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In Vitro and In Vivo Activity of Polyclonal and Monoclonal Human Immunoglobulins G, M, and A against *Pseudomonas aeruginosa* Lipopolysaccharide

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We evaluated the in vitro opsonophagocytic killing activity of monoclonal human immunoglobulin G (IgG), IgM, and IgA specific for *Pseudomonas aeruginosa* lipopolysaccharide and the in vivo protective capacity in neutropenic mice of both monoclonal and purified polyclonal IgG, IgM, and IgA. Monoclonal IgM was efficacious in mediating opsonophagocytic killing only in conjunction with complement, whereas monoclonal IgG opsonic killing was potentiated by complement, and monoclonal IgA opsonic killing was independent of complement. These findings are similar to those previously reported for purified polyclonal IgM, IgG, and IgA. The monoclonal and polyclonal immunoglobulins had comparable 50% protective doses in neutropenic mice (range, 0.28 to 0.46 μg per mouse). The protective activity of IgM in neutropenic mice was abolished by cobra venom factor treatment, whereas IgG and IgA maintained efficacy in cobra venom factor-treated mice. These data indicate that all three major human serum immunoglobulin isotypes have opsonophagocytic and protective activities against *P. aeruginosa*, with a critical role for complement in the function of IgM.

The recent marketing of immunoglobulin G (IgG) preparations for intravenous delivery of antibody has spurred interest in the therapeutic potential of these preparations for prophylaxis and treatment of infectious diseases. Their use in passive therapy of *Pseudomonas aeruginosa* infections is an area of interest. However, IgG isolated from normal donors does not appear to possess much activity against *P. aeruginosa* as measured by in vitro opsonic killing or animal protection (2, 14), even when the donors have been selected because of naturally occurring high titers of antibody to lipopolysaccharide (LPS). Immunoglobulin obtained from the sera of individuals immunized with *P. aeruginosa*-specific vaccines or monoclonal antibodies may be required for maximal therapeutic efficacy. Determination of the antigenic specificity and characteristics of antibodies that would provide maximal therapeutic efficacy against *P. aeruginosa* infection is critical. A number of studies have suggested that IgG specific for the LPS O side chain is an important component in any efficacious passive therapy (23, 27). IgM provided suboptimal protection in a number of studies (1, 3), whereas IgA has not been evaluated as an immunotherapeutic reagent for *P. aeruginosa* infection; however, monoclonal murine IgA has been reported to protect mice against mucin-enhanced infection by group B streptococci (9) but not against infection by *Streptococcus pneumoniae* (6).

The production of human monoclonal antibodies specific for *P. aeruginosa* LPS O side chains affords the opportunity to compare different isotypes of antibody for in vitro and in vivo therapeutic efficacy. Polyclonal IgG, IgM, and IgA purified from the sera of humans immunized with a high-molecular-weight polysaccharide preparation from the Fisher immunotype 1 (IT-1) strain of *P. aeruginosa* were all able to mediate in vitro opsonophagocytic killing by polymorphonuclear leukocytes (PMN) or peripheral blood monocytes (20). The effect of complement on phagocytic killing mediated by these three different immunoglobulin classes varied. However, in some of these experiments it was not possible to conclusively determine the activity of a given immunoglobulin class in opsonic killing because of the potential of low-level contamination by a heterologous isotype (20). In vitro-derived monoclonal antibodies avoid this problem and are useful for determining the effect of isotype variation on bacterial killing. In this study we have compared human monoclonal IgG, IgM, and IgA antibodies specific for *P. aeruginosa* LPS in in vitro opsonic killing and both monoclonal and isotype-purified polyclonal antibodies in in vivo protection of neutropenic mice and have attempted to determine whether superior protection was associated with a particular immunoglobulin isotype.

MATERIALS AND METHODS

Polyclonal and monoclonal immunoglobulin preparations. Purified preparations of human IgG and IgA specific for the LPS O side chain of the Fisher IT-1 strain of *P. aeruginosa* were obtained from the pooled sera of volunteers given a single 100-μg dose of high-molecular-weight polysaccharide (17, 19, 20). IgG was purified by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) followed by removal of any residual IgM and IgA by affinity chromatography on columns containing goat anti-human IgM or IgA. IgA was purified by using a combination of ion-exchange chromatography on DEAE Zeta-prep cartridges (LKB Instruments, Inc., Rockville, Md.) as described by the manufacturer, molecular sieve chromatography on Sepharose CL-4B columns, and affinity chromatography on anti-human IgM columns to remove IgM and protein A-Sepharose chromatography to remove IgG1, IgG2, and IgG4. Polyclonal IgM specific for the LPS O side chain of the Fisher IT-2 strain was purified, as described previously (20), from the serum of a single individual immunized with 100 μg of IT-2 high-molecular-weight polysaccharide (19) who responded by producing high levels of IgM. This was the only

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individual with this response out of 19 tested. As a negative control we used a polyclonal IgG preparation purified from a pool of human serum obtained prior to immunization and prepared as described above.

Monoclonal IgG antibody P8-4, specific for the IT-1 LPS, was kindly provided by Shuzo Sawada, Teijin Institute for Biomedical Research, Tokyo, Japan, and prepared as described previously (23). P8-4 antibody was obtained as a lyophilized powder (2 mg) containing 10 mg of bovine serum albumin. It was reconstituted in sterile saline and diluted in tissue culture medium (RPMI 1640 with 10% fetal calf serum) to a concentration of 100 μg/ml. Monoclonal IgA antibody C1G8, specific for the IT-1 LPS, was obtained from Epstein-Barr virus-transformed human lymphocytes as described previously (24). Supernatants of this antibody concentrated three- to fivefold by ultrafiltration were used. Monoclonal IgM antibody RM-5 specific for the IT-2 LPS was also obtained from Epstein-Barr virus-transformed human lymphocytes (30, 31; Zweerink et al., manuscript in preparation) and concentrated fivefold in tissue culture medium for use. Controls for experiments with these monoclonal antibodies contained an irrelevant (unknown antigenic specificity) human IgM monoclonal antibody in tissue culture medium identical to that used to grow the C1G8 and RM-5-producing cells.

Serologic assays. The purity and isotype of the polyclonal and monoclonal immunoglobulin were evaluated by Ouchterloney immunodiffusion (13) with goat or sheep antisera specific for human immunoglobulin classes or IgG subclasses (Miles Laboratories, Inc., Elkhart, Ind.) and quantitated by single radial immunodiffusion (LC Partigen and NorPartigen plates; Behring Diagnostics, La Jolla, Calif.). The lower limit of detection for the various immunoglobulin isotypes by radial immunodiffusion was 0.1 μg of IgM per ml, 0.008 μg of IgG per ml, and 0.008 μg of IgA per ml. Antibody specific for the IT-1 and IT-2 LPS O side chains was quantitated by using a previously described radioactive antigen-binding assay (16), with a lower limit of detection of 0.26 μg of specific antibody per ml. Comparisons of the specific antibody level determined in the radioactive antigen-binding assay with the concentration of the monoclonal antibodies determined by radial immunodiffusion showed agreement within 5%. Enzyme-linked immunosorbent assays with heat-killed (100°C for 2 h) whole cells of Fisher IT-1 to IT-7 strains were used to check the specificity of the monoclonal antibody preparations. These were performed as described previously (5).

Binding of the antibodies to the LPS O polysaccharide side chains was determined after separation of the LPS in 10% polyacrylamide-sodium dodecyl electrophoresis gels as described previously (27). LPS was blotted to nitrocellulose by the method of Sturm et al. (28), and then individual nitrocellulose strips were incubated with the different human antibody preparations diluted in blocking buffer. Bound antibody was visualized by using alkaline phosphatase conjugates of goat antibody to individual human immunoglobulin isotypes as described by Blake et al. (4).

In vivo protection assays. A modification of the neutropenic mouse model of *P. aeruginosa* infection as described by Cryz et al. (7) was used. Outbred CD-1 male and female mice, 6 to 8 weeks old, were given 200 mg of cyclophosphamide (Cytoxan; Mead-Johnson Oncology Products, Syracuse, N.Y.) per kg of body weight once every other day for 3 days. On the day of the last dose of drug, leukocyte counts were made with blood obtained from the tail vein; the various immunoglobulin preparations or control tissue culture medium was administered intraperitoneally 2 h after the final dose of cyclophosphamide; and then 2 h later, the bacterial challenge was administered intraperitoneally. Deaths were recorded for 10 days after challenge. Mice were challenged with either a Fisher IT-1 strain, 15921, or an IT-2 strain, 9882-80, both of which were clinical isolates from septicemic patients. The immunotypes of these strains were determined by agglutination, with immunotype-specific antisera raised in rabbits to the LPS extracted from the Fisher IT strains. The 50% lethal dose (LD_{50}) for these strains was calculated by probit analysis (11), with five mice per group and 10-fold dilutions of bacteria for these determinations. The 50% protective doses (PD_{50}) of the different immunoglobulin preparations were also calculated by probit analysis (11), with twofold dilutions of immunoglobulin and 10 mice per group. Cobra venom factor (CVF; Sigma Chemical Co, St. Louis, Mo.) was administered intraperitoneally to neutropenic mice in 2-unit amounts 24, 20, and 2 h prior to challenge with bacteria. Prior to CVF administration, and 1 h after the final dose, mice were bled from the tail vein, and serum was prepared and stored at −80°C for analysis of hemolytic complement activity. This was performed as described previously (18).

Opsonophagocytosis assays. Opsonophagocytic killing of 2×10^6 cells of either the IT-1 or IT-2 *P. aeruginosa* strain was performed as previously described (17, 20), with either purified human PMN or peripheral blood monocytes as the phagocytic cell source and the polyclonal and monoclonal immunoglobulin preparations. The PMN and monocyte preparations were prepared by dextran sedimentation of whole blood followed by Ficoll-Hypaque (Winthrop Laboratories, Div. Sterling Drug Inc., New York, N.Y.) gradient separation of PMN and mononuclear cells as described previously (20). Purity was determined by treating a sample with Wright stain and counting 100 to 200 cells. All preparations had >98% purity. All reagents were diluted to their final concentration in tissue culture medium. In our assays, fresh human serum was used as the complement source at a final dilution of 1:10. This serum was adsorbed with Formalin-fixed and lyophilized homologous whole bacterial cells at a concentration of 1 mg of bacterial cells per ml of serum at 4°C for 30 min prior to use. The complement source was filter sterilized after adsorption. Killing was calculated as follows: $\log_{10}$ CFU reduction = 100 − [($\log_{10}$ CFU viable at 120 min × 100)/$\log_{10}$ CFU at zero time].

Statistics. The significance of the differences in mouse survival over a 10-day period was calculated by the Kruskal-Wallis test (12). A *t* test was used to determine the significance of $\log_{10}$ reductions in CFU in the opsonophagocytic assay.

RESULTS

Characterization of immunoglobulin preparations. The various human monoclonal and polyclonal immunoglobulin preparations used, along with some of their properties, are listed in Table 1. The unavailability of a human IgM monoclonal antibody to the IT-1 strain required us to compare polyclonal and monoclonal IgM against an equally virulent (see below) IT-2 strain. The polyclonal antibody preparations were not contaminated with heterologous immunoglobulin down to the lower limit of detection of the radial immunodiffusion assays. Thus, all polyclonal antibody preparations were >99.9% pure as assessed by radial immunodiffusion. Each *P. aeruginosa* monoclonal antibody preparation reacted in the radioactive antigen-binding assay and

TABLE 1. Human monoclonal and polyclonal antibody preparations used

Preparation	Specificity	Antibody concn (μg/ml) to *P. aeruginosa*[a]	Designation and/or source
Polyclonal			
IgG	Nonimmune	<0.26	Purified from preimmune human serum
IgG	IT-1 LPS	16.2	Purified from high-titer immune serum
IgA	IT-1 LPS	7.0	Purified from high-titer immune serum
IgM	IT-2 LPS	5.4	Purified from high-titer immune serum
Monoclonal			
IgG2	IT-1 LPS	98.7	P8-4, human-murine heteromyeloma (Teijin Pharmaceuticals)
IgA	IT-1 LPS	68.2	C1G8, EBV-transformed human B cells (Genetic Systems, Inc.)
IgM	IT-2 LPS	20.9	RM-5, EBV-transformed human B cells (Merck Sharp & Dohme)

[a] Determined by radioactive antigen-binding assay.

whole-bacterial-cell enzyme-linked immunosorbent assay only with a single serotype of radiolabeled O side chain or *P. aeruginosa* bacterium, respectively. Western immunoblot analysis indicated that the monoclonal antibody preparations bound to the O-polysaccharide side chains of their respective IT LPS (not shown), as did the purified polyclonal preparations.

Opsonophagocytic killing. The ability of each monoclonal antibody preparation to mediate in vitro opsonophagocytic killing of 2×10^6 bacteria is shown in Fig. 1. We have previously reported on the opsonophagocytic activity of polyclonal human IgG, IgM, and IgA to IT-1 high-molecular-weight polysaccharide (20). The opsonophagocytic killing activity of the monoclonal antibodies paralleled that of the corresponding polyclonal preparation. IgG mediated a high level of killing in the presence of complement and good killing in the absence of complement. Monoclonal IgA also exhibited a high level of opsonophagocytic killing, which was unaffected by the deletion of complement from the reaction mixture. Monoclonal IgM mediated a high level of opsonophagocytic killing activity, which was abolished when complement was deleted from the reaction mixture. Preparations of an irrelevant human IgM monoclonal antibody in tissue culture medium failed to mediate in vitro opsonophagocytic killing, and no killing was observed if phagocytic cells were omitted (i.e., there was no killing by antibody and complement alone). When the monoclonal antibodies were titrated out in opsonophagocytic assays in which comparable concentrations of specific antibody were tested, there were no significant ($P > 0.4$, t test) quantitative differences in killing activity among these preparations.

Characteristics of the neutropenic mouse model. When mice were given cyclophosphamide as specified for the regimen described above, they became extremely susceptible to *P. aeruginosa* infection. Both the IT-1 and IT-2 strains used in these experiments were lethal to 100% of unprotected neutropenic mice when as few as 25 organisms were administered via intraperitoneal challenge (not shown). The

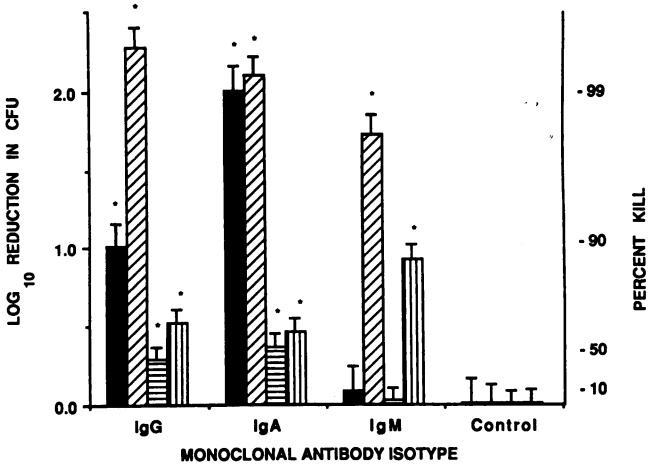

FIG. 1. Killing of *P. aeruginosa* IT-1 (by IgG and IgA) and IT-2 (by IgM) strains by monoclonal human antibody in the presence of human PMN (■), PMN plus complement (▨), monocytes (▤), and monocytes plus complement (▥). Control indicates killing in the absence of added antibody. The bars represent means of quadruplicate samples, and error bars represent the standard deviations. *, Significant ($P \leq 0.035$, t test) reduction in the number of CFU from time zero.

calculated LD_{50} for both strains was 8 organisms per mouse (95% confidence interval, 2.3 to 15.2). On the day of the third dose of cyclophosphamide, the total leukocyte counts of treated mice were about 10% of the pretreatment level (Fig. 2), with mononuclear cells accounting for 90% of these cells. Almost all of the mononuclear cells (>80%) had the appearance of small lymphocytes, with only a minority of the cells appearing like phagocytic mononuclear cells. At 24 h later, the total leukocyte count was further depressed in both infected and uninfected mice, with no detectable neutrophils for 72 h. In the uninfected animals the total leukocyte count remained low for 72 h, after which recovery was noted (Fig. 2). Infected mice showed a similar course of leukocyte counts, except that most of these animals were dead by 72 h postchallenge (data not shown). When LD_{50} determinations were carried out in the neutropenic mice on days 0 to 3 following the final cyclophosphamide dose, the calculated values were always <20 organisms per mouse, indicating fairly uniform susceptibility for at least 4 days. By day 4 after

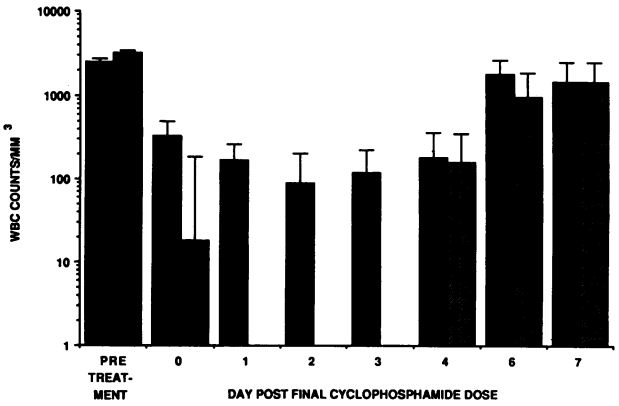

FIG. 2. Monocytic (■) and granulocytic (▨) cell counts in neutropenic, noninfected mice following three doses of cyclophosphamide (200 mg/kg).

TABLE 2. Comparative PD_{50} of human polyclonal and monoclonal antibodies for neutropenic mice

Preparation	Challenge strain	Dose/mouse (CFU)	PD_{50} (95% confidence interval) of specific antibody (μg/mouse)
Polyclonal IgG	IT-1	320	0.28 (0.16–0.41)
Polyclonal IgA	IT-1	320	0.41 (0.31–0.54)
Polyclonal IgM	IT-2	354	0.52 (0.40–0.69)
Monoclonal IgG	IT-1	500	0.46 (0.35–0.66)
Monoclonal IgA	IT-1	500	0.41 (0.31–0.54)
Monoclonal IgM	IT-2	354	0.32 (0.25–0.40)
Nonimmune IgG	IT-1	320	>100[a]
Nonimmune IgG	IT-2	354	>100[a]

[a] Represents total antibody, since nonimmune IgG contained <0.01% *P. aeruginosa*-specific antibody in the total antibody.

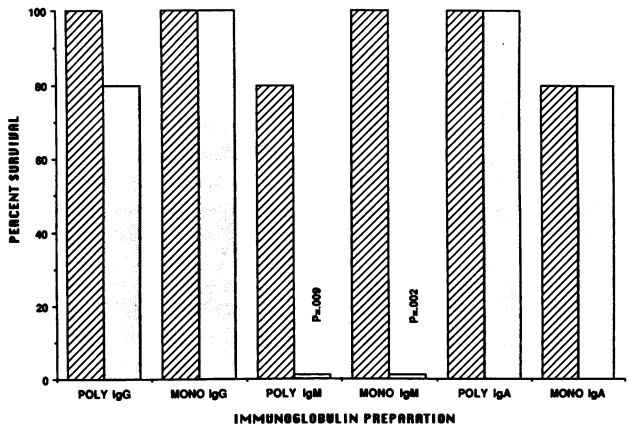

FIG. 3. Survival of neutropenic mice either not treated (▨) or treated (▩) with CVF and the indicated polyclonal (POLY) or monoclonal (MONO) immunoglobulin preparation. *P* values for IgM were determined by a Kruskal-Wallis test (12) and indicate significance of difference between CVF-treated and untreated groups. All other preparations showed no significant ($P > 0.6$) differences.

cyclophosphamide administration, the mice had recovered their resistance to *P. aeruginosa* challenge to a large degree (LD_{50}, 5×10^5 CFU per mouse).

Protection of neutropenic mice by human antibodies. To determine the PD_{50} against a standard challenge inoculum (320 to 500 organisms), we gave each polyclonal and monoclonal immunoglobulin preparation to neutropenic mice in various doses 2 h after the third cyclophosphamide dose and 2 h prior to bacterial challenge. Table 2 shows the PD_{50} determinations. The PD_{50} values (range, 0.28 to 0.46 μg per mouse) all had overlapping 95% confidence intervals by probit analysis, indicating that there was no statistically significant difference among these protective levels. The preparation of nonimmune polyclonal human IgG in tissue culture medium, which lacked in vitro opsonic-killing activity, failed to protect neutropenic mice at doses more than 200 times the PD_{50} of monoclonal human IgG (Table 2). In addition, determinations of PD_{50} of polyclonal and monoclonal IgG for mice passively immunized and challenged with 488 organisms 2 days after the final cytoxan dose showed that these PD_{50} values were comparable to those determined above for mice immunized and challenged on the day of the final cytoxan dose (0.32 and 0.41 μg per mouse for polyclonal and monoclonal IgG, respectively).

Effect of CVF treatment. The opsonophagocytic data indicated a varying role for complement in mediating killing by the different immunoglobulin isotypes. An in vivo correlate was investigated by treating neutropenic mice with CVF prior to challenge. These results (Fig. 3) showed that reduction in hemolytic complement activity to 17 ± 13% of the pretreatment level was accompanied by a loss of protection mediated by both polyclonal and monoclonal IgM ($P = 0.002$ and 0.009, respectively), whereas IgG and IgA maintained protective efficacy in the face of reduced complement activity. Control uninfected, neutropenic mice treated with CVF all survived the experimental period.

DISCUSSION

P. aeruginosa-specific monoclonal human IgG, IgM, and IgA were compared for their ability to mediate in vitro opsonophagocytic killing, and both monoclonal and purified polyclonal IgG, IgM, and IgA were tested for in vivo protection of neutropenic mice. Consistent with previous results with polyclonal immunoglobulin preparations (20), the three major human serum antibody classes were effective opsonins for in vitro phagocytic killing. Human IgA mediated this effect independent of complement, whereas human IgG had enhanced activity in the presence of complement and human IgM absolutely required complement. All three isotypes were equally effective in protecting neutropenic mice from *P. aeruginosa* challenge. IgM-mediated protection was abrogated when complement levels were lowered by treatment with CVF, whereas this degree of hypocomplementemia had no effect on the protection mediated by IgG and IgA. The complement requirement of human monoclonal IgM for in vivo protection against *P. aeruginosa* infection is similar to that reported by Shigeoka et al. (26), who used a murine monoclonal IgM in the neonatal rat model of group B streptococcus infection. Thus, using in vitro opsonophagocytosis and in vivo protection of neutropenic mice, we were unable to document any advantage for the specific human immunoglobulin isotypes tested in prophylaxis of *P. aeruginosa* infection, except when a hypocomplementemic state was induced.

These results indicate that all major immunoglobulin classes are potential passive therapeutic agents. Furthermore, our results with the P8-4 preparation are comparable to those recently reported by Sawada et al. (22), who found comparable levels of opsonic-killing activity and in vivo protection against *P. aeruginosa* infection when using human monoclonal antibodies. Similarly, Zweerink et al. (30, 31) also demonstrated the ability of human IgM monoclonal antibody RM5 to protect neutropenic mice against *P. aeruginosa* infection. The complement requirement for in vitro opsonic killing and in vivo protection of human IgM may be important, since complement can be inactivated by *P. aeruginosa* proteases (25) as well as host factors (29), potentially rendering the IgM ineffective. Alternatively, the inability of IgA to activate complement could be important in terms of generating chemotactic factors (C3a and C5a) that would enhance the migration of phagocytic cells to an infected site. This may not be critical in a septicemic model such as the one we used in neutropenic mice, or in septicemic patients, but may be more important in local infections, such as pneumonia. The ability of IgA to mediate protection and opsonic killing in the absence of complement supports the notion that phagocytic cells have Fc_α receptors (10). Alternatively, it is possible that in vitro opsonic killing in the presence of IgA was due to agglutination of the bacteria, making them more susceptible to phagocytosis, as opposed to a specific interaction of IgA-coated bacteria and Fc_α

receptors on phagocytes. Furthermore, we examined only a limited number of monoclonal antibody preparations specific for *P. aeruginosa* LPS, which may obscure differences due to antibody affinity. Ideally, one needs to compare monoclonal antibodies from cells induced to undergo an isotype switch while conserving the same variable regions of their light and heavy chains. When these reagents become available, they should be very useful in defining the advantages or disadvantages of particular immunoglobulin isotypes in the therapy and prophylaxis of infection. Finally, antibody quality other than affinity may be important. Bjornson and Michael (2) have shown that natural antibody to *P. aeruginosa* requires complement for phagocytosis, whereas immune antibody does not. Thus, the source of the prophylactic antibody could be critical. However, the monoclonal antibodies used were derived from nonimmune subjects, indicating that selecting antibodies with good binding to *P. aeruginosa* LPS may be sufficient for use in immunotherapy.

Other considerations regarding the significance of our findings are seen in relation to the route of infection. For example, infection beyond the vascular space might be more amenable to IgG prophylaxis, owing to its ability to penetrate to these areas. However, except for lung infections, most local *P. aeruginosa* infections are not fatal until the organisms gain access to the blood. Secretory IgA does not appear to offer much protection against *P. aeruginosa* on mucosal surfaces (21), making it likely that transudation of serum immunoglobulin is required for effective antibacterial activity at these sites.

A recent study of pneumonia in neutropenic guinea pigs (15) failed to demonstrate protective efficacy of intravenously administered human hyperimmune IgG, prepared from the plasma of donors with elevated levels of naturally occurring antibody to Fisher IT-1, IT-2, IT-4, or IT-6 LPS. This preparation acted synergistically with antibiotics to protect these animals. Our results showing protection of intraperitoneally challenged neutropenic mice by fairly low levels of intraperitoneally administered human antibodies indicate that this model is more amenable to the use of immunotherapeutic reagents alone. This may be due to the much lower challenge inocula used here (about 500 organisms used here versus 5×10^4 used previously [15]), the use of intraperitoneal infection as opposed to intratracheal instillation of organisms, the greater ability of the few available leukocytes to attack bacteria in the peritoneum and blood as opposed to the lungs, and the use of monoclonal and immune polyclonal antibodies as opposed to naturally occurring antibody. A study by Dunn and Kamp (8) showed that mice made neutropenic following immunization with killed whole cells of *P. aeruginosa* exhibited enhanced clearance of 10^6 bacteria administered intrabronchially. Curiously, there were no differences between immune and nonimmune neutropenic mice in their ability to clear a lower challenge dose (10^4 per animal), and bacteria grew in the lungs of all neutropenic mice.

In summary, we have confirmed that human IgG, IgM, and IgA are all capable of mediating in vitro opsonic killing and in vivo protection of neutropenic mice against *P. aeruginosa* infection. Since human susceptibility and resistance to *P. aeruginosa* infection involves many factors not replicable in an animal model, cautious conclusions must be drawn about the potential of a particular isotype as an immunotherapeutic agent. We did not test IgG subclasses other than IgG2, and so the possibility remains that immunoglobulins of these other subclasses have advantages or disadvantages in protective immunity. Nonetheless, in vitro opsonophagocytosis and in vivo passive protection are generally regarded as critical tests for the potential efficacy of immunotherapeutic reagents for *P. aeruginosa*. Our study indicates that immunoglobulin class is either not critical or not testable in these models for evaluating passive reagents for *P. aeruginosa* therapy.

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