent and mutant proteins were incubated with Ar:SRBC. The antibody-sensitized SRBC were then washed and incubated with FC-1, a macrophage cell line (34) that had adhered to cover slips. After washing away the unattached SRBC, the coverslips were scored for rosettes (Table VI). Only the parent and ArM17 formed rosettes. Facilitated hemagglutination assays showed that approximately the same amount of each mutant bound to the SRBC. SRBC sensitized with sixfold less parent protein formed rosettes efficiently (Table VI).

IgG2b fixes complement by the classical pathway and mediates complement-dependent cytolysis, although less efficiently than IgM antibodies. C1q, the first component of complement, is reported to associate with the CH2 domain (6). We examined the ability of the parent and mutant proteins to lyse Ar:SRBC in the presence of guinea pig complement absorbed with protein A to remove guinea pig antibodies. ArM1, ArM2, ArM7, and ArM17 all lysed SRBC, though not always as efficiently as the parent. When ArM1 H2L2 and HL molecules were separated, the HL molecules could not detectably lyse the RBC, whereas the fully assembled molecules did. A control IgG1 anti-Ar hybridoma was also unable to lyse Ar:SRBC. ArM16, ArM18, and ArM20 were the only mutants that did not lyse the SRBC. This was true of ArM16, even when the cells were incubated with over 16 times as much ArM16.
Table VI

| Immunoglobulin coating Ar:SRBC | Percent of macrophages bearing five or more SRBC |
|-------------------------------|-----------------------------------------------|
| Ar13.4                        |                                               |
| 200 µg                        | 95                                            |
| 75 µg                         | 95                                            |
| 20 µg                         | 80                                            |
| 5 µg                          | 5                                             |
| ArM1(125 µg)                  | 5                                             |
| ArM2(125 µg)                  | 5                                             |
| ArM7(125 µg)                  | 5                                             |
| ArM17(125 µg)                 | 85                                            |
| ArM16(125 µg)                 | 5                                             |

250 µl of freshly prepared Ar:SRBC (not gluteraldehyde fixed) were incubated with the indicated amounts of purified anti-Ar immunoglobulins for 1 h at 25°C. Antibody-sensitized RBC were washed twice and resuspended as a 10% suspension in fresh culture medium. The macrophage-like cell line FC-1 had been allowed to adhere at a low density to glass coverslips for 2 h, after which unattached cells were rinsed off and the sensitized RBC (2% suspension, 0.5 ml/coverslip) were allowed to settle for 20 min at 25°C onto the coverslips. Coverslips were rinsed by immersing several times in DME and scored for rosettes. A rosette was defined as a macrophage bearing five or more RBC (34), but the rosetted macrophages were usually completely covered and had often phagocytized RBC as well. The background reported as 5% is felt to be due to RBC occasionally trapped between adjacent macrophages. Ar:SRBC with no immunoglobulin attached and unhaptenated SRBC incubated with 200 µg Ar13.4 immunoglobulin produced similar backgrounds.

Protein as the minimum amount of parental protein required for lysis. This result is consistent with the monoclonal serology, suggesting that ArM16 lacks a large portion of the C_H2 domain.

Discussion

The hybridoma technology has made it possible to generate homogeneous antibodies to many biologically and medically important antigens (2). Because large amounts of monoclonal antibodies can be produced and the supply renewed as needed, many investigators are examining their diagnostic and therapeutic uses. The potential availability of human monoclonals (36–38) has led to a reexamination of the usefulness of passively administered antibody in the prevention, diagnosis, and treatment of disease. Among the significant advances already achieved are the generation of antibodies that recognize many human differentiation antigens (39). Antibodies that recognize antigens on tumor cells could provide a means of identifying sites of metastatic spread or even delivering cytotoxic agents specifically. Monoclonal antibodies coupled to plant or bacterial toxins have been shown to kill carcinoma cells, specifically in vitro (40, 41). In a particularly dramatic example of the therapeutic use of monoclonal antibody, Miller et al. (42) recently reported successfully treating a patient with a widespread B cell lymphoma unresponsive to current chemotherapy by injecting a mouse monoclonal antibody that recognized the idiotype of the lymphoma immunoglobulin.

Using antibodies both in vivo and in vitro is potentially complicated by the unique
structural and functional properties of immunoglobulins. Bivalent antibodies often modulate surface antigens so that they are no longer present on the cell. Monoclonals bearing cytotoxic agents or radioactive labels will combine not only with the intended target cells but also with cells of the immune system bearing Fc receptors either through cytophilic interactions or by forming immune complexes with circulating antigens. Circulating immune complexes might initiate dangerous allergic reactions, and deposition of immune complexes can damage nonlymphoid tissues, such as kidney.

A solution to these problems analogous to preparing Fab fragments from conventional antisera is to identify hybridoma mutants producing structurally altered immunoglobulins that form only HL half-molecules or do not exhibit the normal immunoglobulin functions. Such mutant monoclonal antibodies would provide a homogeneous reagent that would be continuously renewed, thus, retaining the major benefits of the hybridoma technology. The techniques we had used to identify such immunoglobulins produced by mutant mouse myeloma cells were slightly modified and applied successfully to hybridomas. Out of ~17 × 10^3 clones examined, eight (0.05%) were producing structurally altered immunoglobulins that did not react with the selecting antiserum but still bound antigen. This number does not include variants that produced little or none of either immunoglobulin chain or mutants that had lost the ability to bind antigen because these would have been discarded in screening. This frequency is at least 10-fold lower than the frequency with which similar mutants have been isolated from mouse myeloma cells (4), although it is still high enough to make it relatively easy to obtain mutants. The difference could reflect a difference in the stability of the immunoglobulin genes in these cells, but it is more likely due to the use of different selecting antisera and the more stringent requirements imposed by the double overlay technique. For example, it is very likely that some mutants went undetected because they retained enough determinants to react with the selecting antiserum. Others might have been sufficiently altered not to react with the second antiserum and were discarded as heavy chain nonproducers or nonsecretors. Even a mutant frequency of 0.05% reflects a high degree of instability for genes in animal cells.

Using an IgM-producing hybridoma, Kohler and Schulman (43) also identified mutants producing structurally altered immunoglobulins. However, their cell line generated mutants at much lower frequencies. Schulman et al.2 recently examined another IgM-producing hybridoma and found mutants at frequencies similar to those reported here. We have done a few experiments with another IgG2b-producing hybridoma, and it also generates mutants at high frequencies (28).

Although we used mutagenesis in these experiments in the hope of increasing the yield of mutants, we do not have any evidence that it was necessary or effective. The recovery of two pairs of phenotypically identical mutants (ArM17 and ArM19; ArM16 and ArM18) in this small sample suggests that some mutants could have existed in the population before mutagenesis.

Most of the mutants isolated with IgG2b- and C4b3-specific antisera appeared to contain deletions in the C-terminal portion of the heavy chain. It is not surprising that these two different antisera should select such similar mutants. In our experience,

2 Shulman, M. J., C. Heusser, C. Filkin, and G. Kohler. Mutations affecting the structure and function of immunoglobulin M. Manuscript submitted for publication.
many of the IgG2b-specific determinants appear to reside in CH₃. M3.11, the myeloma mutant that lacks exclusively CH₃, does not react with either commercial anti-IgG₂b antisera prepared in goats or the rabbit IgG₂b-specific antisera used in these experiments. Herzenberg et al. (44) have shown that most of the polymorphic determinants recognized by mouse alloantisera are confined to the carboxy-terminal half of the immunoglobulin molecule. Most of the rat anti-mouse IgG₂b monoclonal antibodies that we generated also react with the C₃ domain. Mutants with alterations in other parts of the molecule presumably could be selected with antisera directed at different portions of the constant region. Preparation of such antisera might be facilitated by immunizing with mutant proteins lacking the normally immunodominant determinants.

We do not know the molecular mechanisms responsible for generating the mutant phenotypes that we identified. Some mouse myeloma mutants with C-terminal deletions are the result of point mutations or frame shifts that lead to premature terminations (4). Preliminary studies examining the size of the H-chain mRNA of the mutants reported here suggest that more complex events are occurring in some mutants (Sau-Ping Kwan, unpublished observations). The origin of the multiple H-chains in some mutants (ArM2 and ArM17) might provide more information about the expression of immunoglobulin genes.

As noted above, mutant monoclonal antibodies could be useful in further characterizing the structural bases of effector functions. All but one of the mutant monoclonals reported here contain large deletions. This is probably because the loss of a single antigenic determinant would not be detected by the antisera used. In addition, the mutagen used (if effective) favors production of frame shift and deletion mutants (4). Our aim in examining the effector functions of these deletion mutants was primarily to support the inferences made from the serological mapping of the deletions in the Ar13.4 mutants. Even this small group of grossly altered proteins, however, turned out to contain members (ArM17 and ArM1) that upon further study might yield new information about the structural basis of biological activity. For example, protein A has been reported to inhibit the uptake of IgG₂b by macrophages, suggesting that protein A and Fc receptors might recognize the same part of the immunoglobulin molecule (45). ArM17 does not bind protein A but does bind the macrophage Fc receptor, indicating that protein A and Fc receptors do not bind to identical sites. If ArM17 proves to be a IgG₂b-2a hybrid, it would be interesting to know whether it binds the IgG₂b or IgG₂a Fc receptor (34). ArM1 does not bind to macrophage Fc receptors. As has been described above, this mutant is very similar to M3.11 in its serology and assembly, but its heavy chain may be slightly smaller (Fig. 6). Because M3.11 does bind to Fc receptors when artificially aggregated (8), it is conceivable that ArM1 is missing a functionally important part of the molecule retained by M3.11. Studies are now under way to compare these proteins functionally and structurally.

Although more mutants like these could be produced in an attempt to find ones that defined functionally important regions, it seems preferable to identify mutants with point mutations or very small deletions. These may make it possible to identify precise sequences responsible for each effector function. In addition, these mutants with large deletions have shortened half-lives in the circulation (Susan B. Roberts and D. E. Yelton, unpublished observations) and accumulate poorly in ascites, making
it difficult to prepare large amounts of mutant proteins. One solution to these problems is to select mutants with small mutations using antisera made specific for small regions by sequential absorption on multiple mutant proteins. However, the observation that monoclonal 196 competes with protein A for binding to IgG2b suggests a strategy for obtaining mutants with small mutations at sites of greatest biological interest. If a large battery of monoclonal anti-immunoglobulins is generated, those that interfere with a particular effector function can be identified. These can then be mapped with respect to each other by cross-inhibition studies and those that recognize independent antigenic determinants used to identify mutants. Based on studies with influenza virus (46), many of these are likely to be point mutations resulting in single amino acid substitutions. The mutant immunoglobulin can then be examined for its ability to carry out effector functions. Once a monoclonal that identifies mutations in important sequences has been identified, it can be used to identify similar mutations in any monoclonal of that particular subclass.

Summary

Somatic cell mutants with deletions in the immunoglobulin constant region were isolated from an IgG2b Ar-binding hybridoma. Rat anti-mouse immunoglobulins were used to identify the sites of the deletions. The mutant monoclonal antibodies differ from the parental molecule in their assembly states and are defective in various immunoglobulin activities, making them potentially more useful reagents.

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