

TRUNCATED μ (μ') CHAINS IN MURINE IgM

Evidence that μ' Chains Lack Variable Regions

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The first immunoglobulin (Ig) chain to be expressed in developing B cells is the μ chain (1–4). Light (L)¹ chain expression follows and results in the assembly of μ and L chains to form IgM monomers (μ_2L_2) (5, 6). IgM is secreted as a pentamer, (μ_2L_2)₅ (reviewed in 7). The formation of pentameric IgM depends on disulfide bonding of one or more secretory μ chains with another cellular component, the J chain (8–10), and involves the penultimate cysteine residue in the μ chain terminus (11).

Whether all IgM pentamers are structurally uniform is open to question, as recalled by an old controversy concerning the number and possible heterogeneity of antigen-binding sites in individual IgM antibodies. Reports of 10 equivalent antigen-binding sites (12–14) are at variance with reports of 10 nonequivalent antigen-binding sites; i.e., 5 low and 5 high affinity binding sites (15–17). A number of laboratories have reported only 5 binding sites per IgM pentamer (18–22). Some results of less than 10 binding sites may be attributed to steric hindrance of antigen binding and/or heterogeneity of IgM antibodies (20, 23, 24); however, other results appear free of this criticism, particularly those of Giles et al. (22) in which two monoclonal antidinitrophenyl (anti-DNP) IgM antibodies were shown to display an average of only 5 high affinity binding sites for DNP.

Relevant to possible irregularities in IgM structure and valence, the present work provides evidence that some μ chains of secreted IgM lack antigenic determinants characteristic of Igh variable regions (Igh-V). The truncated μ (μ') chains have an apparent molecular weight of $\sim 55,000$, display antigenic determinants in common with the μ constant region ($C\mu$), and are detectable in preparations of specifically purified IgM of different IgM-producing tumors and of normal serum IgM (a brief account of these findings was reported earlier [25]). μ' Chains are also detectable in IgM-producing tumor cells, in a hybridoma cell line that lacks a productive μ allele, and in bone marrow cells of normal mice. The possible significance of μ' chains is discussed.

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¹ *Abbreviations used in this paper:* $C\mu$, constant region of μ chain; DNP, dinitrophenyl; HBSS, Hanks' balanced salt solution; H chain, heavy chain; IEF, isoelectric focusing; L chain, light chain; LPS, lipopolysaccharide; μ' , truncated μ ; NP, (4-hydroxy-3-nitrophenyl)acetyl; PAGE, polyacrylamide gel electrophoresis; PC, phosphorylcholine; SDS, sodium dodecyl sulfate; Tm, tunicamycin; 2-ME, 2-mercaptoethanol.

Materials and Methods

Animals and IgM-producing Tumors. Pooled serum of normal mice and serum of mice bearing IgM-producing plasmacytomas and hybridomas served as sources of IgM. The plasmacytomas were MOPC-104E (26), TEPC-183 (26), and M104-76 (27). Three hybridomas were used: K6100.16 (28), HPCM3, and HPCM27 (29). All of the tumors were grown in BALB/c mice except K6100.16, which was grown in (NZB × BALB/c)_{F1} mice. The inbred mouse strains used in this study included BALB/c, C57BL/6, C.B-17, and an Ig-deficient mutant of C.B-17 (C.B-17scid) (30). All mouse strains were bred and maintained at this Institute.

Cells. Bone marrow, spleen, and lymph node cell suspensions of 12-wk-old BALB/c mice were made up aseptically in cold Hanks' balanced salt solution (HBSS) containing 1% bovine serum albumin. Red blood cells were lysed with NH₄Cl (31). Nucleated cells were counted in a hemacytometer; cell viability was determined by trypan blue exclusion.

Cultured cell lines included the IgM-producing tumors, M104-76 and M27, and a hybridoma (C2) (32) that deleted its productive μ allele (33). M27 produces IgM molecules that specifically bind phosphorylcholine (PC) (29). All cell lines were grown in RPMI 1640 (Whittaker M. A. Bioproducts, Walkersville, MD) supplemented with glutamine (2 mM), gentamycin (100 μ g/ml), 2-mercaptoethanol (2-ME) (5×10^{-5} M), and 20% fetal calf serum (Whittaker M. A. Bioproducts). This growth medium is hereafter referred to as RPMI 1640 medium.

Cell Incubations. All incubations were at 37°C in an atmosphere of 5% CO₂. Untreated cells (controls) were simply incubated in RPMI 1640 medium. Tunicamycin (Tm)-treated cells ($2-4 \times 10^6$ /ml) were incubated 4-6 h in RPMI 1640 medium containing 5 μ g/ml Tm (a gift of Dr. Robert Hamill, Eli Lilly Co., Indianapolis, IN). Stimulation of cells ($\sim 4 \times 10^6$ /ml) with *S. typhosa* 0901 lipopolysaccharide W (LPS) (Difco Laboratories, Inc., Detroit, MI) was carried out for 48 h in RPMI 1640 medium containing 20 μ g/ml LPS. For internal labeling of cell proteins, cells were washed twice in HBSS and then incubated for 4 h at $\sim 10^7$ cells/ml in RPMI 1640 medium containing 50 μ Ci/ml [³⁵S]methionine (New England Nuclear, Boston, MA).

Cell Lysates and Supernatants. Cells were washed twice in HBSS and suspended for 20 min in cold lysis buffer (0.5% Nonidet P-40 [NP-40], 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.02% Na₃, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin [Sigma Chemical Co., St. Louis, MO], and 10 mg/ml soybean trypsin inhibitor [Millipore Corp., Freehold, NJ]). Nuclei and debris were removed by centrifugation (10,000 g) for 10 min at 4°C. The supernatant was removed, frozen, and stored in liquid nitrogen.

M27 cell lysates and salt-precipitated [at 50% saturated (NH₄)₂SO₄] proteins of M27 cell supernates were specifically enriched for IgM by incubation with PC-Affigel (kindly supplied by Dr. A. Feeny, Medical Biology Institute, La Jolla, CA). IgM was specifically eluted from the PC-Affigel with PC (10^{-3} M).

Nonspecific Purification of Serum IgM. Serum Ig was precipitated and washed in 50% saturated (NH₄)₂SO₄. The precipitate was dissolved in 0.1 M PO₄ (pH 7.4), reprecipitated by extensive dialysis against distilled water, washed twice, and again dissolved in 0.1 M PO₄. After clarification by centrifugation (5,000 g), the Ig solution was size fractionated over a 1 × 90 cm column of Bio-Gel A (1.5m, 100-200 mesh; Bio-Rad Laboratories, Richmond, CA) using 0.1 M PO₄ as the eluting buffer. The IgM fraction, which eluted just after the column void volume, was concentrated and in some cases fractionated a second time.

Specific Purification of Serum IgM. The tumor IgM proteins of MOPC-104E (104E), K6100.16 (6100), HPCM3 (M3), and HPCM27 (M27) were specifically purified on the basis of their known binding specificities: 104E (14) was precipitated with α 1-3 dextran; 6100 (28) was precipitated with NP [(4-hydroxy-3-nitrophenyl)acetyl]-conjugated bovine serum albumin; and M3/M27 (29) were affinity purified by absorption onto PC-conjugated Affigel beads followed by elution with 10^{-3} M PC. These preparations are designated 104E(dx), 6100(NP), M3(PC), and M27(PC), respectively.

Electrophoretic Separation of IgM Heavy (H) Chains. Polyacrylamide gel electrophoresis (PAGE) of reduced IgM subunits in sodium dodecyl sulfate (SDS) was done according to

Laemmli (34). Cell lysates, cell supernatant preparations, serum, or purified protein samples were diluted in sample buffer (0.05 M Tris-HCl, pH 6.8 containing 4% SDS, 10% glycerol, and 1% pyronin Y) to twice the desired concentration and then added to an equal volume of sample buffer containing 0.04 M dithiothreitol. All samples were boiled for 5 min and then loaded onto a 5% polyacrylamide stacking gel (2 cm in length, containing 0.1% SDS in 0.125 M Tris-HCl, pH 6.8). The separating gel of 7.5 or 10% polyacrylamide was 8 cm in length and contained 0.1% SDS in 0.375 M Tris-HCl, pH 8.8. The electrolyte buffer (pH 8.8) contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Samples were electrophoresed for 2–3 h at constant power (~ 0.2 W/cm). Prestained molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) were routinely applied to all gels.

Two-dimensional gel electrophoresis was according to the technique of O'Farrell (35) except for minor modifications in the electrode solutions for isoelectric focusing (IEF). The cathode solution consisted of NaOH (0.2 M) and CaO (0.2 M) (to precipitate dissolved CO_2). This was prepared in advance, stored under vacuum, and filtered immediately before use. The anode solution consisted of 0.4 M aspartic acid and was prepared as needed. These modifications greatly reduced "cathode drift" (36) and resulted in a stable gradient over a pH range of 3–9. Samples of purified serum IgM were diluted in IEF sample buffer (9.2 M urea, 2% NP-40, 2% ampholine [pH 3.5–9.5], 5% 2-ME) and loaded on the basic end of tube gels consisting of 6% acrylamide, 15% *N,N'*-diallyltartardiamide (37), 2% NP-40, 2% ampholine (pH 3.5–9.5), and 9.2 M urea. Samples were electrophoresed for 5500 volt-hours. Afterwards, the tube gels were equilibrated with SDS sample buffer and electrophoresed in a second dimension in 10% acrylamide slab gels.

Western Blot Analysis. After gel electrophoresis, the separated proteins were electrophoretically transferred onto nitrocellulose in the manner described by Towbin et al. (38). This was done for 16–20 h at room temperature at constant current (60 mA). The electrolyte buffer (pH 8.3) contained 0.125 M Tris, 0.192 M glycine, and 20% (vol/vol) methanol. The nitrocellulose blot was subsequently incubated at room temperature with inert protein (2% casein in 0.075 M Tris-HCl, pH 7.5) for 3–4 h, then with antibody for 16 h, and finally with ^{125}I -labeled IgM of 104E (^{125}I -104E) or ^{125}I -labeled protein A (^{125}I -protein A) for 3 h. (Proteins were radiolabeled with ^{125}I [Amersham Corp., Arlington Heights, IL] using the chloramine T method of Hunter [39].) Between incubations, the nitrocellulose blot was washed with 0.1 M PO_4 buffer (pH 7.4). After the last incubation, the blot was again washed and then dried. A sandwich of the blot, x-ray film (X-Omat AR film; Eastman Kodak, Rochester, NY), and an intensifier screen (Du Pont Cronex Lightening-Plus XD; Du Pont Co., Wilmington, DE) was loaded into a film holder cassette and exposed at -70°C . The film was developed in a Kodak automatic x-ray film developer. The autoradiographs are referred to as Western blots. In some instances, the separated proteins were not transferred out of the gel, but instead fixed and stained with Coomassie Brilliant Blue or with silver nitrate (40).

Antibodies. Several independent antisera to mouse IgM were used to visualize IgM H chains. These included goat anti-IgM (Cappel Laboratories, Cochranville, PA), rabbit anti-IgM (Litton Bionetics, Inc., Kensington, MD), and our own rabbit anti-IgM. The latter was made against BALB/c IgM and absorbed with Sepharose 4-B beads conjugated with IgG2a (λ) of HOPC-1 (26) and IgG1 (κ) of MOPC-31c (26). 100 μl of a given antisera in a 10 ml solution of 0.075 M Tris/2% casein (pH 7.5) contained sufficient antibody for successive coating of eight or more nitrocellulose blots.

Rat monoclonal antibodies specific for μ chains (R1.42.22, R33.24.12, R33.60.2) were a gift of Dr. T. Imanishi-Kari (Massachusetts Institute of Technology, Center for Cancer Research, Boston, MA). Affinity-purified rabbit antibodies (28) specific for Ig H chain variable regions (anti-Igh-V) of 6100 or HPC 52 (41) were generously supplied by Dr. K. Karjalainen (Basel Institute for Immunology, Basel, Switzerland).

Results

Detection of μ' Chains in Secreted IgM. Electrophoretic separation of IgM subunits in SDS-containing acrylamide gels resolved two distinct sizes of H chains.

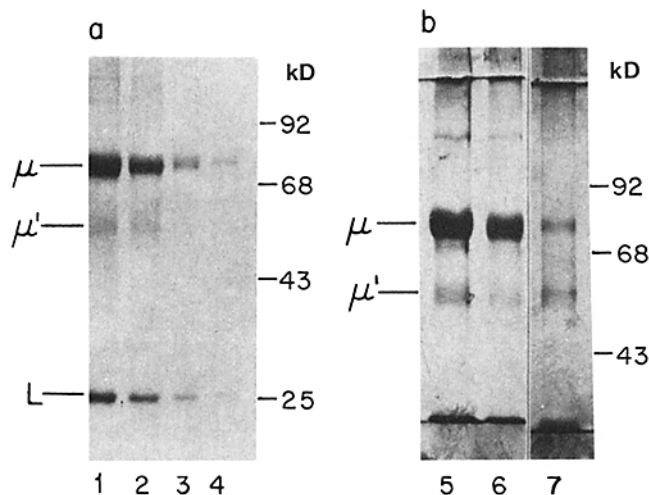


FIGURE 1. SDS-PAGE of reduced IgM subunits resolves two apparent sizes of heavy chain, denoted μ and μ' . (a) Polyacrylamide gel (10%) containing decreasing quantities of M3(PC) in lanes 1-4 (20, 10, 5, and 2 μ g). Proteins were stained with Coomassie Brilliant Blue. (b) Polyacrylamide gel (7.5%) containing purified serum IgM of TEPC-183-bearing BALB/c mice (30 μ g in lane 5; 15 μ g in lane 6) and of normal BALB/c mice (10 μ g in lane 7). Proteins were stained with silver nitrate.

This is illustrated in Fig. 1a for the PC-binding IgM of M3. PC affinity-purified IgM was reduced, and different concentrations were applied to a 10% gel that was subsequently stained with Coomassie Blue. Lanes 1 and 2, containing 20 and 10 μ g of M3(PC), respectively, show an H chain band labeled, μ' (~55 kD), in addition to the expected μ and L chain bands. The silver-stained 7.5% gel of Fig. 1b shows a similar result for purified serum IgM of normal BALB/c mice (lane 7) and of BALB/c mice bearing TEPC-183, an IgM-producing plasmacytoma (lanes 5 and 6). Ig L chains are not resolved in a 7.5% gel and they run in the solvent front. Note that the relative intensities of μ' and μ chains are comparable for normal serum IgM but disproportionate for TEPC-183 and M3.

μ' Chains Display $C\mu$ Antigenic Determinants. We also detected μ' chains with anti-IgM antibody, as shown by Western blot analysis. Nitrocellulose blots of acrylamide gels were sequentially overlaid with 2.5% casein, anti-IgM antibody, and ^{125}I -104E. Representative results are shown in Fig. 2. Two bands corresponding in size to μ and μ' chains are clearly seen for reduced IgM samples of TEPC-183 (Fig. 2, lane 3), M27(PC) (lane 4), BALB/c serum (lane 5), and C57BL/6 serum (lane 6). Controls for specificity included Ig-deficient serum of a C.B-17scid mouse (lane 7) and purified mouse IgG (lane 8). Nonreduced samples of TEPC-183 (lane 1) and M27(PC) (lane 2) gave only one band at the top of the 5% stacking gel.

Serologic detection of μ' chains was not dependent on a particular anti-IgM serum or labeled IgM indicator. In fact, three different monoclonal antibodies (R1.42.22, R33.24.12, and R33.60.2) specific for $C\mu$ determinants recognized μ' chains (see Fig. 3). Moreover, when isolated ^{125}I - μ chains were eluted from acrylamide gels and used as the labeled indicator (in lieu of ^{125}I -104E), the same

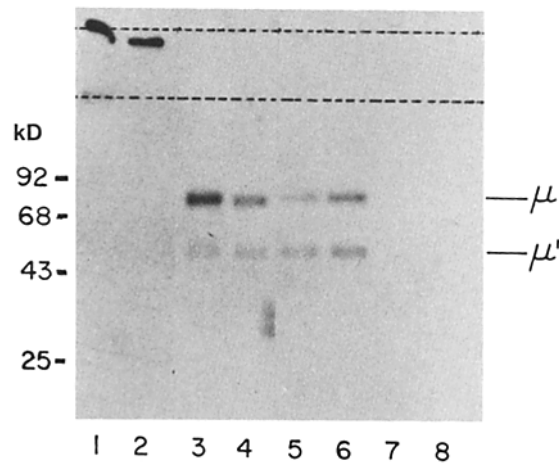
TRUNCATED μ CHAINS

FIGURE 2. SDS-PAGE (10%) Western blot overlaid with anti- μ and ^{125}I -104E, showing that μ' chains display $\text{C}\mu$ antigenic determinants. All samples were reduced except in lanes 1 and 2. (1 and 3) IgM of TEPC-183 (1 μg), (2 and 4) M27(PC) IgM (0.5 μg), (5), BALB/c serum (10 μl diluted 10^{-2}), (6) C57BL/6 serum (5 μl diluted 10^{-2}), (7) C.B-17scid mouse serum (10 μl diluted 5×10^{-1}), and (8) IgG of BALB/c (10 μg).

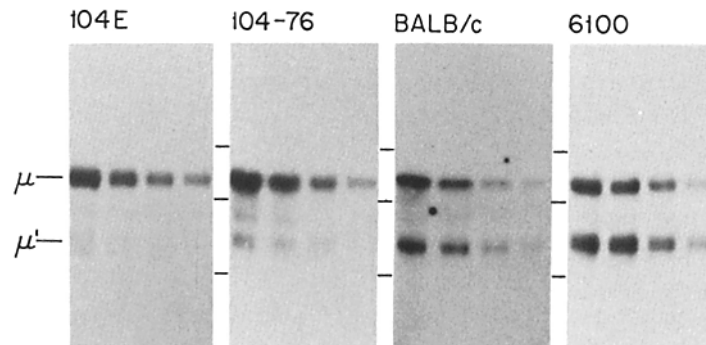


FIGURE 3. SDS-PAGE (7.5%) Western blots overlaid with monoclonal anti- μ (R33.24.12) and ^{125}I -104E, indicating variable proportions of μ' to μ chains in IgM of different IgM-producing tumors. Each of the four lanes, from left to right in each blot, containing decreasing quantities (10, 5.0, 2.5, 1.25 μg) of reduced 104E(dx), M104-76, BALB/c IgM, and 6100(NP). The positions of the molecular mass standards (92, 68, and 43 kD) are indicated between each blot.

results were obtained (data not shown). Thus, we infer that μ' and μ chains share similar (if not identical) $\text{C}\mu$ antigenic determinants.

Variable Proportions of μ' and μ Chains in Different IgM Populations. To estimate the proportion of IgM H chains corresponding to μ' chains, serial dilutions of mouse serum and various IgM preparations were reduced and electrophoresed in 7.5% gels. Nitrocellulose blots of the gels were overlaid with monoclonal anti- μ (R33.24.12) and ^{125}I -104E, and exposed to x-ray film. By superimposing the developed film over the blot, we were able to cut out nitrocellulose strips corresponding to radioactive bands of μ' and μ . The strips were counted in a gamma counter, and the percentage of counts (cpm) associated

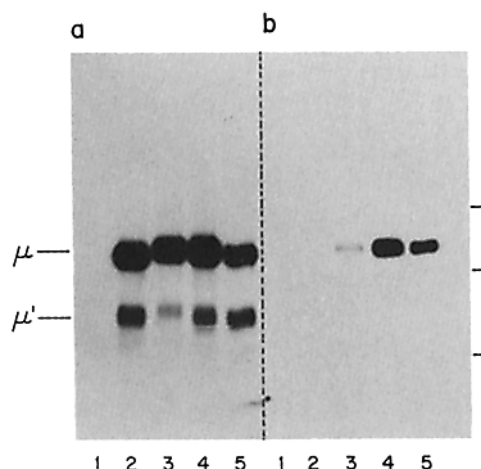


FIGURE 4. Evidence that μ' chains lack Igh-V determinants. Duplicate halves of an SDS-PAGE (7.5%) Western blot overlaid (a) with rabbit anti- μ and ^{125}I -protein A and (b) with affinity-purified rabbit anti-6100 Igh-V and ^{125}I -protein A. All samples were reduced. (Lane 1) 10 μg of IgG2a (λ) (from HOPC-1); (2) 5 μg of M3(PC) IgM; (3) 10 μg of 104E IgM; (4) 5 μg of TEPC-183 IgM; and (5) 2 μg of 6100(NP) IgM. The positions of the molecular mass standards (92, 68, and 43 kD) are indicated to the right of lane 5.

with the μ' band was calculated from the relationship: $[\text{cpm } \mu' / (\text{cpm } \mu' + \text{cpm } \mu)] \times 100$.

In Fig. 3, we show the autoradiographs of four such titrations. As can be seen, μ' chains were barely detectable in IgM of MOPC-104E (<1%) and relatively low amounts were in the IgM of M104-76 ($\leq 5\%$). In contrast, μ' chains accounted for $\geq 30\%$ of the H chains in the normal serum IgM and for $\sim 50\%$ in the IgM of 6100. A strictly quantitative interpretation of these results, however, is not possible as the ^{125}I cpm associated with μ' (or μ) chains was not linearly related to the concentration of IgM added to the gel (slope of \log_2 cpm vs. \log_2 concentration of IgM = 0.6–0.7). Moreover, the implicit assumption in these analyses that monoclonal anti- μ binds to μ' and μ chains with the same stoichiometry and affinity is unproven.

The estimated yield of L chains relative to IgM H chains was reasonably constant for all preparations and independent of wide variations in the estimated proportion of μ' chains. This conclusion is based on an analysis similar to that above except that for this we used anti-L chain-specific antisera and the appropriate ^{125}I -labeled indicator (data not shown). This suggests that L chains are able to associate with μ' chains.

μ' Chains Lack Igh-V Antigenic Determinants. To test for the presence of Igh-V determinants on μ' chains, affinity-purified anti-Igh-V sera and anti- μ antibody were applied separately, to duplicate halves of a given nitrocellulose blot containing a panel of reduced Ig proteins. An appropriate ^{125}I -labeled indicator was subsequently added to each blot. Figs. 4 and 5 show the results obtained with two anti-Igh-V sera, anti-Igh-V6100 and anti-Igh-V HPC 52. Neither of these antisera reacted with μ' chains, although they did recognize the μ chains of all but two IgM preparations. Anti-Igh-V6100 crossreacted with μ chains of TEPC-

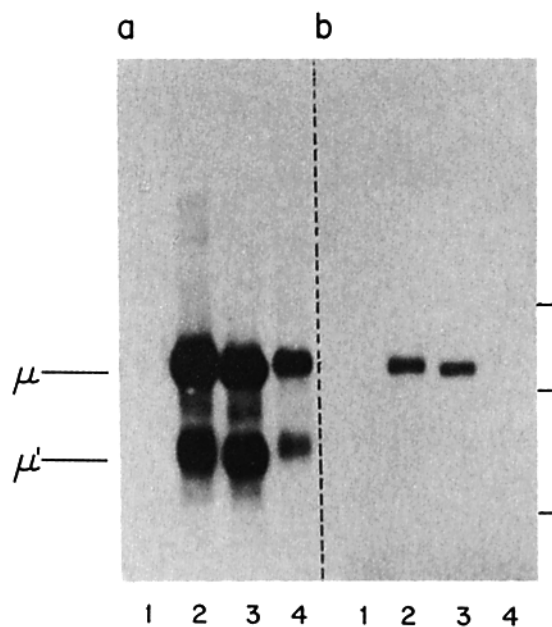


FIGURE 5. Evidence that μ' chains lack Igh-V determinants. Duplicate halves of an SDS-PAGE (7.5%) Western blot overlaid (a) with rabbit anti- μ and ^{125}I -protein A and (b) with affinity-purified anti-HPC-52 Igh-V and ^{125}I -protein A. All samples were reduced. (Lane 1) 20 μg of BALB/c IgG; (2) 30 μg of TEPC-183 IgM; (3) 30 μg of M3(PC) IgM; and (4) 10 μg of BALB/c IgM. The positions of the molecular mass standards (92, 68, and 43 kD) are indicated to the right of lane 4.

183 and MOPC-104E (Fig. 4b, lanes 3 and 4) but not with those of M3 (lane 2). Similarly, anti-Igh-V HPC 52 showed a detectable crossreaction with μ chains of TEPC-183 and M3 (Fig. 5b, lanes 2 and 3) but not with μ chains of normal IgM of BALB/c mice (lane 4). These results clearly demonstrate the Igh-V specificity of the antisera and the apparent absence of Igh-V determinants on μ' chains.

Two broadly reactive anti-Igh-V sera (anti-Igh-V104E and anti-Igh-Vnp) (28) also failed to reveal μ' chains (data not shown). Therefore, we infer that μ' chains are missing Igh-V determinants and probably all of the variable region. Consistent with this inference is the restricted charge heterogeneity of μ' chains. This was demonstrated by two-dimensional gel analysis of serum IgM of normal C57BL/6 mice. IgM subunits were subjected to IEF in one dimension and to SDS-PAGE (10%) in the second dimension. Western blot analysis of the gel (with anti- μ and ^{125}I -104E) showed μ' chain banding from pH 5.5 to 6.0, whereas that of μ chains extended from pH 5.5 to 7.8 (Fig. 6).

Detection of Intracellular μ' Chains. Low levels of intracellular μ' chains were detected in both of the IgM-producing tumors examined, M104-76 and M27. Results are shown only for the M27 cell line with the molecular mass (kD) of μ' and μ chains indicated in parenthesis (Fig. 7). M27 cell lysates, prepared from cells that had been incubated 4 h in medium containing unlabeled methionine or labeled [^{35}S]methionine, were specifically enriched for IgM by adsorption onto PC-Affigel, followed by elution with PC (10^{-3} M). Both polyclonal and monoclonal anti- μ readily detected μ (78) and μ' (55) chains in this material, as

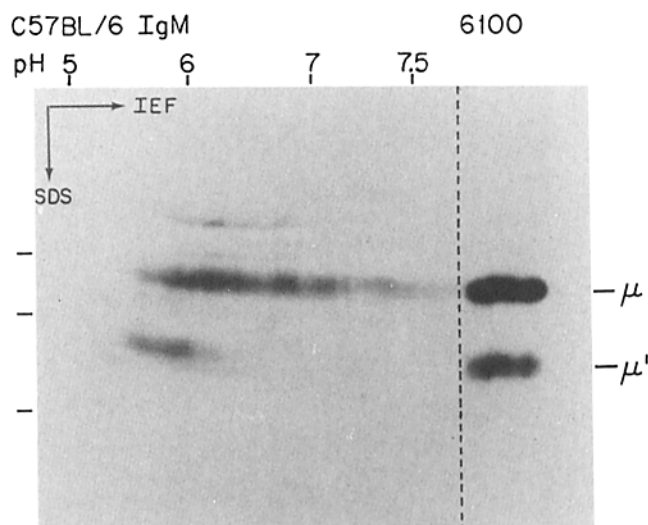


FIGURE 6. Western blot of two-dimensional gel of reduced C57BL/6 IgM showing restricted charge heterogeneity of μ' vs. μ chains. To serve as marker for μ and μ' chains, purified 6100 IgM (10 μ g) was added at the alkaline end of the IEF tube gel before electrophoresis in SDS-containing polyacrylamide (10%). The nitrocellulose blot was overlaid with anti- μ and 125 I-104E. The positions of the molecular mass standards (92, 68, and 43 kD) are indicated on the left.

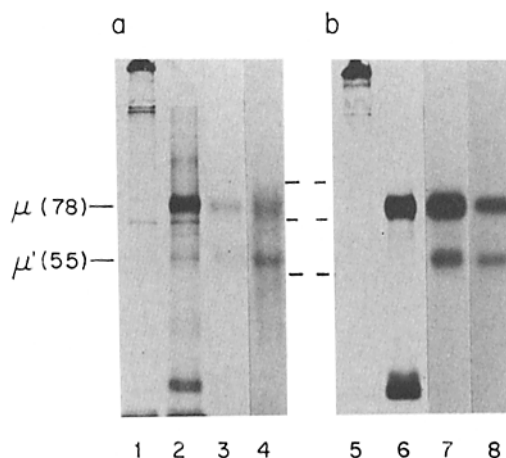


FIGURE 7. SDS-PAGE (10%) blots of reduced and nonreduced M27(PC) cell lysate and medium. (a) Nonreduced (lane 1) and reduced (2) M27(PC) lysate from 2×10^7 cells labeled with [35 S]methionine. (3 and 4) Reduced cell lysate from unlabeled cells overlaid with anti- μ / 125 I-104E and with monoclonal anti- μ (R33.24.1)/ 125 I-104E, respectively. (b) Nonreduced (lane 5) and reduced (6) M27(PC) medium of [35 S]methionine-labeled cells. (7 and 8) Overlaid with anti- μ / 125 I-104E and containing reduced M27(PC) medium of unlabeled cells (2×10^7) and reduced serum-free medium of M27 cells, respectively. The positions of the molecular mass standards (92, 68, and 43 kD) are indicated between lanes 4 and 5.

shown by Western blot analysis (Fig. 7, lanes 3 and 4). In addition, a blot of the internally labeled polypeptides of [35 S]methionine-labeled cells showed bands corresponding to μ (78) and μ' (55) chains (lane 2); neither of these bands were detected in lane 1 containing the unreduced sample. Clearly, μ' (55) accounted

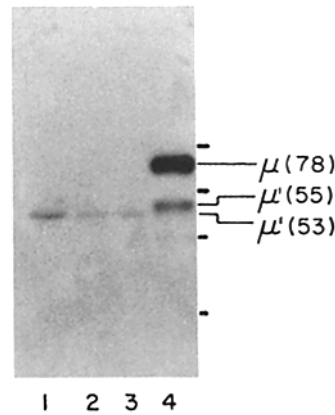


FIGURE 8. SDS-PAGE (10%) Western blot of cell lysates from the C2 cell line. The blot was overlaid with anti- μ ¹²⁵I-104E. (Lane 1) Reduced C2 lysate; (2) reduced lysate of Tm-treated C2 cells; (3) nonreduced C2 cell lysate; and (4) reduced BALB/c serum. The positions of the molecular mass standards (92, 68, 43, and 25 kD) are indicated to the right of lane 4.

for only a small proportion (~5%) of the recently synthesized intracellular IgM H chains. None of the recently synthesized μ' (55) chains could be detected in the PC affinity-purified IgM of the cell medium (Fig. 7, lane 5). Note, however, that the cell medium of the unlabeled control cells did in fact contain secreted μ' (55) chains (Fig. 7, lane 7) as detected by Western blot analysis; μ' (55) chains were also detected in serum-free medium of M27 cells (lane 8) (defined serum-free medium [42] was kindly supplied by Dr. D. Mosier, Medical Biology Institute, La Jolla, CA).

Truncated μ chains were also detected in a hybridoma cell line (C2) lacking a productive μ allele (33) (Fig. 8, lane 1). The size of the C2 μ' chains [μ' (53)] was slightly smaller than that of M27 but equivalent to that of bone marrow cells, as shown later. However, unlike bone marrow cells, C2 cells failed to produce detectable unglycosylated μ' chains in the presence of Tm (Fig. 8, lane 2); also, the μ' chains of C2 were apparently not crosslinked by disulfide bonds since their migration of SDS-PAGE was unaffected by the presence or absence of DTT (Fig. 8, lane 1 vs. 3).

Tissue-specific Differences in the Production of μ' Chains. Western blots of normal bone marrow cell lysates showed a prominent μ' (53) band in addition to that of μ (76) (Fig. 9a, lane 2). Incubation of bone marrow cells in tissue culture medium for 6 h (Fig. 9a, lane 3) resulted in the faint appearance of μ (80) and μ' (57) chains as well. After 48 h incubation, the μ (80) and μ (76) bands were each of comparable intensity, as were the μ' (57) and μ' (53) bands (Fig. 9b, lane 9). Inclusion of LPS in the medium during the 48 h incubation did not alter the result (Fig. 9b, lane 10). In the presence of Tm, bone marrow cells produced substantial quantities of unglycosylated μ [μ (62)] and μ' [μ' (42)] chains (Fig. 9a, lane 4); interestingly, μ' (53) was no longer detectable, suggesting a fast turnover of μ' chains in bone marrow cells (Fig. 9a, lane 4). This contrast differs from our results with Tm-treated C2 cells, which contained μ' (53) but no detectable μ' (42) (see Fig. 8, lane 3). Also, unlike C2 cells, the μ' (53) chains in bone marrow

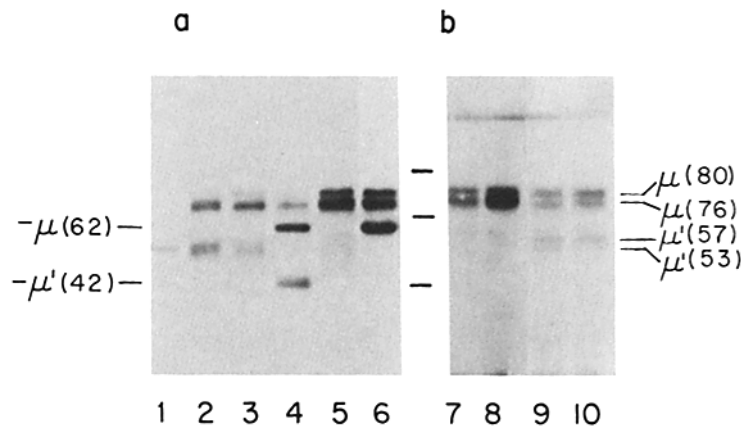


FIGURE 9. SDS-PAGE (10%) Western blots of reduced bone marrow and spleen cell lysates of BALB/c mice. Blots *a* and *b* were overlaid with anti- μ - ^{125}I -104E. The lysates in each lane were from: C2 cells (1); fresh bone marrow cells (2); bone marrow cells incubated for 6 h in the absence (3) or presence (4) of Tm; spleen cells incubated for 6 h in the absence (5) or presence (6) of Tm; spleen cells incubated for 48 h in the absence (7) or presence (8) of LPS; bone marrow cells incubated for 48 h in the absence (9) or presence (10) of LPS. Cell viability (73–87%) remained reasonably constant throughout the incubation period for all cell preparations. The positions of the molecular mass standards (92, 68, and 43 kD) are indicated between lanes 6 and 7.

cells were disulfide bonded (presumably with μ chains) so that their separation in SDS-PAGE required reduction (data not shown).

In striking contrast to bone marrow cells, spleen cells that were incubated for 6 h in RPM1 1640 medium contained little or no detectable μ' chains despite the production of relatively large quantities of $\mu(80)$ and $\mu(76)$ chains (Fig. 9*a*, lane 5). Similar results were obtained for nonincubated spleen and lymph node cells (data not shown) and for spleen cells incubated 48 h, with or without LPS in the medium (Fig. 9*b*, lanes 7 and 8). Spleen cells incubated in the presence of Tm for 6 h showed only one species of unglycosylated μ chain [$-\mu(62)$ chains] (Fig. 9, lane 6).

Quantitation of the radioactivity associated with the $\mu'(53)$, $\mu(76)$, and $\mu(80)$ bands of several Western blots of bone marrow, spleen, and lymph node lysates showed that $\mu'(53)$ chains accounted for $\geq 30\%$ of the detected IgM H chains in bone marrow vs. $\leq 1\%$ of IgM H chains in spleen and in lymph node cells. This analysis was done as described for Fig. 3.

Discussion

The preceding results indicate the presence of μ' chains in serum IgM, IgM-producing tumor cells, and in normal bone marrow cells. In the following discussion we consider (a) the evidence that μ' chains are in fact truncated μ chains lacking variable regions; (b) the possible basis of μ' chains; and (c) the implications of μ' chains for the valence of IgM.

Evidence that μ' Chains Are Truncated μ Chains. μ' Chains were serologically detected by Western blot analysis: nitrocellulose blots of SDS-PAGE gels containing reduced IgM were overlaid sequentially with anti- μ and ^{125}I -IgM of

MOPC-104E. Detection of antigen by this procedure depends on antibody crosslinking of labeled antigen (reference antigen) to the bound antigen. This requires high affinity antibodies and close homology between the crosslinked antigens, as shown previously in this laboratory (43, 44) with polystyrene as the solid phase absorbent. Crossreactions between low affinity antibodies and unrelated antigens are not measured. Using the above procedure, μ' chains were found in secreted IgM and in cell lysates of IgM-producing cell lines, but not in purified preparations of IgG nor in serum of IgM-deficient C.B-17*scid* mice (30). Further, μ' chains were detected by three independent monoclonal antibodies specific for $C\mu$ determinants. We conclude that μ' chains have $C\mu$ determinants in common with μ chains and are produced by IgM-synthesizing cells.

A second indication that μ' chains are truncated μ chains was their association with specifically purified IgM molecules. Since μ' chains appeared to lack variable regions (as discussed below), the copurification of μ' and μ chains would not be expected unless both were part of the same molecule. Further, when affinity-purified IgM was not reduced, μ' chains were not detected in the separating gel. This suggests that μ and μ' chains are linked together by disulfide bonds.

Evidence that μ' Chains Are Missing Variable Regions. Antisera specific for Igh-V antigenic determinants failed to react with μ' chains. Four distinct anti-Igh-V sera were tested: anti-Igh-V6100, anti-Igh-V HPC 52, anti-Igh-V104E, and anti-Igh-Vnp. The first three antisera were made against purified Igh-V fragments, affinity purified, and shown to be specific for Igh-V determinants (28). In all of the examples in which the μ chains of a given IgM-producing tumor reacted with a particular anti-Igh-V sera, the corresponding μ' chains were unreactive. From this we infer μ' chains are lacking variable regions. Consistent with this, μ' and μ chains from serum IgM of normal mice showed striking differences in charge heterogeneity; μ' chains displayed very little charge heterogeneity compared with that of μ chains (see Fig. 6). This too indicates μ' chains lack a variable region since charge heterogeneity of a given class of Ig chains primarily reflects variable region diversity (45).

Possible Basis for μ' Chains. The proportion of μ' to μ chains varied widely according to the particular source of IgM H chains. For example, $\geq 30\%$ of the H chains in secreted IgM of normal mice corresponded to μ' chains [$\text{cpm } \mu' / \text{cpm } \mu' + \text{cpm } \mu \times 100$] whereas, for most IgM-producing tumors, this value was $\leq 5\%$. Intracellular μ' chains generally accounted for $\geq 30\%$ of the detected IgM H chains in bone marrow lysates as opposed to $\leq 1\%$ in spleen lysates.

Differences in the proportion of μ' to μ chains could represent the extent of μ chain breakdown into μ' chains. If true, intracellular proteases would be implicated since μ' chains were shown to be present in serum-free medium of M27 cells. Moreover, the activity of such proteases would have to be under tissue-specific control to account for the prevalence of μ' chains in bone marrow vs. spleen cells. Evidence for protease degradation of μ chains in bone marrow pre-B cells was recently reported by Thorens et al. (46). These investigators noted that most μ chains were degraded into a μ fragment of ~ 48 kD when cell lysis was performed in the absence of protease inhibitors. However, our results with lysates of unfractionated bone marrow cells differ from theirs in that we detected μ' chains in the presence of protease inhibitors and in quantities comparable to

those of conventional-size μ chains. Moreover, our detection of μ' chains in the C2 cell line suggests that they are not breakdown products of μ chains. The C2 cell line deleted its productive μ allele (33) and could not have synthesized μ chains; indeed, none were detected. Thus, we are led to consider that μ' chains may derive from a truncated species of μ mRNA.

Evidence consistent with this latter possibility is the recent finding (47) that some pre-B cells transformed by Abelson murine leukemia virus (A-MuLV) contain mRNA transcripts that encode truncated μ chains. Analysis of many A-MuLV-transformed cell lines has indicated that the joining of diversity (D) and heavy chain joining region (J_H) gene segments represents the first *Igh* gene rearrangement in pre-B cells (48), and that most D gene segments have upstream initiation sites such that in-phase joining of D and J_H results in synthesis of truncated μ chains ($D\mu$ proteins) (47). $D\mu$ proteins contain a variable DJ_H peptide, four $C\mu$ domains, and a μ secretory (μ_s) or μ membrane (μ_m) terminus; secondary joining of a V gene to the DJ_H complex (productive or nonproductive) may result in the loss of $D\mu$ protein synthesis (47).

The question arises whether productive $C\mu$ RNA transcripts may be included in the class of $C\mu$ RNA known as $S\mu$ -RNA (for sterile). Various sizes of $S\mu$ -RNA, 1.9–3.0 kb, have been reported in pre-B, B, T, and other cell types (33, 49–51). $S\mu$ -RNA occur independently of productive μ mRNA (49) and may originate from unrearranged μ loci (51). $S\mu$ -RNA lack *Igh*-V sequences (49, 51) and appear not to be translated into $C\mu$ protein (52). Our findings, however, indicate that some $S\mu$ -RNA may in fact encode μ' chains. Western blot analysis of cell lysate from the C2 cell line, which has deleted its productive μ allele and contains only the so-called $S\mu$ -RNA, showed detectable μ' chains (Fig. 8). This is consistent with previous findings (33) indicating that $S\mu$ -RNA in the 70Z/3, 38C-13, and C2 cell lines have different possible initiation sites, one of which is located ~0.3 kb upstream of the DQ52 gene segment. Also, $S\mu$ -RNA show the same regulated termination at μ_s or μ_m polyadenylation sites as do productive μ -mRNA (33, 49, 51). In the case of the C2 cell line, all the $S\mu$ RNA terminate at the μ_s site (33), consistent with the idea that μ' (53) corresponds to an incompletely glycosylated μ' chain.

Implications of μ' Chains for IgM Valence. The presence of μ' chains in serum IgM implies that many IgM antibodies have fewer antigen-binding sites than theoretically predicted. Relevant to this point are many conflicting reports concerning the valence of IgM molecules. First, it has long been known that reductive 7 S subunits of IgM antibodies (H2L2) appear univalent; i.e., they bind but do not crosslink antigen (as reviewed in reference 7). Indeed, the first reported measurements of the valence of IgM indicated there were about five antigen-binding sites per IgM molecule and about one binding site per reductive 7S subunit (15). One study noted that only 50% of the reductive halfmers (HL) of IgM anti-Forssman antibody would bind antigen (19). Some early reports suggested there were 10 nonequivalent antigen-binding sites per IgM pentamer (16–18); i.e., 5 high-affinity binding sites (10^6 to 10^7 liters/mol) and 5 low-affinity binding sites (10^4 to 10^5 liters/mol). Other reports (12–14) described 10 equivalent binding sites for each IgM pentamer; interestingly, in the cases studied therein, the binding affinities were relatively low ($\sim 10^4$ liters/mol). While some

examples of <10 (equivalent) binding sites may be attributed to steric hindrance of antigen binding and/or heterogeneity of IgM antibodies (23, 24), other examples appear free of this criticism. This is especially true in the case of a recent report (22) that two independent populations of monoclonal IgM antibodies specific for DNP each contained an average of only one antigen binding site per IgM monomer.

The apparent inconsistencies between the above-mentioned reports may reflect variations in individual IgM populations. For instance, we estimated IgM of MOPC-104E to have $<1\%$ of its H chains as μ' chains. This is consistent with previous data showing each IgM pentamer of 104E to have 10 homogeneous binding sites (14). Further, H chains of the 104E IGM have been isolated and sequenced, and no truncated μ chains were noted (11). In contrast to the 104E results, the estimated percentage of μ' H chains in serum IgM of normal mice was $\geq 30\%$. This suggests that many IgM subunits contain a pair of μ - μ' chains and only one high affinity antigen-binding site, consistent with a number of the reports cited above.

Whether truncated μ chains are of any functional consequence to B cell differentiation is, of course, open to debate (47, 53). The mere existence of intracellular μ' chains and their prevalence in normal bone marrow cells suggests a possible regulatory role in early B cell development. However, as pointed out elsewhere (53), μ' chains need not serve an obligate role in B cell development since their synthesis is not a prerequisite for productive VDJ_H joining (54), which in most plasmacytomas and hybridomas appears to occur on μ alleles, with the primary DJ_H join not in phase (55-57).

While μ' chains may not significantly affect the function of secreted pentameric IgM, they could affect the ability of antigen to crosslink monomeric IgM on the cell surface. In addition to μ' (53) chains, we detected the appearance of μ' (57) chains in bone marrow cells after their in vitro incubation in tissue culture medium for ≥ 24 h (see Fig. 9 a, lane 6). The latter could correspond to truncated μ_m chains. Accordingly, some B cells may insert a high proportion of μ - μ' -L₂ molecules on their surface. This would mean relatively few divalent IgM molecules and less efficient cross-linking of IgM by antigen; this might render affected B cells less responsive (or unresponsive) to antigen activation. One is left with the enigma of how IgM molecules containing μ' chains ever get to be secreted at all. These and other issues concerning the basis and significance of μ' chains remain to be resolved.

Summary

Secreted IgM was shown to contain truncated μ (μ') chains with an apparent molecular mass of ~ 55 kD. The estimated percentage of IgM heavy (H) chains in the μ' form ranged from $\leq 1\%$ in the case of one tumor IgM protein (104E) to $\geq 30\%$ in normal serum IgM. Serum μ' chains lacked antigenic determinants characteristic of immunoglobulin variable regions and showed a restricted isoelectric focusing pattern compared with that of conventional μ chains. Intracellular μ' chains were readily detected in bone marrow cells but not in spleen or lymph node cells; μ' chains were also detected in IgM-producing tumor cells and in a hybridoma cell line that deleted its productive μ allele. These results predict

irregularities in IgM structure and recall an old controversy concerning the valence of IgM molecules.

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