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Functional Properties of Isotype-Switched Immunoglobulin M (IgM) and IgG Monoclonal Antibodies to *Pseudomonas aeruginosa* Lipopolysaccharide

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Controversy exists regarding isotype-related differences in the antibacterial and protective properties of lipopolysaccharide (LPS)-specific antibodies of the immunoglobulin M (IgM) class and various IgG subclasses. To clarify this issue, a murine hybridoma secreting IgM monoclonal antibody (MAb) specific for the O polysaccharide of *Pseudomonas aeruginosa* serogroup O6 LPS was class switched, by sib selection, to produce an IgG3 MAb with identical specificity and variable region heavy and light chain nucleotide sequences. This IgG3-secreting cell line was further switched to the production of O-specific, variable region-identical IgG1, IgG2b, and IgG2a MAbs. Functional comparisons of these LPS-specific IgM and IgG MAb isotypes revealed similar LPS binding, opsonic, and protective activities. Relatively minor isotype-related differences in levels of efficiency of MAb-mediated, complement-dependent opsonophagocytic killing (IgM > IgG2a > IgG3 > IgG2b > IgG1) were not associated with corresponding differences in in vivo functions. These findings, in conjunction with previously published data, support a cautious approach to generic conclusions regarding the immunotherapeutic superiority of LPS-specific antibodies belonging to either the IgM or IgG class or to a particular IgG subclass.

Antibodies to bacterial lipopolysaccharides (LPS) mediate antibacterial, endotoxin-neutralizing, and protective functions (1, 15). These antibodies play a critical role in natural and acquired immunity to gram-negative bacterial disease and are therefore appropriately considered possible therapeutic agents in gram-negative bacterial infections. LPS-specific monoclonal antibodies (MAbs) may be the most effective vehicles for such therapy (31). Yet, there is ongoing debate concerning the practicality of developing either highly specific MAbs directed toward the hypervariable O polysaccharide portion of the LPS macromolecule or broadly cross-reactive MAbs that recognize phylogenetically conserved substructures in the core or lipid A region of LPS (2, 4, 7, 28).

Experimental interest in the functional properties of anti-LPS antibodies in relation to epitope specificity and bacterial cross-reactivity has been supplemented by a growing interest in the functional implications of antibody isotype, particularly with respect to the therapeutic potential of LPS-specific immunoglobulin M (IgM) versus IgG (3, 8, 9, 11, 14, 17, 20, 21–23, 32). The scientific literature is confusing and somewhat contradictory on this subject, however, in part because published studies have utilized diverse bacteria, polyclonal or idiotypically distinct MAbs, and a variety of different in vitro and in vivo assays of antibody function.

It was the purpose of this study to switch the isotype of a murine hybridoma secreting IgM MAb reactive with the O polysaccharide of *Pseudomonas aeruginosa* serogroup O6 LPS to produce various IgG subclasses having identical specificities and variable region heavy and light chain nucleotide sequences. Further characterization of this variable region-matched set of LPS O side chain-specific IgM and IgG MAb isotypes, it was hoped, would serve as a basis for delineating possible isotype-related variations in antibody functions in relation to this particular bacterium, LPS, and antibody idiotype. It was anticipated that this sharply focused yet definitive analysis, interpreted in conjunction with previously published data, would generate broader insights regarding the influence of Ig class and subclass on LPS-specific antibody function.

MATERIALS AND METHODS

***P. aeruginosa* strains.** The Fisher immunotype 1 (It-1) strain (also referred to here as antigenic subtype 6a of *P. aeruginosa* serogroup O6) was obtained from the American Type Culture Collection, Rockville, Md. (ATCC 27312). The sources of other serogroup O6 subtype strains (referred to as 6ab, 6ac, 6ad, and Habs 6) were previously described (12).

LPS. LPS was extracted from *P. aeruginosa* strains by the phenol-water method (29). The extracted material was digested with nucleases and pronase, ultracentrifuged, and chromatographed over a Sepharose CL4B column (13). The resulting material contained 3 to 5% 2-keto-3-deoxyoctulosonic acid and <1% contaminating nucleic acids and proteins.

ELISA. The enzyme-linked immunosorbent assay (ELISA) protocol used in connection with initial hybridoma screening and cloning (see below) was the same as that previously described (18) except that LPS purified from subtype 6ad of *P. aeruginosa* serogroup O6 was used as the coating antigen at a concentration of 25 μg/ml and the antibody probes employed were rabbit anti-mouse IgG plus IgM plus IgA. In subsequent analyses by ELISA of binding activities of various MAb isotypes, we compared microtiter plates coated with either 25 μg of purified It-1 LPS per ml or 10^4 heat-killed It-1 bacteria per ml. Two types of antibody probes were used in these binding comparisons: (i) a rabbit antiserum specific for heavy and light chains of mouse IgG, IgM, and IgA; and (ii) a rabbit anti-mouse κ light chain-specific antiserum. Mouse IgM and IgG negative control MAbs were employed in ELISA and opsonophagocytic killing assays; both MAbs are specific for the outer membrane immobilization antigen D of *Paramecium multimicronucleatum.*

A capture ELISA was employed for sib selection with 96-well polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) coated overnight at 4°C with 7.5 to 10 μg of γ chain-specific rabbit anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, Calif.) per ml. Subsequent 30- to 60-min incubations, separated by washing steps, were carried out sequentially at room temperature in the presence of the following additives: hybridoma culture supernatant; biotinylated rabbit anti-mouse IgG1 (gamma 1 specific), IgG2a (gamma 2a specific), IgG2b (gamma 2b specific), or IgG3 (gamma 3 specific) (all from Zymed; all used at dilutions of 1:1,000 to 1:5,000); alkaline phosphatase-streptavidin

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(Zymed); and *p*-nitrophenylphosphate substrate (Sigma 104), 1 g/ml in 10% diethanolamine, pH 9.8. The A_{405} was read with a Titertek Multiscan Micro-ELISA spectrophotometer (Flow Laboratories, Inc., McLean, Va.).

Preparation of hybridoma 1F6. The murine hybridoma designated Ld3-4E6-1F6, or simply 1F6, was prepared as previously described (19). The 1F6 hybridoma secretes an IgM MAb reactive with the LPS O side chains from all five *P. aeruginosa* serogroup O6 subtype strains, including Fisher It-1 and subtype 6ad (19). The spleen cell donor for the cell fusion giving rise to the 1F6 hybridoma was immunized by two intraperitoneal (i.p.) injections, 3 months apart, of 5×10^6 live *P. aeruginosa* serogroup O6 subtype 6ad bacteria, suspended in saline; the second injection 2 months later by a single i.p. booster shot consisted of 10^8 heat-killed bacteria of the same strain. The cell fusion protocol was implemented 3 days after the final immunization. Hybridomas from this fusion were screened by ELISA with 96-well microtiter plates coated overnight with 25 μg of LPS purified from serogroup O6 subtype 6ad bacteria per ml. The 1F6 hybridoma cell line was cloned by limiting dilution and adapted to mouse ascites.

Isotype switching: mutagen treatment of hybridomas and sib selection (25, 26). Hybridoma Ld3-4E6-1F6 was grown in a 150-cm^2 tissue culture flask (Costar, Cambridge, Mass.) and adjusted to a cell density of 10^6 cells per ml in fresh Dulbecco's modified Eagle medium (Hazleton Biologics, Inc., Lenexa, Kans.) supplemented with 5% fetal calf serum (HyClone Laboratories, Logan, Utah), 10% NCTC 109 (BioWhittaker, Walkersville, Md.), nonessential amino acids, sodium pyruvate, and gentamicin. The acridine mutagen ICR-191 (Raylo Chemicals) was added to the hybridoma cells at a concentration of 0.5 to 3.0 μg/ml, and the mixture was incubated in six-well culture plates (Costar), 1 ml per well, overnight at 37°C in an atmosphere containing 5% CO_2. The cells were then adjusted to 10^4 cells per ml in fresh medium, distributed in 96-well tissue culture plates (Costar) at a density of 1,000 cells per well, and incubated at 37°C for 7 days. At the end of this incubation period, cell supernatants were tested by capture ELISA (see above) for the presence of IgG antibodies, with appropriate subclass-specific antibody probes. Positive wells were expanded into new 96-well plates at a density of 100 cells per well, and positive cultures from these plates were subsequently cloned by limiting dilution.

The process of mutagenization and sib selection was repeated sequentially with appropriate IgG subclass-specific antibody probes for each of the IgG subclasses downstream of the IgG3-secreting class-switched hybridoma originally produced from the parent IgM-secreting 1F6 cell line. The isotypes of secreted MAb products were confirmed by capture ELISA (see above) performed on tissue culture supernatants from cloned cell lines with biotinylated class- and subclass-specific rabbit anti-mouse probes. All isotype-switched hybridomas, like the parent cell line, were adapted to growth as mouse ascites tumors. Monoclonal antibodies present in mouse ascites fluid were quantified by radial immunodiffusion with mouse isotype-specific agarose plates (Tago, Inc., Burlingame, Calif.).

Determination of MAb variable region cDNA sequences. Total RNA from each subcloned mutagenized hybridoma was extracted from 10^7 hybridoma cells with the rapid total RNA isolation kit (5 Prime-3 Prime, Boulder, Colo.) and mRNA purified on an oligo(dT) affinity column. Next, cDNA was prepared with avian myeloblastosis virus reverse transcriptase and random hexamer primers. The variable regions were then amplified from the cDNA with a PCR GeneAmp core reagent kit (Perkin-Elmer, Danbury, Conn.) for 20 to 25 cycles. Previously described degenerate primers (5) were used to amplify κ light chain and IgG1, IgG2a, and IgG2b heavy chain variable region cDNA. The 5′ primers hybridized within the leader region, and the 3′ primers hybridized within the heavy chain constant regions at amino acids 121 to 129 and within the κ chain constant region at amino acids 116 to 122. For amplification of the IgG3 and IgM cDNAs, the following 3′ primers were used: IgG3, 5′-AGC GGA TCC GTC ACT GCA GCC AGG GAC CAA GGG ATA GAC; and IgM, 5′-GGG GGA TCC GCT CTC GCA GGA GAC GAG GGG GAA GAC ATT.

PCR products were cloned into the pCRII vector (TA cloning kit, InVitrogen Corp., San Diego, Calif.) for direct cloning. Plasmid DNA was prepared from clones with apparently correct DNA inserts and sequenced by the Molecular Medicine Unit at Beth Israel Hospital, Boston, Mass., with an automated sequencer. A minimum of three clones from separate PCRs were sequenced in both directions to document the fidelity of the *Taq* DNA polymerase and to rule out potential sequencing errors or ambiguities. Although minor discrepancies were noted among the individual PCR products of particular heavy or light chain variable region sequences, these were determined to be either *Taq* DNA polymerase-induced or sequencing errors, since no discrepancies occurred at the same position in more than one of the three sequences derived for each variable region from the parental and four isotype-switched hybridoma cell lines. Thus, the variable region sequences from the five hybridoma cell lines were identical.

Immunoblots. As previously described (12, 16), whole bacteria (6×10^6 CFU/10 μl) or purified LPS (2.5 mg per lane) was boiled in sample buffer for 5 min and run at 30 mA per gel at 4°C for 3 h in a Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, using a dual slab apparatus (Bio-Rad, Richmond, Calif.) with a 4% stacking gel and a 15% separating gel. The gels were blotted onto 0.45-μm-pore-size nitrocellulose membranes (Bio-Rad) and reacted with a 1:16 dilution of MAb-containing tissue culture supernatant. The blots were further developed as described previously (16).

Bactericidal and opsonophagocytic assays. These assays were performed essentially as previously described (19). Briefly, bacteria were grown overnight at 37°C in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.), washed twice in normal saline, and resuspended to an appropriate bacterial density, determined spectrophotometrically, in Mueller-Hinton broth. Fresh normal human serum previously absorbed with the test organism to remove specific antibody (AbsNHS) and MAb-containing mouse ascites fluid, both appropriately diluted in Mueller-Hinton broth, were added to the bacterial suspension to achieve a final volume of 0.5 ml, a bacterial density of 10^6 CFU/ml, and a serum concentration of 10% (vol/vol). In opsonic assays, polymorphonuclear leukocytes (PMNs) were isolated from human blood by dextran sedimentation and Ficoll-Hypaque density gradient centrifugation; washed cells were suspended in Mueller-Hinton broth and added to the culture mixtures described above to achieve a final PMN concentration of 10^6 cells per ml. Test samples contained, in addition to bacteria, MAb plus 10% AbsNHS or MAb plus 10% AbsNHS plus PMNs. Control test samples comprised bacteria plus 10% AbsNHS previously heat inactivated at 56°C for 30 min (ΔAbsNHS). Additional control samples contained bacteria plus IgM or IgG MAbs specific for irrelevant antigens plus AbsNHS with or without PMNs. Capped plastic culture tubes containing the reaction mixtures were incubated with continuous rotation at 37°C for 2 h. Twenty-microliter aliquots were removed from the tubes at the end of the incubation period, added to 180 μl of sterile distilled water, allowed to stand for 10 min, and diluted in normal saline, and the number of viable bacteria was determined by quantitative culture on Mueller-Hinton agar. Results, expressed as the mean percentage (± standard error of the mean) of control CFU remaining at the end of the 2-h incubation period, based upon triplicate samples, were derived by the following calculation: (CFU of MAb-containing test samples/CFU of control samples containing ΔAbsNHS only) × 100.

In vivo protection. The mouse burn infection model (27) as modified by Pavlovskis et al. (10) was used. Six-week-old outbred female Swiss-Webster mice (Hilltop Laboratory Animals, Inc., Scottsdale, Pa.) were divided into five groups of five mice; each group was injected intravenously with a single dose of MAb (0.2 ml of mouse ascites fluid diluted in saline) 30 min before undergoing burn injury and infection. The following MAb doses were employed (in micrograms): 0.01, 0.1, 1.0, 10, and 100. After receiving MAb, the mice were subjected to a 2.5-cm^2 third-degree alcohol flame burn on the midportion of the back, and the fresh burn site was immediately inoculated subcutaneously with 50 50% lethal doses (10^7 CFU) of washed, log-phase *P. aeruginosa* It-1 bacteria. Cumulative rates of mortality were evaluated 5 days postinfection, and 50% protective doses (PD_{50}s) of MAbs were calculated by the Spearman-Karber method (6).

The protocol for the study of i.p. infections was the same as that of the burn infection model except that normal, unburned mice were injected i.p. with 50 50% lethal doses (4×10^4 CFU) of *P. aeruginosa* It-1 bacteria suspended in 0.5 ml of 2.5% hog gastric mucin in normal saline. The dose, timing, and route of administration of MAb were the same as those of the burn infection model, as were the assessment of cumulative rates of mortality at 5 days and the calculation of PD_{50}s of administered MAbs.

Mouse model protocols were reviewed and approved by the Laboratory Animal Review Board of the Uniformed Services University of the Health Sciences.

Nucleotide sequence accession numbers. The variable region heavy and light chain nucleotide sequences of the IgM-producing parental hybridoma cell line (1F6) were deposited in GenBank under accession numbers U20143 (heavy chain) and U20144 (light chain).

RESULTS

A class-switched, IgG3-secreting hybridoma cell line (2E12) was prepared from the parental, IgM-producing mouse hybridoma (1F6) by exposure to the frameshift reagent, ICR-191, followed by sib selection. Additional isotype switch variants—IgG1 (1E1), IgG2b (2H3), and IgG2a (4H1)—were subsequently isolated through a repetition of these procedures with appropriate IgG subclass-specific screening reagents (Fig. 1).

Monoclonal antibodies produced by the isotype-switched cell lines exhibited ELISA reactivity with purified *P. aeruginosa* It-1 LPS and with heat-killed It-1 bacteria that was nearly indistinguishable from the binding activity demonstrated by MAb secreted by the parental 1F6 cell line (Fig. 2). The similar binding activities of various MAb isotypes were apparent whether the antibody probe used for detection was a heavy and light chain-specific rabbit anti-mouse IgG plus IgM plus IgA or a κ light chain-specific rabbit anti-mouse antiserum (Fig. 2).

Comparative immunoblot analyses of the IgM and IgG3 MAbs expressed by the parental 1F6 and class-switched 2E12 cell lines, respectively, revealed similar patterns of reactivity with heat-killed bacteria and LPS isolated from various *P. aeruginosa* serogroup O6 subtypes (Fig. 3). Both MAbs reacted with purified LPS from all five serogroup O6 subtypes; both exhibited strong reactivities with faster-migrating molecular

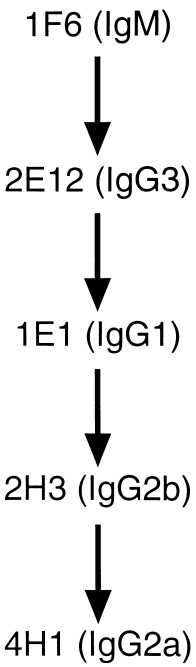

FIG. 1. Isotype-switched hybridoma clones, derived by mutagenization and sib selection from the parental IgM-producing murine 1F6 cell line, secreted MAbs representing the various IgG subclasses indicated. The isotype-switched variants retained specificity for the O polysaccharide of *P. aeruginosa* serogroup O6 LPS.

species; both recognized slowly migrating material containing O polysaccharides of various chain lengths, especially evident in immunoblots of subtype 6ad bacteria; and neither MAb reacted with heat-killed Habs 6 bacteria. MAbs produced by the IgG3 isotype-switched variant did not react with more slowly migrating elements from purified LPS. Immunoblots produced with isotype-switched MAbs representing other IgG subclasses (data not shown), while differing somewhat in intensity, were similar to those produced with MAb from the class-switched 2E12 (IgG3) cell line.

The cDNA sequences of variable region heavy and light chains were identical among the parental IgM-secreting and four isotype-switched IgG-secreting hybridoma cell lines. The sequences are shown in Fig. 4. These sequences were different from all other antibody variable region sequences deposited in the Kabat Sequences of Proteins of Immunologic Interest or GenBank database.

MAbs from the parental IgM-producing 1F6 hybridoma and all isotype-switched IgG-secreting cell lines mediated concentration-dependent opsonophagocytic killing of *P. aeruginosa* It-1 bacteria (Fig. 5, panel 1). MAb-mediated killing, documented by a 50% reduction in CFU following 2 h of incubation at 37°C, compared with that of a control sample containing bacteria plus AbsNHS but no antibody or PMNs, occurred at a concentration of 0.09 μg of the IgM MAb per ml and at concentrations of 0.13, 0.67, 3.70, and 9.08 μg of the isotype-switched IgG2a, IgG3, IgG2b, and IgG1 MAbs per ml, respectively (Fig. 5, panels 2 to 6). Less than 50% opsonophagocytic killing was observed in the presence of MAbs, PMNs, and ΔAbsNHS (data not shown). Negative control MAbs displaying the IgM and IgG2a isotypes produced no bacterial killing (<50% reduction in CFU) in the presence or absence of PMNs (Fig. 5, panels 7 to 8).

Appropriate doses of parental IgM MAb (1F6) or isotype-switched IgG MAb (2E12, 1E1, 2H3, or 4H1), administered to mice prior to an otherwise uniformly fatal challenge with 50 50% lethal doses of *P. aeruginosa* It-1 bacteria, provided pro-

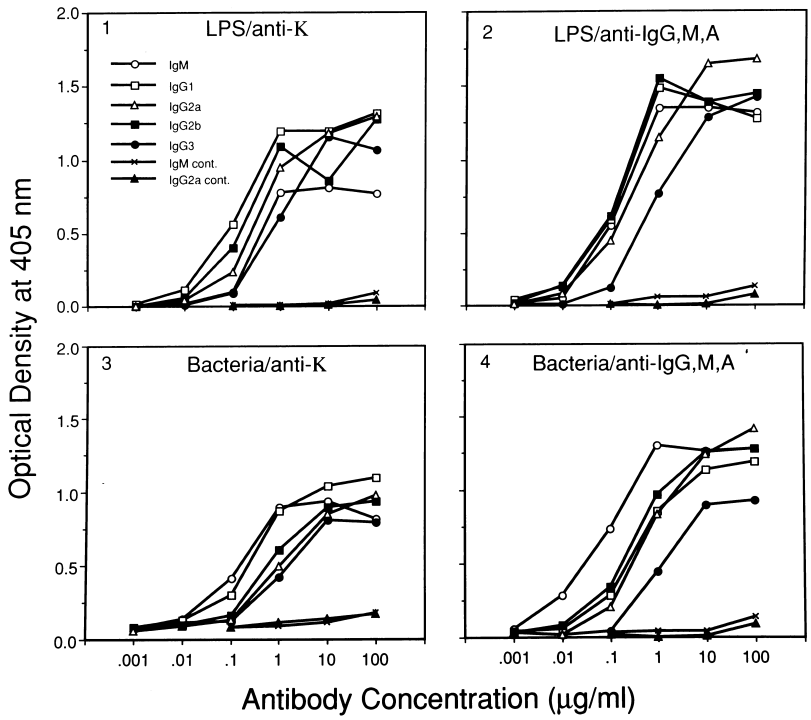

FIG. 2. ELISA reactivity of isotype-switched MAbs with *P. aeruginosa* Fisher It-1 LPS (panels 1 and 2) and *P. aeruginosa* It-1 heat-killed bacteria (panels 3 and 4). Bound MAb was detected with κ light chain-specific rabbit anti-mouse antiserum (anti-K) (panels 1 and 3) and with heavy and light chain-specific rabbit anti-mouse IgG plus IgM plus IgA antiserum (panels 2 and 4). The negative control MAbs, 13-17 and 50-9 (IgG and IgM, respectively), recognize irrelevant antigens.

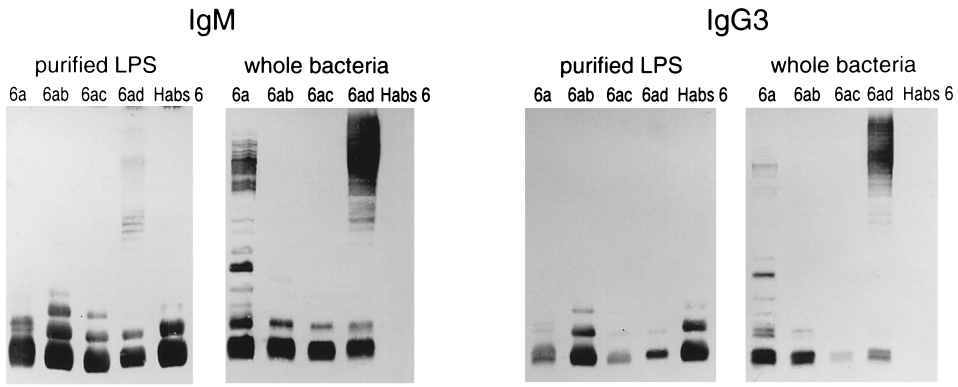

FIG. 3. Comparative immunoblot analyses of binding by parental IgM (clone 1F6) and class-switched IgG3 (clone 2E12) MAbs to various *P. aeruginosa* serogroup O6 subtype LPS and whole, heat-killed bacteria. Fisher It-1 is designated 6a.

tection against infection-related mortality in burn wound sepsis and i.p. infection models (Table 1). With few exceptions, MAb doses of 100 or 10 μg per mouse produced sustained 100% survival rates for mice in both models, 1 μg of MAb produced variable protection, and doses of 0.1 or 0.01 μg failed to elicit any survival (data not shown). PD_{50}s based upon the combined results of four separate experiments employing the burn infection model ranged from 1.1 to 3.2 μg per mouse for various MAb isotypes (Table 1). In comparison, PD_{50}s ranged from 0.2 to 3.2 μg per mouse in the i.p. infection model (Table 1). There was no apparent relation, in either infection model, between MAb isotype and PD_{50}, as evidenced by similar isotype-associated PD_{50}s and overlapping 95% confidence intervals (Table 1). Thus, all MAb isotypes appeared to protect equally well against infection-associated mortality in these two murine models.

DISCUSSION

A murine hybridoma secreting IgM MAb specific for the O polysaccharide of *P. aeruginosa* serogroup O6 LPS was successfully class switched to produce an IgG3 MAb with identical specificity and variable region heavy and light chain nucleotide sequences. This IgG3-secreting cell line was further switched to produce O-specific, variable region-identical IgG1, IgG2b, and

HEAVY CHAIN SEQUENCE

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1    CAGGTCCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGGTCTCAGTGAAGATT   60
     Q  V  Q  L  Q  Q  S  G  A  E  L  V  R  P  G  V  S  V  K  I

61    TCCTGCAAGGGTTCTGGCTACACATTCATT GATTATGCTATGCAC TGGGTGAAGCAGAGT   120
      S  C  K  G  S  G  Y  T  F  I   D  Y  A  M  H   W  V  K  Q  S
                                 CDR1↑

121   CATGCAAAGAGTCTAGAGTGGATTGGC GTTATTAATACTTACTATGGTGATGCTAGCTAC   180
      H  A  K  S  L  E  W  I  G   V  I  N  T  Y  Y  G  D  A  S  Y
                                          CDR2↑

181  AACCAGAAGTTCAAG GGCAAGGCCACAATGACTGTAGACAAATCCTCCAGCACAGCCTAT   240
     N  Q  K  F  K   G  K  A  T  M  T  V  D  K  S  S  S  T  A  Y
       CDR2↑

241  ATGGAACTTGCCAGACTGACATCTGAGGATTCTGCCATCTATTACTGTGCCCTC TATAGG   300
     M  E  L  A  R  L  T  S  E  D  S  A  I  Y  Y  C  A  L   Y  R
                                                      CDR3↑

301  TACGACGGCTATGCTATGGACGAC TGGGGTCAGGGAACCTCAGTCACCGTCTCCTCA   357
     Y  D  G  Y  A  M  D  D   W  G  Q  G  T  S  V  T  V  S  S
           CDR3↑
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LIGHT CHAIN SEQUENCE

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1    GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCC   60
     D  V  V  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S

61    ATCTCTTGC AGATCTAGTCAGAGTCTTGTACACAGTAATGGAAACACCTATTTACAT TGG  120
      I  S  C   R  S  S  Q  S  L  V  H  S  N  G  N  T  Y  L  H   W
                      CDR1↑

121  TTCCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTAC AAAGTTTCCAACCGATTT   180
     F  L  Q  K  P  G  Q  S  P  K  L  L  I  Y   K  V  S  N  R  F
                                          CDR2↑

181 TCT GGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATC   240
    S   G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K  I

241  AGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGC TCTCAAAGTACACATGTTCCA   300
     S  R  V  E  A  E  D  L  G  V  Y  F  C   S  Q  S  T  H  V  P
                                          CDR3↑

301 TTCACG TTCGGCTCGGGGACAAAGTTGGAAATAAAA  336
    F  T   F  G  S  G  T  K  L  E  I  K
```

FIG. 4. Variable region heavy and light chain nucleotide and (single letter) amino acid sequences of MAbs produced by parental and isotype-switched hybridoma cell lines specific for *P. aeruginosa* serogroup O6 LPS. The complementarity determining regions (CDR1, CDR2, and CDR3) are indicated in boldface type.

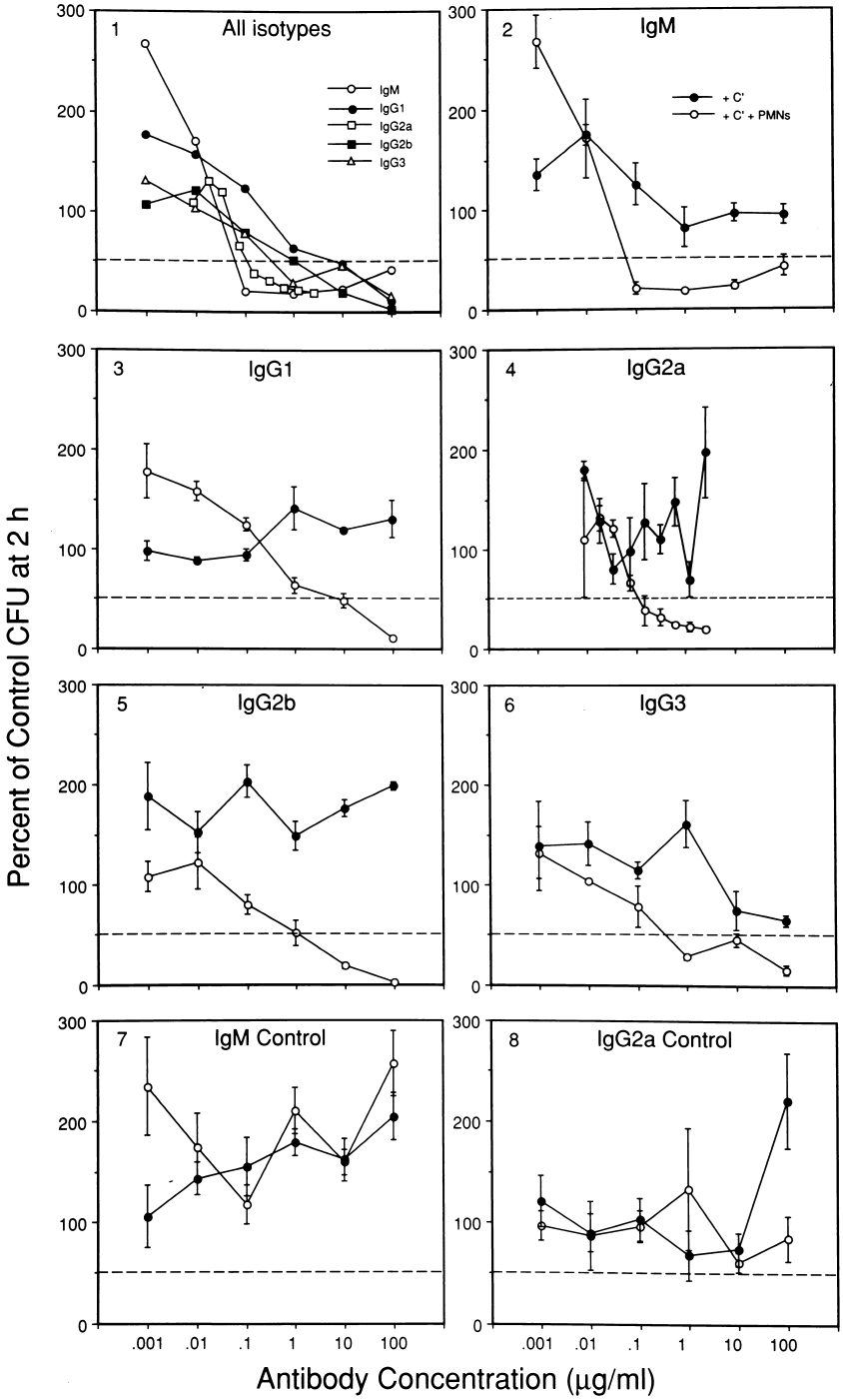

FIG. 5. In vitro complement-dependent bacteriolysis and opsonophagocytic killing of *P. aeruginosa* It-1 bacteria by isotype-switched MAbs following a 2-h incubation at 37°C. Data are expressed as percentages (mean ± standard error of the mean of triplicate samples) of control CFU, the latter measured after a 2-h incubation of bacteria in the presence of 10% heat-inactivated ΔAbsNHS alone. In vitro killing curves generated with various MAb isotypes in the presence of 10% AbsNHS and PMNs are shown in panel 1. In vitro bacterial killing by individual MAb isotypes, in the presence and absence of PMNs and 10% AbsNHS (C′), is shown in panels 2 to 6. Killing curves generated with IgM and IgG control MAbs, which react with irrelevant antigens, are shown in panels 7 and 8, respectively. The horizontal, broken lines in each panel indicate 50% bacterial killing.

IgG2a MAbs. Together, these MAbs represented a matched set of IgM and IgG isotypes presumably sharing a single idiotype corresponding to a common epitope on the O side chain of LPS from *P. aeruginosa* serogroup O6, the *Pseudomonas* serogroup most commonly implicated in human infections. These reagents provided an opportunity to examine the influence of Ig class and subclass on LPS-specific antibody function in relation to previously published information.

A comparison of these five LPS-specific IgM and IgG MAb isotypes revealed remarkably similar LPS binding activities and

TABLE 1. Protective activities of isotype-switched LPS O side chain-specific MAbs against live challenge with *P. aeruginosa* Fisher It-1 in murine burn wound and i.p. infection models

MAb isotype	Burn infection model[a]		i.p. infection model	
	MAb PD_{50} (μg/mouse)	95% confidence interval (μg/mouse)	MAb PD_{50} (μg/mouse)	95% confidence interval (μg/mouse)
IgM	2.0	0.98–4.05	0.2	0.04–1.12
IgG1	1.1	0.63–2.00	2.0	0.79–5.01
IgG2a	3.2	3.16	2.0	0.79–5.01
IgG2b	1.5	0.75–2.89	3.2	3.16
IgG3	2.0	1.17–3.40	0.8	0.26–2.45

[a] Data represent combined results from four separate experiments.

statistically indistinguishable levels of MAb-mediated protection against live bacterial challenge. Although the IgM MAb and all four IgG subclass MAbs were opsonic in vitro in the presence of serum complement, the IgM MAb proved most efficient (with a 50% bacterial kill at 0.09 μg of MAb per ml), the IgG1 MAb was least efficient (with a 50% bacterial kill at 9.08 μg of MAb per ml), and the other IgG MAb subclasses exhibited intermediate levels of efficiency in supporting opsonophagocytic bacterial killing. This isotype-based functional hierarchy was reminiscent of that previously described in connection with the MAb-mediated in vitro killing of *Escherichia coli* O111:B4 (9). In the case of the *Pseudomonas* MAbs, however, isotype-related differences in in vitro function did not appear to be matched by corresponding differences in in vivo protection against live bacterial challenge.

Two previous studies provided comparative functional analyses of polyclonal antibodies and/or MAbs of different isotypes specific for *P. aeruginosa* It-1 (serogroup O6) LPS.

Pier et al. (14) evaluated in vitro opsonophagocytic killing and in vivo protection against live challenge with *P. aeruginosa* It-1 in neutropenic mice by independently derived O side chain-specific, human IgM, IgG, and IgA MAbs and by the corresponding Ig fractions derived from pooled hyperimmune sera from subjects immunized with the high-molecular-weight polysaccharide component of *P. aeruginosa* It-1 LPS. In results somewhat reminiscent of ours, no functional advantage was associated with any of the Ig classes tested, particularly with regard to the prophylaxis of infection. These investigators concluded that "all three major human serum immunoglobulin isotypes have [similar] opsonophagocytic and protective activities against *P. aeruginosa*" (14). It was also documented that the antibacterial properties of LPS O side chain-specific IgM antibodies were more complement dependent than those of IgG (or IgA) antibodies with similar specificities.

More recently, Schreiber et al. (23) used sequential sublining and sib selection to isolate a murine IgG1 MAb with variable regions identical to those of a parent IgG3 MAb specific for a high-molecular-weight component of the O side chain of *P. aeruginosa* It-1 LPS. The IgG3 MAb exhibited superior binding to LPS-derived high-molecular-weight polysaccharide and opsonized *P. aeruginosa* It-1 more efficiently for uptake by murine or human phagocytes. The authors concluded that the IgG subclass of anti-LPS MAbs is an important determinant of avidity for multivalent antigen as well as biological function. There was no attempt in this study to correlate isotype-related differences in in vitro function with possible differences in in vivo activity.

Other investigators have evaluated Ig class- and subclass-related differences in the functional properties of antibodies reactive with *Salmonella typhimurium* and *E. coli* LPS, with various results.

Robbins et al. (21) found that while the LPS-specific IgG fraction of rabbit serum hyperimmune to *S. typhimurium* LPS was more avid for homologous antigen than the IgM fraction, the latter produced more efficient bacterial agglutination, complement-mediated bacteriolysis, and opsonization. Moreover, Saxén et al. (22) reported that the IgM fraction of antiserum produced to *S. typhimurium* LPS was 10 to 100 times more protective than the IgG fraction against mouse salmonellosis. These investigators attributed the greater protective efficacy of LPS-specific IgM to its greater complement-fixing efficiency. Carlin et al. (3) took this observation one step further by demonstrating that IgG3 MAbs specific for the so-called O4 epitope on the *S. typhimurium* O polysaccharide were 2,500-fold more protective than IgG3 MAbs specific for the less active O12 epitope. In contrast, IgM MAbs to either epitope conferred protection equivalent to that mediated by IgG3 MAbs specific for the more active O4 epitope. The authors concluded that epitope specificity and isotype are critical in determining the protective properties of LPS-reactive MAbs.

Pelkonen and Pluschke (11) employed a rat model for neonatal sepsis to define the functional properties of isotype-switched LPS-reactive rat MAbs in live challenge experiments with *E. coli* O18K1. They found that all MAb isotypes except IgE promoted bacterial clearance from the circulation, hepatic sequestration, and bacterial killing. However, all four rat IgG subclasses (and IgA) exhibited a more efficient in vivo function than IgM, while the latter was inactive in animals depleted of complement. Moreover, among various IgG subclasses, IgG2b and IgG2c produced a more efficient bacterial clearance than variable region-identical MAbs bearing the IgG1 and IgG2a isotypes.

Finally, Oishi and his coworkers (9) documented that in the presence of fresh normal human serum, LPS-reactive MAbs attached C3 fragments to *E. coli* O111:B4 bacteria in a dose-dependent manner. Bacterial killing occurred only in the presence of antibody and complement and required an intact alternative pathway. Levels of efficiency of antibody-mediated bacteriolysis and opsonophagocytic killing varied according to MAb isotype (IgM > IgG2a > other IgG subclasses) and correlated with complement-fixing activities. The levels of efficiency of MAb-mediated protection against live i.p. challenge exhibited a similar isotype-related rank order. It was thus concluded that the complement-fixing, antibacterial, and protective properties of MAbs reactive with *E. coli* O111:B4 LPS were interrelated and isotype dependent.

It is clear from our findings, interpreted in conjunction with other published data, that isotype-related differences in the antibacterial activities of LPS-reactive MAbs are susceptible to considerable variation on the basis of phylogenetic differences in the antigenic structure of LPS and the epitope specificities of the antibodies themselves. Such variation is accentuated by studies, such as those cited above, that utilize diverse bacteria, polyclonal antibodies or idiotypically discrete MAbs, and a variety of different in vitro and in vivo assays of antibody function.

As discussed above, a comparison of isotype-related differences in the antibacterial and protective properties of MAbs specific for the O polysaccharides of *E. coli* O111:B4 and *P. aeruginosa* serogroup O6 LPS reveals apparent discrepancies in the functional effects of Ig class and subclass. A possible explanation for these bacterial species-related discrepancies in antibody functions may reside in the exquisite susceptibility of *E. coli* O111:B4 to MAb-mediated, complement-dependent bacteriolysis (9), which is driven by the antibody-directed dep-

osition of C3 at critical sites on the bacterial cell surface and the formation of lytic C5b-9 membrane attack complexes (15). Isotype-associated differences in the efficiencies with which LPS-specific MAbs fix complement are critical to this process and are reflected in corresponding differences in bactericidal and protective functions (9).

P. aeruginosa, on the other hand, is relatively resistant to antibody- and complement-mediated bacteriolysis (15, 19, 30). LPS-reactive MAbs serve what is primarily an opsonic function toward *P. aeruginosa* wherein isotype-related differences in complement-fixing efficiencies may be offset by class- or subclass-associated discrepancies in corresponding Fc or complement receptor functions. These countervailing factors may mean that the complement-fixing efficiencies of LPS-reactive MAbs are less critical in relation to the opsonophagocytic killing of *P. aeruginosa* than in connection with the complement-dependent bacteriolysis of *E. coli* O111:B4. In the case of *P. aeruginosa* this less critical complement-fixing efficiency could account for the smaller functional differences noted among LPS-specific MAb isotypes, including IgM and various IgG subclasses, compared with the complement-fixing efficiency of *E. coli* O111:B4 (9), especially with respect to in vivo protection against live bacterial challenge.

Many of the quantitative, isotype-related differences in antibody functions documented here and in previous studies may be of limited biological significance to the infected or potentially infected host since these differences tend to occur with a low range of absolute antibody concentrations likely attained through immunization or as a result of natural infection.

These analyses support a cautious approach to generalizations regarding the comparative antibacterial and protective functions of LPS-reactive IgM and IgG antibodies, particularly when such views involve overly simplistic assertions concerning the functional superiority of one Ig class over another. The need for caution regarding putative isotype-related differences in antibody functions is further emphasized by the complexity of in vivo protection, in which LPS-reactive antibodies likely mediate antiendotoxic (1) as well as antibacterial functions (15). Moreover, the very different pharmacokinetic properties of IgM and IgG antibodies, particularly with regard to tissue distribution and serum half-life (24), must be considered in any analysis of therapeutic potential.

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