

## Mutations Affecting the Structure and Function of Immunoglobulin M

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Using a hybridoma cell line which secretes hapten-specific immunoglobulin M (IgM), we have isolated a variety of mutants which produce abnormal immunoglobulin. Immunoglobulin was tested for the size and composition of the component heavy and light chains and for variable and constant region related functional and serological activities. Some mutants secrete IgM which seems to be defective in hapten binding; others make IgM which appears not to activate complement. Many of the mutants secrete monomeric as opposed to pentameric IgM. In some cases, the defect apparently correlates with structural alterations in the  $\mu$  heavy chain: partial deletion, polypeptide addition, and abnormal glycosylation have been observed. These mutant cell lines provide a means of identifying the structural basis of IgM function and of studying the biochemistry of IgM synthesis and processing.

Several features of the mouse immunoglobulin system facilitate the analysis of the structural basis of immunoglobulin protein and gene function. First, there are hybridoma and myeloma cell lines from which it is comparatively easy to isolate mutants in which immunoglobulin production is qualitatively or quantitatively altered. Second, the mouse immunoglobulin system has been extensively characterized at both DNA and protein levels, thus providing a framework for the biochemical analysis of the mutant defects. Third, immunoglobulin comprises a relatively large fraction of the protein made by these cells, so that adequate material for biochemical study is available.

Here we describe a variety of mutants which appear to affect several different properties of the immunoglobulin M (IgM) molecule: structure of the antigen binding site, interaction with complement, size of the  $\mu$  polypeptide chain, formation of pentamers, and glycosylation of the  $\mu$  chain.

### MATERIALS AND METHODS

**Cell culture media.** Cells were grown in Dulbecco modified Eagle medium H21 (GIBCO Laboratories) supplemented with penicillin, streptomycin, fetal bovine serum (15%), and 2-mercaptoethanol, as described previously (30). Phosphate-buffered saline (PBS) contained (per liter) 8 g of NaCl, 0.2 g of KCl, 0.2 g of  $\text{KH}_2\text{PO}_4$ , and 1.15 g of  $\text{Na}_2\text{HPO}_4$  and, where indicated, was adjusted to pH 8.5 with NaOH.

**Monoclonal antibodies used to characterize mutant immunoglobulins.** Monoclonal T15 idiotype-specific antibody (MaId 5-4) is an IgG<sub>1</sub> antibody secreted

by a hybridoma derived by fusing the myeloma NS-1 (29) and spleen cells from A/J mice hyperimmunized with the TEPC 15 (T15) myeloma protein. The MaId 5-4 cell line does not produce the NS-1 light chain. Antibodies specific for T15 idiotype activity were scored by inhibiting T15-induced agglutination of A/J anti-T15-coated erythrocytes. Antibody at 1 mg/ml has a titer of  $5 \times 10^5$  for T15-coated erythrocytes and no detectable titer for MOPC 511-coated erythrocytes. MaId 5-4 was found to recognize antibodies produced by that population of phosphorylcholine (PC)-specific B cells which were termed T15 idiotype positive by the A/J anti-T15 idiotype antisera (C. Heusser, J. Johnson, and M. Julius, Abstr. 4th Int. Cong. Immunol. 1980, abstr. no. 2.1.07).

For monoclonal mouse  $\mu$  chain-specific antibody, hybridomas b-7-6 and c-2-23 are derived from a fusion of the cell line Sp2/0 Ag14 (45) and spleen cells from Lewis rats immunized with a mixture of mouse myeloma proteins of various isotypes (C. Heusser, unpublished data). The immunoglobulins of cell lines b-7-6 and c-2-23 react with mouse IgM, recognizing determinants located in the second and fourth domains, respectively (M. Potash et al., manuscript in preparation).

**Immunoglobulin-dependent hemolysis and hemagglutination tests.** IgM production was assayed with the protein A-coupled sheep erythrocyte lysis test (21), using a rabbit anti- $\mu$  serum [raised against the IgM( $\lambda$ ) myeloma protein MOPC 104E (40)] as a developing antibody and guinea pig serum (Behringwerke) as a complement source.

For PC-specific lysis tests, we coupled the PC hapten to sheep erythrocytes, using *p*-diazonium phenylphosphorylcholine prepared from aminophenyl phosphorylcholine (Biosearch) as described previously (7, 8).

Immunoglobulin secretion by individual cells was

assayed as plaque formation, as described by Cunningham and Szenberg (13). Alternatively, the immunoglobulin concentration in culture medium was measured by its hemolytic titer on protein A- or PC-erythrocytes. Hemolytic titer of culture supernatants was measured by diluting the supernatant in PBS and testing whether a 2- $\mu$ l droplet could lyse PC- or protein A-erythrocytes which were immobilized in agarose in the presence of 5% guinea pig serum as a complement source and, where appropriate, rabbit anti-mouse  $\mu$  serum.

PC-binding immunoglobulin was also measured by the agglutination of PC-coupled erythrocytes. The T15<sup>+</sup> idiotype marker was scored by agglutination of erythrocytes coupled with monoclonal MaId 5-4. PC-specific IgM, determined to be T15<sup>+</sup> by the use of conventional A/J anti-T15 idiotype sera, has at 1 mg/ml a titer of 10<sup>6</sup>, whereas T15<sup>-</sup> PC-specific IgM has no detectable titer. Agglutination of PC but not of anti-T15-coupled erythrocytes could be inhibited by free PC. Monomeric IgM could be distinguished from pentameric IgM by the dependence of PC- and T15-specific agglutination on the addition of anti- $\mu$  serum.

Purified wild-type IgM was prepared by N. Sigal (7). The wild-type cell line, PC700, was grown as an ascites tumor, and the ascites fluid, either untreated or mildly reduced and alkylated, was used as a source of IgM which was then affinity purified on PC-Sepharose.

**Reactivity with rat monoclonal anti- $\mu$  IgG.** The isolation and characterization of the  $\mu$ -specific rat hybridoma cell lines and the enzyme-linked immunosorbent assay will be described elsewhere (M. Potash et al., manuscript in preparation). In brief, the incubations were conducted in plastic 96-well cluster trays. PC coupled to bovine serum albumin was prepared and adsorbed to the plastic. Remaining protein binding sites were blocked with a 1% bovine serum albumin solution. Culture supernatants were incubated in the wells so that the PC-specific IgM bound to the PC-bovine serum albumin. The monoclonal  $\mu$ -specific rat IgG described above was then added to bind to the IgM. Bound rat IgG was detected by introducing an anti-rat IgG rabbit serum coupled to alkaline phosphatase. Bound alkaline phosphatase was measured by the color change associated with the hydrolysis of *p*-nitrophenylphosphate.

**Biosynthetic labeling of immunoglobulin.** Secreted immunoglobulin was labeled with [<sup>14</sup>C]leucine by incubating cells in minimum essential medium with Earle salts, 100 U of penicillin-streptomycin per ml (adjusted to contain 5  $\mu$ Ci/ml), and 10  $\mu$ g of leucine per ml. After overnight incubation, the culture was centrifuged, and the supernatant was used as a source of secreted immunoglobulin. Labeled intracellular immunoglobulin was prepared as described previously (30), except that the labeling medium contained no serum. For labeling of immunoglobulin in the presence of tunicamycin, about 5  $\times$  10<sup>6</sup> cells were washed and suspended in 1 ml of leucine-free medium containing the indicated tunicamycin concentration. After 30 min of incubation at 37°C, either [<sup>14</sup>C]leucine at a final concentration of 5  $\mu$ Ci/ml or [<sup>3</sup>H]leucine at 100  $\mu$ Ci/ml was added, and the incubation continued at 37°C for the indicated time, after which the detergent Nonidet P-40 was added to a final concentration of 1% to lyse the cells.

For sodium dodecyl sulfate-polyacrylamide gel elec-

trophoresis (SDS-PAGE) analysis, the material containing labeled IgM  $\mu$  or  $\kappa$  chains was reacted with either rabbit anti-mouse IgM( $\lambda$ ) (MOPC 104E [40], for anti- $\mu$  precipitation) or with a rabbit anti-mouse IgG<sub>1</sub>( $\kappa$ ) (MOPC 21 [35], for anti- $\kappa$  precipitation). PC700 culture supernatant was used as a source of carrier IgM in the precipitation. After overnight incubation (2 h at room temperature followed by 4°C), the material was centrifuged, washed once in PBS, and suspended in 0.5% SDS.

**Electrophoresis.** Radiolabeled immunoglobulin was analyzed by SDS-PAGE as described previously (11, 31). In some cases, the sensitivity of the autoradiography was enhanced by soaking the gel in salicylate (6).

**Preparation of phosphorylcholine *N*-hydroxysuccinimide-activated ester.** Pohlit et al. (39) reported the preparation and use of the PC *N*-hydroxysuccinimide-activated (PC-ONS) ester to attach PC nontoxically to cells. To prepare the PC-ONS compound, diazonium phenylphosphorylcholine (DPPC) was synthesized by the method of Chesebro and Metzger (7). DPPC in 0.1 M HCl was added slowly with stirring to a 1.2 M excess of *p*-hydroxyphenyl-acetic acid (pH 9.2) and allowed to react at room temperature for 4 h. The monoconjugate of DPPC and *p*-hydroxyphenylacetic acid was then converted to the succinimide ester in dimethyl sulfoxide as described previously (39).

**Origin of cell lines.** The cell lines Sp2/0-Ag14 (45) and X63-Ag8.653 (26) were fused with spleen cells from BALB/c mice which were immunized with PC-coupled keyhole limpet hemocyanin in complete Freund adjuvant and boosted either with the PC-coupled keyhole limpet hemocyanin (regimen 1) or a mixture of pneumococcus R38a and PC-coupled *Brucella abortus* (regimen 2). We then selected hybridomas in HAT (hypoxanthine-aminopterin-thymidine) medium as described previously (45), except that Dulbecco modified Eagle medium was used as culture medium. PC-specific immunoglobulin was scored by the lysis of PC-coupled sheep erythrocytes in the absence of developing sera to detect IgM or in the presence of rabbit sera (Litton Bionetics) specific for each of the mouse IgG classes IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgG<sub>3</sub>. Immunoglobulin from these hybridomas was reduced and analyzed by SDS-PAGE. For IgM, the T15<sup>+</sup> idiotype was initially scored as the inhibition of agglutination of PC-coated erythrocytes by T15<sup>+</sup>-specific mouse antiserum. Subsequently, the T15 idiotype was scored by the direct agglutination of sheep erythrocytes coupled with the monoclonal anti-T15. The cell lines Sp2/0-Ag14 and X63-Ag8.653 each yielded a comparable number of hybridomas expressing a similar spectrum of immunoglobulin classes. One such hybrid (PC7), derived from Sp2/0-Ag14 and the mouse immunized with regimen 1, was recloned twice by limiting dilution (PC7/1-2). From this line, an azaguanine-resistant derivative was isolated and cloned (PC7/1-2Ag4 = PC700). The light chain was identified as  $\kappa$  by its binding to monoclonal  $\kappa$ -specific immunoglobulin secreted by a rat hybridoma cell line (M. Potash et al., manuscript in preparation). The mutants described here were isolated from PC700 and from reclones (PC704, PC705) of this line.

These fusions also yielded hybridomas secreting PC-specific T15<sup>+</sup> immunoglobulin of the IgG<sub>1</sub> (PC59), IgG<sub>2a</sub> (PC55), IgG<sub>2b</sub> (PC28), and IgG<sub>3</sub> (PC51) classes and a PC-specific, T15<sup>-</sup> IgM producer (PC111), which

were used as standards in the mutant testing. The myeloma-derived cell line S107 (40) was obtained from A. Marks.

**Mutagenesis.** Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), ethyl methane sulfonate (EMS), and X rays was carried out as described by Friedrich and Coffino (18) to obtain 10% survival after the mutagenic treatment of an initial population of  $1 \times 10^7$  to  $2 \times 10^7$  cells. PC700 cells were exposed to NTG (2  $\mu\text{g/ml}$ ) for 1 h at 37°C, washed twice in PBS, and suspended in growth medium. PC704 cells were incubated in EMS (250  $\mu\text{g/ml}$ ) for 24 h at 37°C, washed in PBS, and returned to growth medium. PC705 cells were exposed to 400 rads of X-irradiation in growth medium. Approximately 1 week after mutagenesis, the mutagenized populations were subjected to the mutant enrichment protocol.

**Enrichment for mutants.** A volume of  $10^7$  cells from a culture containing about  $5 \times 10^5$  cells per ml was washed in cold PBS and suspended at  $5 \times 10^6$  cells per ml in cold PBS adjusted to pH 8.5. To couple PC to the cell membrane, PC-ONS ester was added to a final concentration of  $2 \times 10^{-4}$  M and incubated on ice for 15 min, at which time the cells were diluted with cold PBS, centrifuged, and suspended in 60 ml of medium containing 5% guinea pig serum (Behringwerke) as a complement source. To effect the suicide selection, 15-ml samples of this cell suspension were placed in 2-liter flasks (approximately  $1.5 \times 10^5$  cells per ml/10  $\text{cm}^2$ ) and incubated for 2 h at 37°C, after which 150 ml of cold PBS was added to each flask. The cells were then recentrifuged, suspended in medium, and incubated at 37°C. After at least 20 h, the cells were tested for their ability to make  $\mu$ -specific and PC-specific plaques. Where indicated, the selection procedure was repeated after cells had grown at least 10-fold to reduce the level of membrane-bound PC. In the subsequent enrichments, where the population contained a correspondingly higher frequency of mutant cells, fewer cells were subjected to the selection.

The enrichment was repeated until the frequency of  $\mu$ -specific plaques exceeded the frequency of PC-specific plaques. The mutants were then cloned by limiting dilution at 1 cell per well in 96-well cluster trays. When the cells had grown to fairly high density, the culture supernatant was scored for  $\mu$ -specific hemolysis. For this purpose, it was convenient to use a

transfer plate (Dynatech Laboratories, Inc.) to deposit 1- $\mu\text{l}$  samples from all wells in one operation. Comparable culture supernatants of wild-type cells diluted up to 10-fold consistently lysed protein A erythrocytes, and on this basis we expected that cells secreting less than 10% of the normal amount of IgM would fail to lyse the protein A-coupled erythrocytes and would be classified as nonsecreters (denoted  $\text{IgM}^-$ ). Almost all of the cells from which the supernatant did lyse the protein A erythrocytes secreted an abnormal IgM (denoted  $\text{IgM}^*$ ), e.g., noncytolytic on PC-erythrocytes. Table 1 gives some of the results of tests used to monitor the course of the enrichment. The frequency of  $\text{IgM}^*$  clones was 50- to 400-fold higher than was indicated by the frequency of the  $\mu$ -specific plaques in the enriched cultures. However, most of the  $\text{IgM}^*$ -producing mutants had a lower than wild-type plaquing efficiency, and we suppose that this effect accounts for the discrepancy.

## RESULTS

**Description of the wild-type cell line.** The variable (V) region of BALB/c mouse immunoglobulin which binds PC and bears the T15<sup>+</sup> idiotype has been well characterized. All PC-binding T15<sup>+</sup> IgMs have the same heavy chain variable region  $V_H$  amino acid sequence (20, 27), and the DNA of BALB/c mice contains only one gene encoding this  $V_H$  amino acid sequence (12, 15). The three-dimensional structure of the PC binding site has been determined (38). The selection method itself is expected to work best with immunoglobulin of the IgM class (see below). To combine these advantages—a V region which is well characterized joined to a  $\mu$  heavy chain which permits the mutant selection—a hybridoma cell line (PC700) secreting T15<sup>+</sup>, PC-specific IgM was generated, as described above.

**Enrichment for mutants.** The enrichment for mutants of this cell line was adapted from a method described earlier (30). The PC hapten was attached covalently to the surface of the hybridoma cells, as described by Pohlit et al.

TABLE 1. Parameters of mutant enrichment<sup>a</sup>

Cell line	Mutagen	Enrichment cycles	Plaques per cell		Frequency of $\text{IgM}^-$ mutants before enrichments	Fraction of cells surviving enrichments	Frequency of $\text{IgM}^*$ mutants after enrichments	Origin of mutants
			PC specific	$\mu$ specific				
PC705		0	0.4	0.2	0.005	0.005	0.13	102, 109, 110, 128, 132, 134
PC705		4	$<10^{-5}$	$3 \times 10^{-4}$				
PC705	X ray	4	$<10^{-5}$	$<10^{-5}$	0.01	0.01	0.17	201, 205, 208, 212
PC704	EMS	5	$2 \times 10^{-4}$	$1 \times 10^{-3}$				
PC700		0	0.7	0.3	0.01	0.01	0.11	1
PC700	NTG	6	$3 \times 10^{-4}$	$2 \times 10^{-3}$				

<sup>a</sup> The frequency of  $\text{IgM}^-$  mutants before enrichment was determined by cloning the cells by limiting dilution (1 cell per well) and measuring the frequency of clones which failed to secrete PC-specific cytolytic IgM. The frequency of  $\text{IgM}^*$  mutants after enrichment is the fraction of clones from which the culture supernatants lysed protein A-erythrocytes, but not PC-erythrocytes.

(39). The cells were then incubated in the presence of complement. Under conditions of limited diffusion, the PC-specific IgM apparently binds preferentially to the PC on the surface of the cell from which it was secreted, where it activates complement, killing that cell. Thus, cells secreting normal amounts of normal IgM are lysed, whereas IgM-defective mutants survive.

Wild-type cells were assessed by measuring the frequency of PC-specific plaques (on PC-coupled erythrocytes). Both wild-type cells and mutants making an altered IgM were detected by a  $\mu$ -specific plaque assay (on protein A-coupled erythrocytes). We repeated the enrichment procedure until the frequency of wild-type cells was substantially below the frequency of mutants making an altered IgM. The mutants in these enriched cultures were then cloned by limiting dilution, and the culture supernatants were tested for IgM production with the protein A-erythrocyte lysis test. For 90% of these clones, the culture supernatants did not lyse the protein A-erythrocytes, indicating that these cells (IgM<sup>-</sup> mutants) secrete either a low amount of IgM (<10% of normal) or none at all. Except for a few wild-type survivors, the remaining 10% of the clones (IgM\* mutants) secreted an altered

immunoglobulin, i.e., immunoglobulin which lyses protein A- but not PC-erythrocytes. The frequency of IgM<sup>-</sup> mutants in the population before enrichment is estimated at  $10^{-2}$  per cell. If IgM<sup>-</sup> and IgM\* mutants survive the selection treatment equally well, these figures indicate that IgM\* mutants constitute 0.1% of the unenriched population (see above).

We have applied a variety of tests to characterize the immunoglobulin made by the mutants. The results of these analyses indicate that in most cases, the state of the heavy chain produced by the mutants is abnormal. Nevertheless, the mutant material is closely related to normal  $\mu$  and for this reason is referred to as  $\mu$  or IgM, despite its novel properties. (See Table 4 for a summary of the properties of the mutants.)

**Analysis of  $\mu$  and  $\kappa$  chains by SDS-PAGE.** Light and heavy chains from mutant and wild-type cells were separated by reducing the disulfide bonds of biosynthetically labeled immunoglobulin, and the size of these chains was analyzed by SDS-PAGE (Fig. 1). All of the IgM\* mutants secreted light chains of the same size (mobility) as wild-type  $\kappa$ . Mutants no. 12, 13, 205, and 209 made  $\mu$  of normal size. In this gel, the  $\mu$  chain from mutant no. 207 appeared to run slightly faster than wild-type  $\mu$ , but this differ-

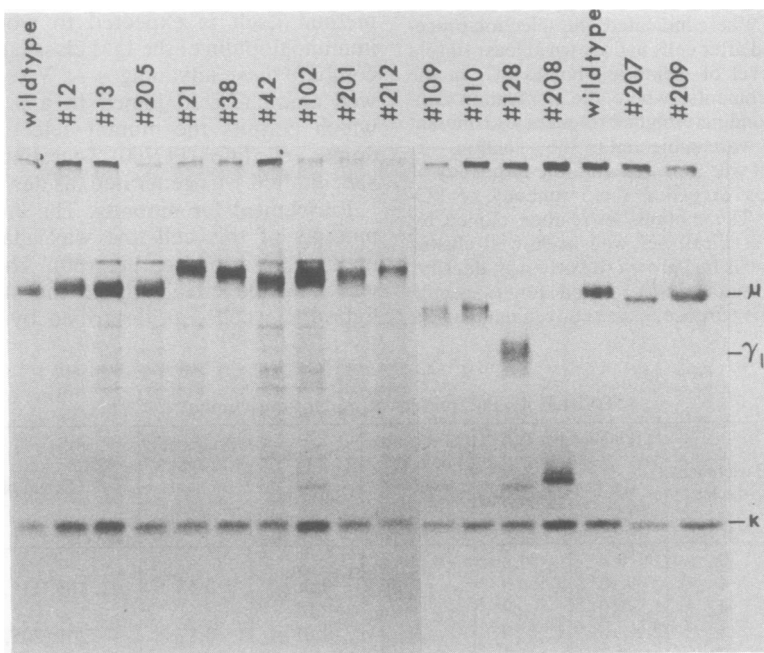


FIG. 1. Analysis of  $\mu$  and  $\kappa$  chains of the IgM\* mutants. Secreted biosynthetically radiolabeled IgM was prepared from the indicated cell lines and precipitated with anti- $\mu$  serum. Culture supernatant (50  $\mu$ l) was used, except for no. 207 and 209, where 200  $\mu$ l was taken. This material was reduced and analyzed by SDS-PAGE. The wild-type cell lines used were PC700 (lane 1) and PC704 (lane 15). The position of the  $\gamma_1$  (MOPC21) heavy chain is indicated.

ence was not always seen. The mutants no. 21, 38, 42, 102, 201, and 212 secreted a heavy chain of slower than wild-type mobility. From the mobility shift in this and other gels, we estimated that these  $\mu$  chains had increased apparent molecular weights of 8,000 (no. 21), 6,000 (no. 38), 2,000 (no. 42), 4,000 (no. 102), 5,000 (no. 201), and 7,000 (no. 212). We have tested whether the slower mobility reflects increased glycosylation or a longer polypeptide chain by analyzing the mobility of the  $\mu$  chains made in the presence of tunicamycin, a compound which blocks glycosylation (23, 49–51). For comparison, we have included the hybridoma cell line SP2/B, which had been found previously to produce a  $\mu$  chain of apparently high molecular weight (28), both in the presence of tunicamycin and in cell-free translation (G. Kohler, unpublished data). With tunicamycin, the mutants no. 21, 38, 42, 201, and 212 make  $\mu$  chains which comigrate with wild-type  $\mu$  (Fig. 2), suggesting that the  $\mu$  chains of these mutants are otherwise abnormally glycosylated. By contrast, the  $\mu$  chain from no. 102 has a slow mobility even in the presence of tunicamycin, suggesting that for this mutant the  $\mu$  polypeptide chain is abnormally long.

The faster than normal mobility of the  $\mu$  chains from the mutants no. 109, 110, 128, and 208 suggests that these mutants might make  $\mu$  chain fragments. The migration of the material labeled in the presence of tunicamycin (Fig. 3) corresponds to apparent polypeptide deletions of 16 (no. 109 and 110), 25 (no. 128), and 36 (no. 208) kilodaltons. Mutants no. 109 and 110 differ

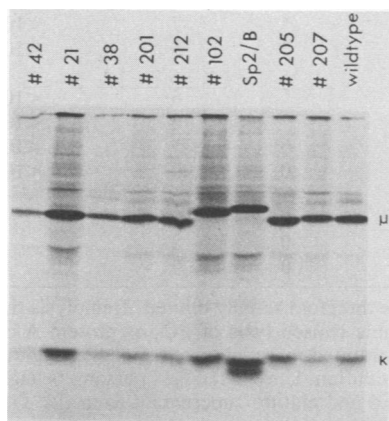


FIG. 2. Analysis of unglycosylated  $\mu$  chains. Unglycosylated [ $^3\text{H}$ ]leucine-labeled  $\mu$  was prepared by incubating cells overnight in the presence of tunicamycin (10  $\mu\text{g}/\text{ml}$ ). The anti- $\mu$  precipitated material was reduced and analyzed by SDS-PAGE. After electrophoresis, the gel was soaked in salicylate to enhance the sensitivity of the autoradiography. The wild-type cell line is PC700.

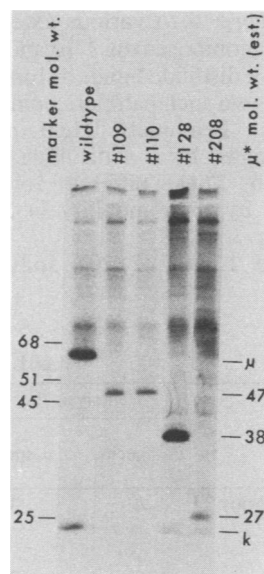


FIG. 3. Molecular weight estimates of  $\mu$  chain fragments. Unglycosylated [ $^{14}\text{C}$ ]leucine-labeled  $\mu$  was prepared by incubating cells for 5 h at 37°C in the presence of tunicamycin (6  $\mu\text{g}/\text{ml}$ ). The anti- $\mu$  precipitated material was reduced and analyzed by SDS-PAGE. After electrophoresis, the gel was soaked in salicylate. The wild-type cell line used here was PC705. The markers used to estimate the  $\mu^*$  molecular weight were bovine serum albumin (68,000), egg albumin (45,000), mouse  $\gamma_1$  (MOFC 21) (51,000), and chymotrypsinogen (25,000).

from each other only slightly, if at all, and are not necessarily independent isolates.

**Reaction of mutant IgMs with  $\mu$ -specific reagents.** Two rat hybridoma lines (b-7-6 and c-2-23) have been isolated which secrete IgG reactive with mouse IgM but not with mouse immunoglobulin of other classes (see above). Mutant IgM\*s bound to PC-coated plates were tested for the presence of the  $\mu$ -specific determinants recognized by these monoclonal anti- $\mu$  reagents. The PC-binding IgM\*s can be divided into two classes on the basis of their reactivity with these monoclonal anti- $\mu$  reagents: (i) no. 12, 13, 21, 38, 42, 102, 201, 205, 207, 209, and 212, which react with both the monoclonal antibodies, and (ii) no. 109 and 110, which react only with IgG from b-7-6 but not with IgG from c-2-23, indicating that the determinant recognized by the c-2-23 antibody is lacking in the  $\mu$  chain fragments of no. 109 and 110. The IgM\* of mutants no. 128 and 208 fails to bind PC (data not shown) and could not be analyzed with this test.

**Mutants making pentamers ( $\mu_{10}\kappa_{10}$ ).** IgM( $\kappa$ ) is composed of  $\mu$ -heavy and  $\kappa$ -light chains which

can be polymerized to various extents. "Halfmer" IgM is composed of 1  $\mu$  and 1  $\kappa$  chain linked by a  $\mu$ - $\kappa$  disulfide bond. "Monomer" IgM is made up of two such halfmers, joined by a  $\mu$ - $\mu$  disulfide link. "Pentameric" IgM includes five monomers, joined by  $\mu$ - $\mu$  disulfide bonds, and one J protein. These different forms can be distinguished by their mobility in SDS-PAGE (53).

Mutants no. 12, 13, 42, 207, and 209 secrete

IgM\*s which migrate as pentamers like the wild-type IgM. We estimated total IgM production by the lysis titer of culture supernatants on protein A-erythrocytes (Table 2) and by the intensity of the  $\mu$  and  $\kappa$  bands in SDS-PAGE analysis (Fig. 1). Mutants no. 12, 13, and 42 secreted approximately normal amounts of IgM, whereas mutants no. 207 and 209 appeared to secrete about 10-fold less IgM than normal.

In accordance with the selection method,

TABLE 2. Functional defects of mutants<sup>a</sup>

Cells	Hemolysis titer		Hemagglutination titer				PC-specific (wild-type) plaques per cell
	PC specific	$\mu$ specific	PC specific		T15 specific		
			-Anti( $\mu$ )	+Anti( $\mu$ )	-Anti( $\mu$ )	+Anti( $\mu$ )	
Wild type (PC700)	4	3	4	6	6	8	0.2
Mutant							
1	0	0	0	0	0	0	$<10^{-5}$
10	0	0	0	0	0	0	$<10^{-5}$
37	0	0	0	0	0	0	$<10^{-5}$
132	0	0	0	0	0	0	$<10^{-5}$
134	0	0	0	0	0	0	$<10^{-5}$
12	0	4	0	0	7	8	$<10^{-5}$
13	0	4	5	6	7	7	$<10^{-5}$
42	0	3	5	6	6	7	$3 \times 10^{-5}$
207	0	2	2	3	4	5	$<10^{-5}$
209	0	1	2	3	4	5	$<10^{-5}$
21	0	4	0	3	*	7	$10^{-3}$
38	0	4	0	3	*	7	$10^{-4}$
102	0	4	0	4	0	8	$<10^{-5}$
201	0	4	0	4	*	7	$3 \times 10^{-5}$
205	0	4	0	4	*	7	$<10^{-5}$
212	0	4	*	3	*	8	$<10^{-5}$
109	0	3	0	*	*	6	$<10^{-5}$
110	0	3	0	*	*	6	$<10^{-5}$
128	0	2	0	0	0	*	$<10^{-5}$
208	0	2	0	0	0	0	$<10^{-5}$
Reference IgM							
(IgM) <sub>5</sub>	6	4	6	8	8	9	
(IgM) <sub>R&amp;A</sub>	0	4	1	5	0	8	
PC111	5	4	5	6	0	0	

<sup>a</sup> Supernatants of cultures grown to about  $10^6$  cells per ml were threefold serially diluted. Hemolysis titer was scored as the number of dilution steps from which a 2- $\mu$ l sample caused lysis of PC- or protein A-coupled erythrocytes. Undiluted culture supernatant was the most concentrated material tested. For hemagglutination titers, the most concentrated material corresponds to a threefold dilution. Unreduced IgM pentamers [(IgM)<sub>5</sub>] or reduced and alkylated IgM [(IgM)<sub>R&A</sub>] purified from ascites fluid and culture supernatant from the T15<sup>-</sup> PC-specific IgM-secreting hybridoma PC111 are included for comparison. For testing here, this material was first diluted to 10  $\mu$ g/ml. The symbol \* indicates that often one well at some intermediate dilution would show partial agglutination. Where indicated, agglutination tests were done in the presence of rabbit anti-mouse  $\mu$  serum diluted 200-fold (for PC-erythrocytes) or 2,000-fold (for anti-T15-erythrocytes). To measure PC-specific plaques,  $10^4$  and  $10^5$  mutant cells were introduced per chamber. Wild-type (PC700) cells had the same plaquing efficiency in the presence and absence of these numbers of mutant cells. Plaques from PC700 cells vary in size, and the plaquing efficiency reported here is based on the large plaques which constitute half the total plaques from PC700. Some of the mutants made minute plaques at a significant frequency: no. 21,  $2 \times 10^{-3}$ ; no. 38,  $4 \times 10^{-4}$ ; no. 42,  $6 \times 10^{-4}$ ; no. 201,  $4 \times 10^{-3}$ ; no. 209,  $1 \times 10^{-2}$ .

these mutants produced IgM\* defective in the cytolysis of PC-erythrocytes (Table 2), but these defects were of two distinct kinds. The IgM\* from mutant no. 12 was defective in PC binding but still bore an apparently wild-type T15<sup>+</sup> determinant, i.e., its IgM was defective in the agglutination of PC-erythrocytes but normal in the agglutination of anti-T15-erythrocytes (Table 2).

In contrast, mutants no. 13 and 42 had normal titers when assayed for agglutination of PC-erythrocytes (Table 2), even when assayed at 37°C, the temperature used for the hemolysis test (data not shown). The affinity for PC of these IgM\*s was estimated by inhibiting agglutination of PC-erythrocytes by free PC. For comparison, we also tested culture supernatants from the hybridomas HPC52 and HPC19, which secrete IgMs differing by 150-fold in their affinity for PC (3). For IgM from the line HPC52, 50% inhibition of the agglutination titer was achieved at a PC concentration of  $5 \times 10^{-5}$  M, 100-fold lower than the PC concentration required to inhibit agglutination by IgM from the line HPC19. For IgM from the wild-type cells and mutants no. 13 and 42, 50% inhibition of agglutination occurred at  $5 \times 10^{-5}$  M PC, suggesting that the IgM\*s of these mutants have a normal affinity for PC.

The T15 idiotype recognized by the monoclonal anti-T15 MalD 5-4 is probably distinct from the PC binding site (see below). We tested whether IgM from mutants no. 13 and 42 was also defective in lysing anti-T15-coupled erythrocytes (Table 3). For comparison, we included the mutant no. 12, which makes IgM\* bearing an apparently normal T15 idiotype and defective PC-binding site, and the hybridoma PC111, which had been found previously to make T15<sup>-</sup> PC-binding IgM (see above). The hemagglutination titers indicated that the IgM\*s of mutants no. 13 and 42 could bind to both PC and anti-T15-coupled erythrocytes. Nevertheless, these IgM\*s could not lyse either type of erythrocyte. These results suggest that IgM\*s from mutants no. 13 and 42 have normal V regions and fail to lyse erythrocytes for some other reason, such as an inability to activate complement.

**Mutants making monomers ( $\mu_2\kappa_2$ ) and halfmers ( $\mu_1\kappa_1$ ).** The unreduced IgM\*s of mutants no. 21, 38, 102, 201, 205, and 212 had a faster than normal mobility in SDS-PAGE (Fig. 4). In general, these mutants produced IgM\* which migrated in two bands, their mobility suggesting that the slower and faster IgM\* bands included  $\mu_2\kappa_2$  and  $\mu_1\kappa_1$ , respectively.

Good agglutination of PC- and anti-T15-erythrocytes by the monomer-halfmer IgM\* of these mutants requires the addition of anti- $\mu$  serum, presumably to generate larger aggregates of the

TABLE 3. Analysis of binding and lysis properties of mutant IgM<sup>a</sup>

Cells	Hemolysis titer		Hemagglutination titer	
	PC	T15	PC	T15
Wild type (PC700)	4	3	5	7
PC111	3	0	6	0
No. 12	0	3	0	8
No. 13	0	0	5	7
No. 42	0	0	6	7

<sup>a</sup> Hemagglutination and hemolysis titers on PC- and anti-T15-coupled erythrocytes were determined for culture supernatants of the indicated cell lines, as described in Table 2 and the text.

IgM\* (Table 2). According to the agglutination testing of these mutant IgM\*s, PC binding and T15<sup>+</sup> idiotype were normal, and by these criteria, the V regions of these IgM\*s appeared normal.

The mutants no. 109 and 110 produced (unreduced) IgM\* which migrated with the mobility expected for the form  $\mu_2\kappa_2$ .

**Analysis of IgM<sup>-</sup> mutants.** Supernatants of the IgM<sup>-</sup> mutants (no. 1, 10, 37, 132, and 134) had no activity in the PC- or  $\mu$ -specific agglutination or lysis tests (Table 2). Nor could we detect radiolabeled  $\mu$  or  $\kappa$  in their culture supernatants (data not shown). Intracellular preparations from these mutants seemed to lack  $\kappa$  light chain, but two of the mutants, no. 132 and 134, synthesized intracellular  $\mu$ , i.e., anti- $\mu$  precipitable material migrating slightly faster than secreted  $\mu$ . Figure 5 shows the results for mutants no. 1 and 132.

**Frequency of revertants.** All of the mutants were impaired in their plaquing on PC-erythrocytes, and the frequency of such plaques could be used as a limit on the frequency of revertants (Table 2). In general, the mutants were stable: after several months in culture, the frequency of revertants was less than  $10^{-4}$  per cell. Mutants no. 21 and 38 had a higher plaquing efficiency, and such plaques might be due to revertants in the population.

**Testing for class switch.** The enrichment procedure should select cells which have switched to the production of another immunoglobulin class which is less cytolytic than IgM. Other workers have found such switches at a frequency of about  $10^{-6}$  per cell generation for an unmutagenized culture (34, 42) or at a frequency approaching 1% after mutagenesis with ICR191 or melphalan (17, 41). To look for such switches, the enriched population derived from PC700 after mutagenesis with NTG was cloned by limiting dilution. Culture fluid from 254 wells

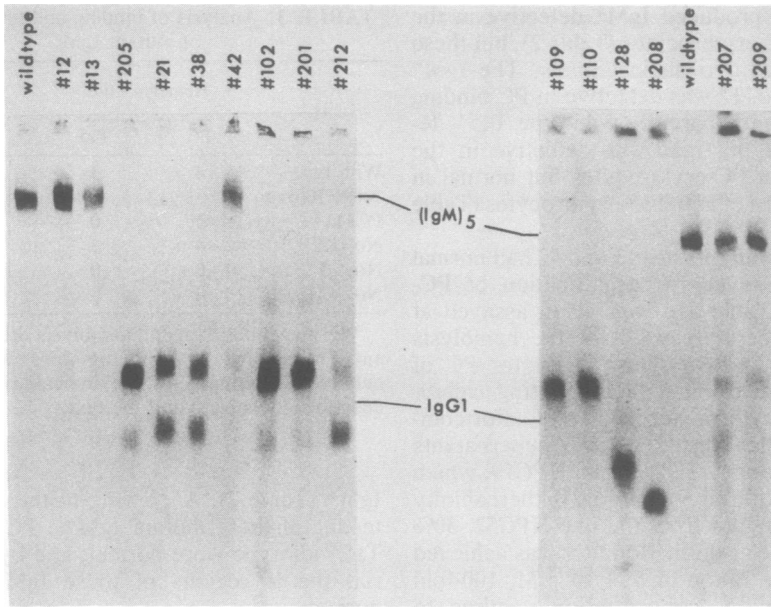


FIG. 4. Analysis of unreduced IgM. Secreted biosynthetically labeled material (200  $\mu$ l for mutants no. 207 and 209; 50  $\mu$ l for all other cell lines) was precipitated with anti- $\mu$  serum and analyzed (without reduction) by SDS-PAGE. The wild-type cell lines were PC700 (lane 1) and PC704 (lane 15). The positions of IgM pentamers [(IgM)<sub>5</sub>] and IgG<sub>1</sub> (MOPC 21) monomers are indicated.

was tested for lysis of PC-erythrocytes in the presence of a mixture of rabbit antisera specific for the IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub>, and IgA classes of mouse immunoglobulin. Various PC-specific T15<sup>+</sup> hybridoma and myeloma cell lines (see above) were used to assess the sensitivity of this test: culture fluids containing immunoglobulins of these classes, diluted up to 10- to 30-fold, lysed the PC-erythrocytes. No switches to any of these IgG or IgA classes were detected among the clones from the selected population. This screening should have been equivalent to testing about  $2 \times 10^4$  unselected cells.

DISCUSSION

Table 4 summarizes the mutant characteristics.

The suicide selection method used here is expected to yield mutants of two general types: (i) those which produce an altered IgM unable to induce lysis, e.g., unable to bind PC or to activate complement, and (ii) those which secrete a reduced amount of normal IgM. Because of the sensitivity of the protein A erythrocyte lysis test used in the screening, cells secreting less than 10% of the normal amount of  $\mu$  chain would have been classified here as  $\mu$  nonsecretors and, by this criterion,  $\mu$  nonsecretors constitute about 1% of the original, unenriched cell population. To date, we have distinguished two types of such mutants on the basis of the pres-

ence (no. 132 and 134) or absence (no. 1, 10, and 37) of intracellular  $\mu$ . We were unable to detect either secreted or intracellular  $\kappa$  in these  $\mu$ -nonsecreting cell lines. This result contrasts with earlier studies in which mutants expressing  $\kappa$  chain in the absence of heavy chain production were frequently obtained (1, 2, 9, 30, 32, 37, 52). Cell lines making only intracellular heavy chain and no light chain have been isolated as Abelson virus-induced tumors (46), fetal liver cell hybridomas (5), and as myeloma mutants (36, 48). The characteristics of these various cell lines suggest that the presence of a light chain might

TABLE 4. Classification of mutants

Characteristics	Mutant no. <sup>a</sup>
<b>Pentameric IgM</b>	
Probable defect:	
PC binding . . . . .	12
Complement activation . . . . .	13, 42*
<b>Monomeric IgM and smaller forms</b>	
$\mu$ polypeptide chain characteristics:	
Partial deletion . . . . .	109, 110, 128, 208
Addition . . . . .	102
Normal length . . . . .	21*, 38*, 201*, 205, 212*
<b>Other</b>	
No $\mu$ no $\kappa$ . . . . .	1, 10, 37
Intracellular $\mu$ , no $\kappa$ . . . . .	132, 134
Low amount of abnormal IgM . . . . .	207, 209

<sup>a</sup> \*, Hyperglycosylated  $\mu$ .



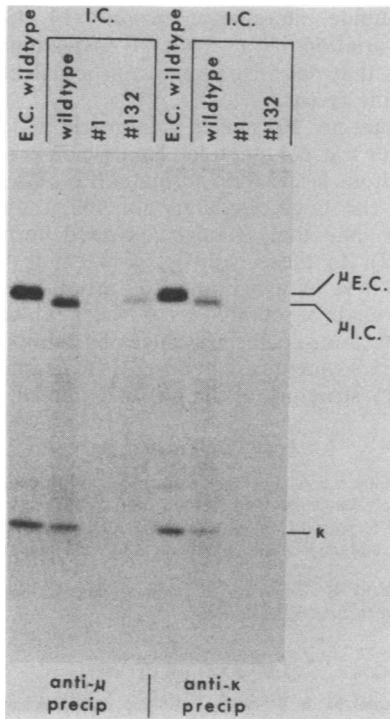


FIG. 5. Analysis of intracellular immunoglobulin of  $IgM^-$  mutants. Intracellular (I.C.) immunoglobulin was labeled and precipitated with anti- $\mu$  and anti- $\kappa$  serum. For comparison, the secreted, extracellular (E.C.) IgM of the wild-type cell line (PC700) was also included. This material was reduced and analyzed by SDS-PAGE.

be required for the secretion of the heavy chain, and the primary defect of mutants such as no. 132 might be the inability to make light chain. It should be noted, however, that Levitt and Cooper have observed the secretion of  $\mu$  heavy chain in the absence of light chain (33).

We do not have a simple explanation for the behavior of mutant no. 1, for which we were unable to detect  $\mu$  or  $\kappa$  chains. In principle, such a phenotype could reflect the mutation of a regulatory gene, the product of which controls both  $\mu$  and  $\kappa$  production. However, mutant no. 1 appears to have lost the  $\mu$  gene (O. Baczynsky et al., unpublished data). This result suggests that deletion of the  $\mu$  gene might be the primary defect and that, in some way, the synthesis or stability of the  $\kappa$  chain in this mutant might depend on the production of the  $\mu$  protein. Alternatively, both  $\mu$  and  $\kappa$  genes might have been inactivated in this mutant.

Mutants making an altered IgM constitute about 0.1% of the cell population before enrichment. This frequency is comparable to that reported by Milstein et al. (35) and by Cook and

Scharff (10) and is 10-fold higher than that observed earlier with a similar selection and screening on a hybridoma line secreting IgM specific for the hapten trinitrophenyl (30). Deletion mutations were isolated in both the PC and trinitrophenyl systems and seemed to be 10-fold more frequent here than in the earlier study. Revertants in most cases were rare ( $<10^{-4}$  per cell), and this result contrasts with the findings of Cook and Scharff (10) that phenotypic revertants of a mutation affecting PC binding are present at a frequency of  $10^{-2}$  per cell.

Several of the  $IgM^*$  mutants make  $\mu$  chain fragments. For each of the mutants no. 128 and 208, the polyvalent rabbit anti-IgM serum precipitates a unique protein of molecular weight 38,000 and 27,000, respectively. A second argument that these proteins are  $\mu$  fragments comes from work in progress which indicates that the  $\mu$  gene is partially deleted in these mutants. The truncated  $\mu$  chains of the mutants no. 109 and 110 (which are not necessarily independent isolates) are about 16 kilodaltons smaller than the 63-kilodalton unglycosylated normal  $\mu$ . Testing with the two monoclonal anti- $\mu$  reagents indicates that their  $\mu$  chains retain one of the two  $\mu$ -antigenic determinants as well as the PC-binding site. The site missing in these fragments has been assigned to the region encoded by the  $C\mu 4$  exon (M. Potash et al., manuscript in preparation). We do not know whether these fragments reflect premature chain termination or arise as degradation products of a larger (i.e., normal sized)  $\mu$  chain.

Mutant no. 12 produced  $IgM^*$  which, when tested for agglutination of PC-erythrocytes, appeared to be defective in PC binding (Table 2). However, PC binding was not wholly impaired, because this  $IgM^*$  binds to PC in the enzyme-linked immunosorbent assay used in measuring its reactivity with the monoclonal anti- $\mu$  reagents. The T15<sup>+</sup> idiotope marker, defined by the monoclonal T15<sup>+</sup>-specific antibody MaId 5-4, is normally found only in association with the PC-binding specificity (45). Nevertheless, this T15<sup>+</sup> determinant appears to be distinct from the PC-binding site in the sense that its binding to the anti-T15<sup>+</sup> reagent is not inhibited by PC. This property is consistent with the finding that the  $IgM^*$  of mutant no. 12 is apparently defective in PC binding but seems to be unaltered in this T15<sup>+</sup> idiotope.

The mechanism of complement activation by IgM is not well characterized. Some studies suggest that parts of the  $C\mu 4$  domain might be necessary (24, 25), but other regions of the  $\mu$  chain have also been implicated (4, 19, 47). Here we have described two mutants (no. 13 and 42) which make pentameric  $IgM^*$ s with apparently normal antigen-binding characteristics, but

which are nevertheless not cytolytic. It will be possible to test directly whether these IgM\*s are in fact deficient in complement activation, and if they are, their structural alterations might identify parts of the IgM required for the interaction with complement.

The mutants no. 207 and 209 (which are not necessarily independent mutants) secrete less than normal amounts of IgM. Low IgM secretion per se might result from low synthesis. However, the IgM of these mutants is abnormal (low lysis titer on PC-erythrocytes), suggesting that the primary defect might be in the IgM structure and that low secretion might reflect increased degradation of the (abnormal) IgM of these mutants.

Some of the mutants (no. 21, 38, 42, 201, and 212) produced apparently hyperglycosylated  $\mu$  chains (slow mobility in SDS-PAGE analysis). We have tested whether the glycosylation also varies among cells producing cytolytic IgM: the wild-type cell lines PC700, PC704, and PC705 and 16 subclones of these lines produce  $\mu$  chains of the same mobility (data not shown), indicating that abnormal glycosylation correlates with the production of defective IgM. We do not yet know whether the change in glycosylation pattern reflects a change in the  $\mu$  chain primary structure or, alternatively, a change in the cellular glycosylation enzymes.

Most of the mutants which were abnormally glycosylated also failed to form pentamers, suggesting that these two phenomena might be related. In different mutants making monomeric IgM, the  $\mu$  chains were glycosylated to different extents. This result argues that abnormal glycosylation does not simply follow from blocking pentamer formation. Experiments are in progress to test the converse hypothesis, that it is the abnormal glycosylation of the  $\mu$  chain which causes the accumulation of monomeric IgM.

Monomeric IgM activates complement much less efficiently than does the pentameric form, and mutants making monomeric and halfmeric IgM would therefore be expected to survive the selection. The  $\mu$  chains produced by such mutants isolated to date (no. 21, 38, 102, 201, 205, and 212) are abnormal in their polypeptide or sugar structure, and these abnormalities might further interfere with IgM function. For example, the interaction with complement might be defective, as seems to be the case for the hyperglycosylated pentameric IgM\* of mutant no. 42. These mutants raise several other questions. Which cysteines are used to link the  $\mu$  chains in the monomeric mutant IgM? Is the failure to form pentamers due to an alteration in the  $\mu$  chain primary structure, or do the mutants have defects in other factors required for polymerization, such as in J protein, polymerizing enzyme,

or disulfide interchange enzyme (14, 43, 44)? The variation in  $\mu$  chain glycosylation again argues that not all mutants can be defective in the same factor.

Mutant no. 102 makes monomeric IgM which includes a  $\mu$  polypeptide chain which is several kilodaltons larger than normal  $\mu$  (i.e., the mobility of the unglycosylated no. 102  $\mu$  chain is slower than that of unglycosylated normal  $\mu$ ) (Fig. 2). In these regards, it resembles membrane IgM in which the 20 carboxy terminal amino acids of secreted  $\mu$  have been replaced by a membrane-specific sequence of 41 amino acids (16). Experiments are in progress to examine the primary structure of the no. 102  $\mu$  chain.

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