INFECTION AND IMMUNITY, Dec. 1990, p. 3819–3828
0019-9567/90/123819-10$02.00/0

Vol. 58, No. 12

Generation and Characterization of Murine Antiflagellum Monoclonal Antibodies That Are Protective against Lethal Challenge with *Pseudomonas aeruginosa*

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Received 5 June 1990/Accepted 2 September 1990

Two murine monoclonal antibodies, IIG5 (IgG3) and IVE8 (IgG2a), that bind to *Pseudomonas aeruginosa* type *a* flagella and type *b* flagella, respectively, were prepared by conventional hybridoma methodology. Specificity of each monoclonal antibody for type *a* or type *b* flagella was demonstrated by enzyme-linked immunosorbent assay, indirect immunofluorescence, and immunoblotting. The percentage of *P. aeruginosa* isolates recognized by each monoclonal antibody was analyzed by enzyme-linked immunosorbent assay. Among a panel of 257 flagellated *P. aeruginosa* clinical isolates, IIG5 bound to 67.7% of the isolates and IVE8 bound to another 30.7%, for a combined coverage of 98.4%. Inhibition of motility of *P. aeruginosa* by the monoclonal antibodies was observed in vitro in a soft agar assay and was dose dependent. The protective efficacy of IIG5 and IVE8 was examined in a mouse burn wound sepsis model. The antiflagellum monoclonal antibodies provided specific and significant prophylactic and therapeutic protection against lethal challenge with *P. aeruginosa* strains.

Pseudomonas aeruginosa, a gram-negative bacterium, is an opportunistic pathogen that causes significant morbidity and mortality in immunocompromised patients (9, 41, 44). The high rate of mortality associated with *P. aeruginosa* infections occurs, in part, because of the resistance of the organisms to many antibiotics (7, 18). Therefore, numerous alternative and supplemental approaches to antibiotic therapy for the prophylaxis and therapy of *P. aeruginosa* infections have been investigated. In particular, immunotherapeutic strategies focusing on the abrogation of one or more virulence factors associated with the pathogenesis of *P. aeruginosa* have been studied in animal models and have been investigated in human clinical trials as well (24). While active or passive immunization targeting extracellular products, e.g., elastase, protease, and exotoxin A, has had limited success in prevention and treatment of *P. aeruginosa* infections in animal models (10, 39, 40), similar therapies to stimulate or augment an antilipopolysaccharide (anti-LPS) immune response have been more effective (10, 11, 42, 46). A significant drawback to anti-LPS immunotherapy, however, is the necessity to develop a multicomponent vaccine, immunoglobulin preparation, or monoclonal antibody (MAb) cocktail to provide protection against *P. aeruginosa* strains that characteristically express any one of at least 17 different serotypes of LPS (34).

Studies in burn wound sepsis models (13, 36, 38) have indicated that motility is important to the virulence of *P. aeruginosa*. When burned mice were challenged with a motile, virulent strain of *P. aeruginosa* or its nonflagellated isogenic mutant, the number of bacteria required to provide a lethal dose with the isogenic mutant was approximately 10^4-fold greater than that with the motile parent strain (13, 38). Moreover, results of murine burn wound sepsis model studies in which mice were actively immunized with flagella (25) or passively immunized with rabbit antiflagellum antisera (12) suggested that antiflagellum antibodies could protect animals from lethal challenge.

Flagella of *P. aeruginosa* have been characterized serologically and exhibit one of two major antigenic types (4, 31, 32). One type antigen, type *a*, is serologically complex, and five subtype antigens, a_0, a_1, a_2, a_3, and a_4, have been identified (4). Even though a strain of *P. aeruginosa* that bears type *a* flagella may express one or more of the subtype antigens, virtually all type *a* strains express the a_0 antigen. The second flagellum type antigen, type *b*, has been described as serologically uniform (4).

Since flagella manifest one of only two major antigenic types, and more than 95% of *P. aeruginosa* clinical isolates are flagellated (4, 5, 31), antiflagellum immunotherapy may be a reasonable approach for treating *P. aeruginosa* infections. Therefore, a study was initiated in our laboratory to determine whether MAbs against each major flagellum serotype could be produced and, if so, whether only two MAbs, antiflagellum type *a* and antiflagellum type *b*, would provide protection against most *P. aeruginosa* strains. In this report, we describe the preparation and characterization of murine anti-*P. aeruginosa* flagellum MAbs and present results of protection studies in a murine burn wound sepsis model.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* International Antigenic Typing Scheme (IATS) strains 1 to 17 (34) (ATCC 33348 to 33364) were purchased from the American Type Culture Collection. *P. aeruginosa* Fisher immunotype (17) reference strains IT-1 to IT-7 (ATCC 27312 to 27318) were received from Michael Collins, Cutter Biological, Miles, Inc., Berkeley, Calif. Clinical isolates of *P. aeruginosa*, *Pseudomonas* sp. other than *P. aeruginosa*, *Escherichia coli*, *Serratia marcescens*, and *Proteus* sp. were obtained from the Oncogen Infectious Disease Therapeutics Biological Resources Organism Bank. All strains were originally isolated from a variety of infection sites and were provided by clinics and hospitals located in

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various regions of the United States. Centers kindly supplying bacterial strains included the Fred Hutchinson Cancer Research Center, Seattle, Wash.; Shriners Burn Institute, Cincinnati, Ohio (I. A. Holder); Brigham and Women's Hospital, Boston, Mass. (G. Pier and J. Pennington); Bristol-Myers Co., Syracuse, N.Y. (R. Goodhines); and Bristol-Myers Co., Wallingford, Conn. (J. Fung-Tomc). Motility of each bacterial isolate was assessed in motility agar by standard bacteriological technique (22). Flagella were visualized by a rapid fuchsin stain of the bacteria performed as described by Forbes (19).

Generation of antiflagellum MAbs. Female BALB/c mice (Fred Hutchinson Cancer Research Center), which were immunized to generate an immune response to a variety of *P. aeruginosa* antigens, were first injected intraperitoneally with viable *P. aeruginosa* IT-6 (8×10^6 organisms) (later shown to bear type *b* flagella), followed 2 weeks later with an injection of viable *P. aeruginosa* IT-5 (4×10^6 organisms), which was nonflagellated. During the following 2 weeks, the two strains were administered together in two weekly intraperitoneal injections. The dosage of each strain was increased for each immunization such that the final dose was 10-fold greater than the first dose. Four days after the last injection of viable bacteria, one mouse received a final intraperitoneal boost with a *P. aeruginosa* IT-6 outer membrane preparation (50 μg of protein) (21). Three days after the last immunization the spleen was removed, and a fusion of spleen lymphocytes with NS-1 myeloma cells was performed as described by Tam et al. (50).

Another group of BALB/c mice was injected intraperitoneally four times over a 6-week period with purified type *a* flagella (10 to 20 μg of protein) from IATS 6 and 8. Three days after the final boost, the spleen was removed from one animal and fused with NS-1 myeloma cells as described above.

Seven to ten days after plating of the fused cells, culture supernatants from each were assayed in an enzyme-linked immunosorbent assay (ELISA) as described below. Hybridomas producing anti-*P. aeruginosa* flagellum antibodies were then minicloned and cloned by limiting dilution techniques (50). The isotypes of the antibodies were determined by ELISA, using the Mouse Monoclonal Sub-Isotyping Kit from American Qualex International, Inc., La Mirada, Calif.

Ascitic fluid containing MAb was prepared in BALB/c mice primed with pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wis.) (50). The concentration of MAb in the ascitic fluid was estimated by agarose gel electrophoresis (Paragon; Beckman Instruments, Inc., Brea, Calif.), using another purified murine MAb as a protein standard. All ascitic fluids that contained more than 5 mg of MAb per ml were pooled, divided into smaller samples, and frozen at −70°C. MAbs were purified from ascitic fluid by affinity chromatography on immobilized protein A (Pharmacia, Piscataway, N.J.) according to the method of Ey et al. (15) as modified by Seppala et al. (47).

Anti-*P. aeruginosa* LPS MAbs. Murine anti-*P. aeruginosa* LPS MAbs were prepared as described by Pennington et al. (42). The following MAbs were included in assays: IIIH10 (IgG2a), anti-IT-1 LPS; VF5 (IgG2a), anti-IT-2 LPS; VF3 (IgG2b), anti-IT-3 LPS; and VD94 (IgG3), anti-IT-4 LPS.

ELISAs. Antigen plates for ELISAs were prepared either by binding viable bacteria to the wells of 96-well microtiter plates (Linbro, Flow Laboratories, Inc., McLean, Va.) coated with poly-L-lysine (PLL) (no. P-1524; Sigma, St. Louis, Mo.) (29) or by adsorption of Formalin-treated bacteria (31) to the wells in the absence of PLL. The latter method permitted storage of the antigen plates at 4°C for as long as 1 month before use in an ELISA.

PLL-coated plates were prepared on the day of assay by adding 50 μl of PLL (1 μg/ml in phosphate-buffered saline [PBS]) to each well and then incubating the plates for 30 min at room temperature. Unadsorbed PLL was flicked out, and the wells were washed with PBS. Bacterial cultures were grown for 16 to 18 h in Trypticase soy broth (TSB) at 37°C, washed once with PBS, and then resuspended in PBS to an optical density at 660 nm (OD_{660}) of 0.2. Bacterial suspensions were added to the wells (50 μl per well) and allowed to bind at 37°C for 1 h. Unbound bacteria were removed by ejecting the supernatant and washing the wells with saline-Tween (0.9% [wt/vol] NaCl containing 0.05% [vol/vol] Tween 20). The ELISA procedure was then performed as described below.

Bacteria for Formalin treatment were grown, washed, and suspended as described above and added to wells of microtiter plates (50 μl per well). The bacteria were pelleted by centrifugation at $1,200 \times g$ for 20 min at room temperature. After removal of the supernatants from the wells, 75 μl of 0.2% (vol/vol) Formalin in PBS was added to each well, and the wells were incubated for 15 min at room temperature. At the end of the incubation, Formalin was removed from the wells and the plates were air dried, sealed with plate sealers (Costar, Corning, N.Y.), and stored at 4°C until use.

Before initiation of the ELISA procedure, nonspecific binding of antibodies was blocked with a 5% nonfat dry milk blocking buffer (27) for 1 h at room temperature. Blocking buffer was then expelled, and the wells were washed with saline-Tween. Hybridoma supernatant or ascitic fluid containing MAb was added to the wells (50 μl per well). Ascitic fluids were diluted 1:1,000 in PBS containing 0.1% (vol/vol) Tween 20 and 0.2% (wt/vol) bovine serum albumin (PTB). After a 30-min incubation at room temperature, the wells were washed with saline-Tween. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM (Tago, Inc., Burlingame, Calif.) was diluted in PTB to a previously determined optimal concentration and added to each well (50 μl per well), and the wells were incubated for 30 min at room temperature. After a saline-Tween wash, reactions were detected and quantitated by standard ELISA procedures (14).

Immunofluorescence assay. Bacteria cultured overnight at 37°C on Trypticase soy agar (TSA) were suspended in PBS to an OD_{660} of approximately 0.2. The diluted bacteria were allowed to stand for 15 min at room temperature to aid the dispersion of small clumps. The bacterial suspension was further diluted 1:100 in PBS, and 20 μl was added to each well of 10-well Carlson slides (Carlson Scientific, Inc., Peotone, Ill.). The slides were placed on a 37°C slide warmer for 20 to 30 min until all but a bare film of liquid had evaporated. MAb (ascites), diluted in cell culture medium containing 15% fetal calf serum, was added to the bacteria (20 μl per well), and the slides were incubated in a closed humid chamber for 30 min at room temperature. The slides were washed in distilled water with gentle agitation for approximately 10 s and then set on the slide warmer (37°C) until the liquid just evaporated from the surface. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG plus IgM (Tago, Inc.), diluted 1:50 in PBS containing 0.1% (wt/vol) bovine immunoglobulins (Miles, Inc., Kankakee, Ill.) (20 μl per well), was then incubated on the slides for 30 min at room temperature in a closed humid chamber. The slides were again washed in distilled water, dried, and mounted with nonfading mounting medium. The mounting medium

was prepared by dissolving 2.5 g of 1,4-diazabicyclo (2,2,2)-octane (Aldrich) in 10 ml of 1 M Tris hydrochloride (pH 8.5) and then mixing the solution with 90 ml of glycerol (28). Slides were viewed with a Leitz fluorescence microscope.

Purification of flagella. Flagella were isolated by differential centrifugation as described by Montie and co-workers (1, 37), with the following modifications. Bacteria were harvested from 16- to 18-h TSB cultures (30°C), and shearing of the flagella from the bacteria was completed in 1 min.

SDS-PAGE. Flagella isolated from various bacterial strains were diluted in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (0.0625 M Tris hydrochloride [pH 6.8] containing 2% SDS, 5% β-mercaptoethanol, and 10% glycerol) (4 μg per lane) and separated by SDS-PAGE in a 5 to 15% acrylamide gradient slab gel with a 4% acrylamide stacking gel as described by Laemmli (30). After electrophoresis, proteins were visualized by staining the gel with 0.125% Coomassie blue R-250. Molecular weight standard proteins (Bethesda Research Laboratories, Gaithersburg, Md.) were included in the gel.

Immunoblot analysis. Flagellum preparations subjected to SDS-PAGE were electrophoretically transferred from the polyacrylamide gel to a nitrocellulose membrane (NCM) (0.45-μm pore size; Schleicher & Schuell, Keene, N.H.) overnight in 25 mM sodium phosphate (pH 7.5) at a constant 75 mA with cooling (10°C) (51). After transfer, the NCM was incubated in blocking buffer (see ELISA method described above) for 2 to 3 h. This step and all subsequent steps were performed at room temperature with rocking agitation of the NCM. After blocking, the NCM was washed with 0.05% (vol/vol) Tween 20 in PBS (PBS-Tween) (6). Purified MAb (10 μg/ml in PBS-Tween) was then incubated with the NCM for 1 h. After washing of the NCM as described above, alkaline phosphatase-conjugated goat anti-mouse IgG plus IgM (Tago, Inc.), diluted as specified by the manufacturer in PBS-Tween, was incubated with the NCM for 1 h. The NCM was washed as before, and bromochloroindolyl phosphate-Nitro Blue Tetrazolium substrate (33) was added for 15 min. The reaction was terminated by washing the NCM with distilled water.

Agglutination. Bacteria for agglutination assays were grown for 16 to 18 h at 37°C on TSA plates and then suspended in PBS to concentrations of 0.5 and 1.0 OD_{660} units. Purified MAbs were diluted in PBS to concentrations of 100, 10, and 1 μg/ml. Equal volumes of a bacterial suspension and MAb dilution were combined, immediately placed on a microscope slide, covered with a cover slip, and viewed by light microscopy (100× magnification).

Motility inhibition assay. In 10-cm plastic culture dishes, an underlayer (6 ml) of 0.6% (wt/vol) agar in motility culture broth (MCB) (1% [wt/vol] Casitone, 0.3% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl) was poured and allowed to solidify for approximately 30 min. Meanwhile, purified MAb was diluted in MCB to a concentration of 80 μg/ml and then titrated in fourfold serial dilutions such that the lowest concentration was 0.02 μg/ml. The final volume of MCB containing MAb was 6 ml for the five highest concentrations and was 18 ml for the two lowest concentrations and for a sample without MAb.

Bacteria grown at 37°C to mid-log phase in MCB were diluted to an OD_{660} of 0.2. The suspension was further diluted 10^{-6} in MCB, and a 10-μl sample was added to the MCB tubes containing antibody and to the tube containing broth without antibody to provide 5 to 20 CFU per tube. Immediately after addition of the organisms to the MCB, an equal volume of MCB containing 0.6% (wt/vol) agar and 0.4% (wt/vol) sodium nitrate was added to the tubes to achieve final concentrations of 0.3% (wt/vol) agar and 0.2% (wt/vol) sodium nitrate (43) and final MAb concentrations ranging from 40 to 0.01 μg/ml. Each culture was gently mixed and then poured onto the solidified agar underlayer. One 10-cm assay plate was prepared for the five highest MAb concentrations, and three plates were prepared for each of the two lowest concentrations and for the control without antibody.

After this layer had solidified (approximately 30 min), it was overlaid with 0.6% (wt/vol) agar in MCB (6 ml). The plates were incubated at 37°C for 13 to 22 h, depending on the migration of the particular *P. aeruginosa* isolate in soft agar, so that the diameters of colonies grown in the absence of MAb were between 9 and 15 mm. The diameters of 6 to 10 colonies were measured per MAb concentration.

Inhibition of motility was assessed by comparing the average diameter of the colonies grown in the presence of MAb with the average diameter of colonies grown without antibody. The percent inhibition was calculated according to the formula $[(D_0 - D_{MAb})/D_0] \times 100$, where D_{MAb} represents the average colony diameter of bacteria grown at a particular MAb concentration and D_0 equals the average colony diameter of bacteria grown in the absence of MAb. The calculation assumes 0% inhibition of motility when bacteria are grown in the absence of MAb.

Mouse burn wound sepsis model. The burn wound sepsis model was performed basically as described by Collins and Roby (8), with the following modifications. At least 1 day but less than 1 week before an experiment, female Swiss-Webster mice (Simonsen Laboratories, Gilroy, Calif.) weighing 24 to 26 g were depiliated as follows. Mice were first anesthetized by intramuscular injection (0.1 ml in each thigh) of a mixture of xylazine (Mobay Corp., Shawnee, Kans.) (20 mg/kg of body weight) and ketamine hydrochloride (Parke-Davis, Morris Plains, N.J.) (1 mg/kg). The right side of each mouse was shaved and then treated with a depilatory agent (Nair; Carter-Wallace Corp., New York, N.Y.) for 3 to 5 min to effectively remove all hair from the shaved area.

Burns were administered to mice after the animals were anesthetized as described above. A woven silicon cloth template (Zetex cloth; 260 by 260 mm; NEWTEX Corp., Vector, N.Y.), with a rectangle (15 by 30 mm) cut from the center of the cloth was used to contain the area of the burn to approximately 5.5 to 6.0% of the body surface (20). Each mouse was laid left side down in a laminar flow hood, and the template was placed over the right side such that the open rectangle was centered between the forelimb and hindlimb and between the dorsal and ventral midlines.

The burn was administered with a grid-top adjustable flame gas burner to which a halo support was attached to aid in positioning the burner at a constant distance, approximately 1.5 cm, from the skin. The flame was applied until the skin just began to blanch, approximately 3 to 5 s. Immediately after removal of the flame, mice were injected subeschar with 0.5 ml of the challenge inoculum. Control animals received cold diluent only. All animals were checked daily for 10 to 14 days for symptoms and mortality. Obviously terminal (moribund) animals were euthanized.

Histopathological examination of the burn site indicated that a full-thickness, third-degree burn was achieved over the entire area of the template opening, with a sharp margin around the burn area. The burn was characterized by destruction of the epidermis, dermal appendages, epithelial

elements, and nerves of the skin (D. H. Gribble, Evergreen Professional Services, Inc., Kirkland, Wash., personal communication). Mortality due to administration of the burn in the absence of bacterial challenge was rare.

Bacteria for each experiment were grown to mid-log phase at 37°C in TSB. The bacteria were harvested by centrifugation, washed once in ice-cold diluent (0.1% [wt/vol] TSB in 0.1% [wt/vol] *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.2], 0.85% [wt/vol] NaCl) and then resuspended in diluent to the appropriate concentration. Each challenge dose was quantitated by plating 10-fold serial dilutions of the inoculum on TSA plates. The 50% lethal dose (LD_{50}) of each strain was determined by probit analysis (16) of mortality data at day 10 for groups of mice that were challenged with increasing two- or fourfold doses of bacteria. For the prophylactic experiments, the challenge dose of each bacterial strain was approximately 10 LD_{50}s, which resulted in 80 to 90% mortality of animals in the negative control groups. The dose was increased to 1,000 LD_{50}s for the therapeutic studies to consistently provide greater than 95% mortality in the negative control groups.

RESULTS

Isolation of antiflagellum monoclonal antibodies. A fusion of NS-1 myeloma cells and spleen cells from a mouse immunized with viable bacteria (IT-5 and IT-6) and outer membrane preparations (IT-6) was first assayed by ELISA on each of the four *P. aeruginosa* reference strains IT-1, IT-2, IT-3, and IT-4. Since the objective of the fusion was to generate anti-*P. aeruginosa* cross-reactive antibodies, four strains other than those used for immunizations were chosen for the initial screen. Master wells containing antibody that bound to any of the four strains were then assayed in a second ELISA in which the individual IATS reference strains 1 to 12 were treated with Formalin and adhered to microtiter wells. Antibody present in one master well, IVE8, which bound only to IT-2 in the first assay, bound to IATS 2, 3, 4, 5, 7, 10, 11, and 12, all of which have type *b* flagella (4, 5). The IVE8 antibody was therefore presumptively identified as an anti-type *b* flagellum antibody. This information also suggested that IT-2 had type *b* flagella. The IVE8 cell line was cloned by limiting dilution techniques, and ascitic fluid was prepared as described in Materials and Methods. The isotype of the IVE8 MAb was shown to be IgG2a.

Isolation of an antibody that bound to flagellum type *b* IATS strains was rare from this fusion and similar fusions in which mice were immunized with viable organisms, likely because of exposure of the mice to more immunodominant antigens, e.g., LPS. Only one other master well contained anti-flagellum type *b* antibody, but the cell line was not cloned because it was unstable. To more readily generate anti-flagellum type *a* MAbs, mice were immunized with purified type *a* flagella, and splenocytes from an animal were fused with NS-1 myeloma cells. The fusion was initially screened by ELISA in which binding of antibody present in culture supernatants to *P. aeruginosa* reference strains IATS 6, IATS 9, and IT-1 was measured and compared. IATS 6 and 9 are type *a* flagellated organisms (4, 5), and IT-1 is nonflagellated, as demonstrated by fuchsin dye staining (data not shown). Only those hybridomas producing antibody that bound to the type *a* flagellated bacteria and not to strain IT-1 were analyzed further by an ELISA with IATS 1 to 12. Several master wells contained antibody that bound to the type *a* strains, IATS 1, 6, 8, and 9 (4, 5), and not to the type *b* strains. The cell line designated IIG5 from one fusion was cloned, and ascitic fluid was prepared. The isotype of the IIG5 MAb was shown to be IgG3. Antibodies produced by the cloned IVE8 and IIG5 cell lines had reactivity patterns on the IATS strains identical to those described for the master wells.

Indirect immunofluorescence assays. Immunofluorescence studies demonstrated that IIG5 and IVE8 bound to the flagella of *P. aeruginosa* strains. A fluorescence pattern of short sinuous lines similar to the shape of flagella observed by fuchsin dye staining was observed when IIG5 was incubated with strain IATS 1 and when IVE8 was incubated with strain IT-2 (Fig. 1A and E). The reactions were specific, since IIG5 did not bind to strain IT-2 and IVE8 did not bind to IATS 1 (Fig. 1D and H). To visualize the flagella in relation to the whole organism, specific anti-*P. aeruginosa* LPS murine MAbs VD94 and VF5, which bound to the outer cell membrane of the IATS 1 and IT-2 strains, respectively (Fig. 1B and F), were incorporated into the assay. Inclusion of the specific anti-LPS MAb with IIG5 or IVE8 showed the outline of the bacterium with a flagellum attached at a polar position characteristic of *P. aeruginosa* (Fig. 1C and G).

Immunoblot analysis. Flagellin, the protein subunit that constitutes the flagellum organelle, was identified as the target antigen of the MAbs by immunoblot analysis. Flagella isolated from *P. aeruginosa* reference strains IATS 6, IATS 8, and IT-2 were separated by SDS-PAGE under reducing conditions and visualized in the gel by staining with Coomassie blue (Fig. 2A). The molecular masses of the major band from the IATS 6 and IATS 8 flagellum preparations were 41,800 and 40,600 Da, respectively, and the molecular mass of the major band from the IT-2 flagellum preparation was 48,500 Da. Molecular masses of all flagellins were lower than those previously reported, 45,000 and 47,000 Da for type *a* flagellins from IATS 6 and IATS 8, respectively, and 53,000 Da for flagellin from type *b* flagella (1). The difference in the molecular masses reported here and by Allison et al. (1) is likely due to differences in migration of proteins in a gradient gel compared with a fixed-percentage acrylamide gel. Other experiments in our laboratory in which flagellin from IT-2 was separated in a 10% acrylamide gel (30) showed that the molecular weight of the type *b* flagellin band was 53,000 (unpublished observations), similar to values in other published reports.

The identity of the low-molecular-weight contaminants present in the IATS 6 flagellum preparation (Fig. 2A, lane 2) is unknown but could represent degradation products of flagellin. Alternatively, one or both of the protein bands might represent pilus contamination, since the molecular mass of each contaminant in the flagellum preparation is similar to that reported for pilin (15,000 Da) (45).

All three flagellum preparations from a gel identical to the one stained with Coomassie blue were transferred to an NCM and immunoblotted. IIG5 bound to the flagellin of both type *a* strains but not to the type *b* flagellin of IT-2 (Fig. 2B), while IVE8 bound to flagellin of the type *b* strain but not to the flagellins of either type *a* strain (Fig. 2C). If the low-molecular-weight contaminants present in the IATS 6 preparation were degradation products of flagellin, the fragments did not include the epitope recognized by IIG5, since the MAb did not identify the bands in the immunoblot.

Flagellum typing of *P. aeruginosa* reference strains and clinical isolates. Binding of IIG5 and IVE8 to each of the 17 IATS reference strains was analyzed by ELISA. As observed in the initial assays, IIG5 bound specifically to the type *a*-bearing strains, IATS 1, 6, 8, and 9 (4, 5), and IVE8 was specific for the type *b* strains, IATS 2, 3, 4, 5, 7, 10, 11,

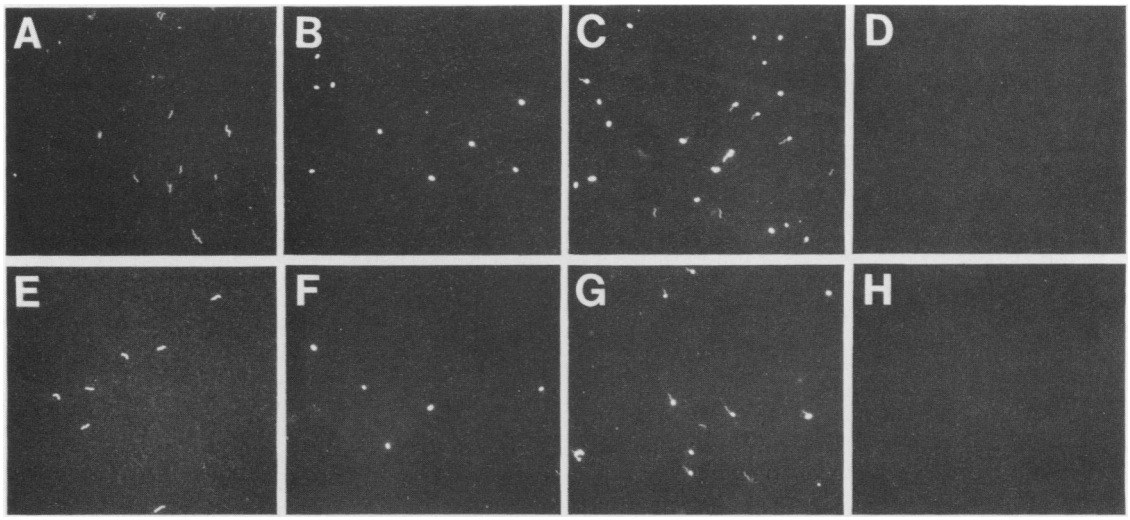

FIG. 1. Indirect immunofluorescence assays. *P. aeruginosa* reference strains IATS 1 (A to D), bearing type *a* flagella, and IT-2 (E to H), bearing type *b* flagella, were gently heat fixed to glass microscope slides. Ascitic fluids containing MAbs were diluted as indicated below in cell culture medium containing 15% fetal calf serum, and binding was detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG plus IgM (see Materials and Methods). MAbs were applied to the slides as follows: (A) IIG5 (diluted 1:125); (B) VD94 (anti-IATS 1 LPS MAb) (1:40,000); (C) IIG5 (1:125) and VD94 (1:40,000); (D) IVE8 (1:125); (E) IVE8 (1:250); (F) VF5 (anti-IT-2 LPS MAb) (1:40,000); (G) IVE8 (1:250) and VF5 (1:40,000); and (H) IIG5 (1:250). The dilutions of ascitic fluids were chosen so the intensities of the fluorescent staining of the LPS and of the flagella were equivalent.

and 12 (4, 5) (Table 1). In addition, IIG5 bound to IATS 14 and 17 and IVE8 bound to IATS 15 and 16, indicating that IATS 14 and 17 bear type *a* flagella and IATS 15 and 16 bear type *b* flagella. Neither MAb bound to IATS 13, which was shown by fuchsin dye staining to be nonflagellated (data not shown).

A similar analysis performed with the seven Fisher immunotype reference strains demonstrated that IIG5 bound only to IT-4 and that IVE8 bound to strains IT-2, IT-6, and IT-7 (Table 1), suggesting that strain IT-4 bears type *a* flagella and strains IT-2, IT-6, and IT-7 bear type *b* flagella. Neither MAb bound to strains IT-1, IT-3, and IT-5, which were shown by fuchsin dye staining to be nonflagellated (data not shown).

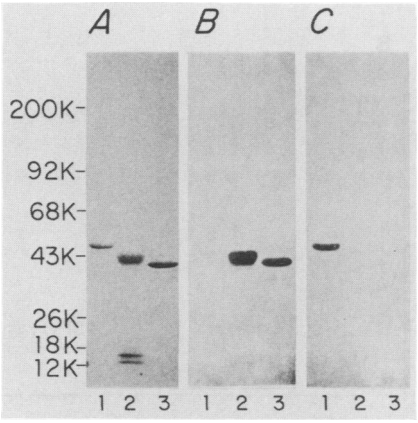

FIG. 2. SDS-PAGE and immunoblot analysis. (A) Coomassie blue-stained SDS-polyacrylamide gel of flagellum type *b* from IT-2 (lane 1), flagellum type *a* from IATS 6 (lane 2), and flagellum type *a* from IATS 8 (lane 3). The proteins from a gel identical to the one shown in panel A were transferred to an NCM and immunoblotted with either IIG5 (10 μg/ml) (B) or IVE8 (10 μg/ml) (C). Molecular weights of marker proteins are indicated at the left (K = thousands).

Binding of IIG5 and IVE8 to a panel of 257 flagellated *P. aeruginosa* clinical isolates was also examined by ELISA. IIG5 bound to 174 clinical isolates (67.7%) and IVE8 bound to 79 other isolates (30.7%), thus identifying the flagellum type of 253 (98.4%) of the 257 strains examined (Table 2). In no instance did both MAbs bind to the same isolate. The expression of epitopes recognized by the antiflagellum MAbs was restricted to *P. aeruginosa*, since the MAbs did not bind to three to five flagellated clinical isolates of each of the following gram-negative organisms: *Pseudomonas* sp. other than *P. aeruginosa*, *E. coli*, *S. marcescens*, and *Proteus* sp. (data not shown).

Agglutination. Agglutination by the antiflagellum MAbs of a type *a* flagellated *P. aeruginosa* clinical isolate (I624) and of a type *b* clinical isolate (F164) was examined by combining bacteria (1.0 and 0.5 OD_{660} units) with increasing concentrations of specific and nonspecific antiflagellum MAbs. Bacteria examined immediately after the addition of 1 μg of specific MAb per ml were still motile, but within 2 to 3 min, bacteria appeared tethered to the glass slide. This phenomenon likely results from the flagellum of an organism binding to MAb nonspecifically bound to the glass, which fixes a bacterium to the slide but does not prohibit rotation of the flagellar motor. Tethering of bacteria was not observed in the presence of nonspecific MAb.

When higher concentrations of specific MAb (10 and 100 μg/ml) were combined with bacteria (0.5 OD_{660} unit), motile bacteria were not observed. Some bacteria had tethered and a few small clumps of bacteria were seen, but the majority of the organisms were single without directed motion. The lack of motility of the bacteria was easily distinguished from the quick darting movement of organisms in the presence of the nonspecific antiflagellum MAb at 100 μg/ml. Similar observations were made with the higher concentration of bacteria (1.0 OD_{660} unit) and 10 μg of specific MAb per ml. When the MAb concentration was increased to 100 μg/ml and added to the higher concentration of bacteria, more clumping of

TABLE 1. Binding of IIG5 and IVE8 to *P. aeruginosa* reference strains[a]

Strain	Flagellum type[b]	OD_{490}	
		IIG5	IVE8
IATS			
1	*a*	0.8	<0.1
2	*b*	<0.1	2.2
3	*b*	<0.1	2.2
4	*b*	<0.1	2.2
5	*b*	<0.1	2.4
6	*a*	0.3	<0.1
7	*b*	<0.1	2.7
8	*a*	1.0	<0.1
9	*a*	1.6	<0.1
10	*b*	<0.1	2.1
11	*b*	<0.1	2.3
12	*b*	<0.1	0.9
13	NF	<0.1	<0.1
14	*a*	2.0	<0.1
15	*b*	<0.1	0.9
16	*b*	<0.1	2.7
17	*a*	1.7	<0.1
Fisher			
IT-1	NF	<0.1	<0.1
IT-2	*b*	<0.1	0.5
IT-3	NF	<0.1	<0.1
IT-4	*a*	0.6	<0.1
IT-5	NF	<0.1	<0.1
IT-6	*b*	<0.1	1.1
IT-7	*b*	<0.1	0.7

[a] Determined by ELISA as described in Materials and Methods. The source of the MAbs was ascitic fluids.

[b] The flagellum types of IATS strains 1 to 12 have been previously described (4, 5). The flagellum types of IATS strains 14 to 17 and the Fisher immunotype strains were inferred from the binding specificity of IIG5 and IVE8 to IATS strains 1 to 12. NF, Nonflagellated (shown by fuchsin dye staining).

organisms was noted. Not all bacteria agglutinated, however, and many single nonmotile organisms were observed.

Motility inhibition assays. A soft agar assay was developed to provide a more sensitive and quantitative in vitro measurement of the effect of antiflagellum MAbs on motility of bacteria. Addition of specific antiflagellum MAb to bacteria grown in soft agar resulted in dose-dependent inhibition of migration. Results obtained when the type *a* flagellum-bearing *P. aeruginosa* clinical isolate I624 was combined with IIG5 in soft agar and when the type *b*-bearing clinical isolate F164 was combined with IVE8 are presented in Fig. 3A and B, respectively. In the absence of specific MAb, the bacteria were freely motile and were able to penetrate the soft agar. Little difference in migration inhibition of I624 by IIG5 at concentrations of 10 and 40 μg/ml was observed, indicating that IIG5 concentrations of 10 μg/ml and greater provided maximal inhibition of migration of I624. Maximum inhibition of migration of F164 by IVE8 was achieved at MAb concentrations of 2.5 μg/ml and greater. All isolates recognized by IIG5 or IVE8 by ELISA that have been tested in this assay have been inhibited by the specific MAb. Furthermore, inhibition of motility of type *a* isolates by IVE8 or inhibition of motility of type *b* isolates by IIG5 has never been observed.

TABLE 2. Binding of anti-*P. aeruginosa* flagellum MAbs to *P. aeruginosa* clinical isolates[a]

MAb	Specificity	No. positive	% Positive
IIG5	Type *a*	174	67.7
IVE8	Type *b*	79	30.7
Combined coverage		253	98.4

[a] A total of 257 *P. aeruginosa* clinical isolates were adhered to microtiter plates and treated with Formalin, and an ELISA was performed as described in Materials and Methods. The source of each MAb was culture supernatant from the cloned cell line.

Prophylactic protection studies. The protective efficacy of the antiflagellum MAbs was studied in a mouse burn wound sepsis model. For these studies, purified MAbs were administered intravenously 1 to 2 h before administration of the burn and challenge with approximately 10 LD_{50}s of *P. aeruginosa* (Table 3). In the first study, mice were challenged with the *P. aeruginosa* clinical isolate G98 (IT-3 LPS), which bears type *a* flagella. All animals that received IIG5 survived, while only 20% of those that received the negative control MAb, anti-IT-1 LPS MAb (IIIH10), lived. In a second experiment, in which mice were challenged with another type *a* isolate, PA220 (IT-1 LPS), the specific MAb IIG5 again provided significant protection, whereas the

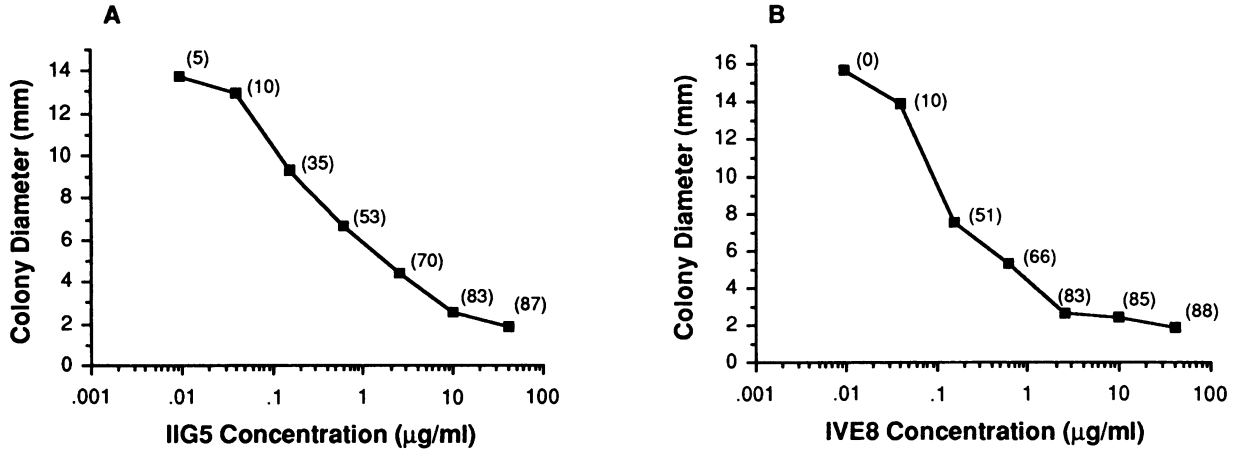

FIG. 3. Motility inhibition assays. Inhibition of migration of the *P. aeruginosa* clinical isolate I624 by IIG5 and inhibition of *P. aeruginosa* clinical isolate F164 by IVE8 were assessed in a soft agar motility assay. (A) Average colony diameter of I624 and percent inhibition (in parentheses) at each concentration of IIG5; (B) average colony diameter of F164 and percent inhibition (in parentheses) at each concentration of IVE8.

TABLE 3. Prophylactic protection by the anti-*P. aeruginosa* flagellum MAbs in a murine burn wound sepsis model

Challenge			Treatment[a]	Survival at day 10[b]	*P* value[c]
Strain	Flagellum type	Dose (CFU)[d]			
G98	*a*	90	IIG5	10/10	<0.001
			Nonspecific MAb[e]	2/10	—
			PBS	1/10	NS
PA220	*a*	95	IIG5	8/10	<0.001
			IVE8	1/9	NS
			Nonspecific MAb[f]	1/10	—
IT-2	*b*	85	IVE8	9/10	<0.001
			IIG5	1/10	NS
			Nonspecific MAb[e]	2/10	—
			PBS	0/10	NS

[a] Purified MAbs (40 μg) diluted in PBS were administered intravenously in a total volume of 0.2 ml 1 to 2 h before burn and bacterial challenge.
[b] Number of animals alive at 10 days compared with the number of animals in each group.
[c] Significance of protection was determined by the Fisher exact test, comparing survival with that of the nonspecific MAb control (—). *P* values of greater than 0.05 were not significant (NS).
[d] Equivalent to 10 LD_{50}s.
[e] IIIH10 (IgG2a), an anti-IT-1 LPS MAb.
[f] VF3 (IgG2b), an anti-IT-3 LPS MAb.

antiflagellum type *b* MAb and negative control anti-LPS MAb (VF3, anti-IT-3 LPS) were not protective. Significant protection by IVE8 of mice challenged with the flagellum type *b*-bearing *P. aeruginosa* IT-2 reference strain was demonstrated in a third study. The specificity of the antiflagellum MAbs was again observed, as IIG5 did not protect animals challenged with IT-2.

Therapeutic protection study. To assess the therapeutic efficacy of the antiflagellum MAbs, administration of the antibodies was delayed for various times postchallenge. Bacterial strains other than those used in the prophylactic studies were chosen to show that protection was not strain dependent. Animal groups were challenged with 1,000 LD_{50}s (20,000 CFU) of the clinical isolate I624 (IT-2 LPS) bearing type *a* flagella. Purified IIG5 was administered intraperitoneally at a dose of 50, 10, or 2 μg per mouse at 1, 7, or 20 h after burn and challenge. In a similar manner, burned mice were challenged with 1,000 LD_{50}s (3,000 CFU) of a type *b* flagellum-bearing isolate, F164 (IT-4 LPS), followed by administration of IVE8. As a negative control for each study, the irrelevant antiflagellum MAb was administered at a dose of 50 μg per mouse at the 1-h time point.

The results of the studies are presented in Fig. 4 and 5. Administration of IIG5 at 50- and 10-μg doses at 1 h and 7 h postchallenge protected 70 to 90% of the animals. Similarly, 50 and 10 μg of IVE8 protected 70 to 90% of mice when administered at 1 and 7 h after challenge with F164. The 2-μg-per-mouse dose of IVE8 administered at 1 h postchallenge was also able to provide significant protection (55%; $P < 0.001$), although the percent survival was less than observed when higher doses of IVE8 were given. In contrast, 2 μg of IIG5 was not sufficient to protect mice against a 1,000-LD_{50} challenge dose of I624. Although total survival of this group was not significant, the delay in time until death compared with that for the negative control group was statistically significant ($P < 0.001$) (35). The difference in protection by the 2-μg dose of each MAb may reflect differences in the functional properties of the antibodies or in the pathogenicity of the challenge organisms.

In both studies, delay of MAb administration to 20 h postchallenge resulted in significant mortality (0 to 10% survival). By 16 h after challenge with this highly lethal dose of bacteria, the mice usually had a systemic infection (unpublished observation) and unprotected animals died within the next 24 h. Even though treatment with antiflagellum MAbs as late as 20 h after bacterial challenge did not result in significant survival of animals, the higher doses did prolong the time until death. Administration of 50 and 10 μg of IVE8 to mice challenged with F164 extended survival of animals ($P < 0.001$ and $P < 0.01$, respectively), as did treatment with 50 μg of IIG5 for animals challenged with I624 ($P < 0.01$) (35).

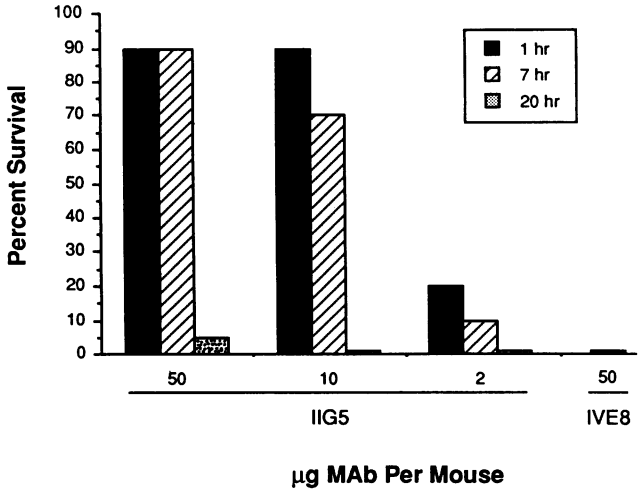

FIG. 4. Therapeutic protection by IIG5 in the murine burn wound sepsis model. Groups of 20 mice were challenged with 1,000 LD_{50}s of *P. aeruginosa* I624. Purified MAbs diluted in PBS were administered intraperitoneally (0.5 ml per mouse) at the times shown postchallenge. Percent survival at day 10 is indicated.

DISCUSSION

Two anti-*P. aeruginosa* flagellum murine MAbs, IIG5, specific for type *a* flagella, and IVE8, specific for type *b*

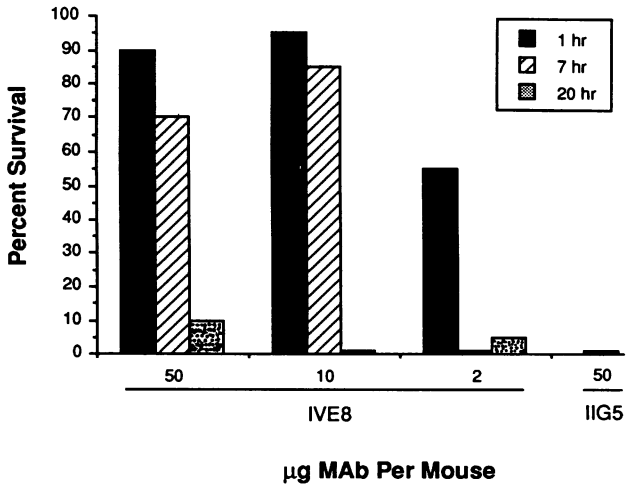

FIG. 5. Therapeutic protection by IVE8 in the murine burn wound sepsis model. Groups of 20 mice were challenged with 1,000 LD_{50}s of *P. aeruginosa* F164. Purified MAbs diluted in PBS were administered intraperitoneally (0.5 ml per mouse) at the times shown postchallenge. Percent survival at day 10 is indicated.

flagella, have been isolated and characterized. Results of prophylactic and therapeutic studies with the antiflagellum MAbs have substantiated the specificity and efficacy of anti-*P. aeruginosa* flagellum immunotherapy suggested by active immunization with purified flagella (25) and passive immunization with rabbit antiflagellum antisera (12).

Of particular interest was the potential value of anti-*P. aeruginosa* flagellum MAbs in treatment of an infected host. A therapeutic study was performed in which three different doses of specific MAb were administered at various times after burn and challenge. Significant protection was dependent on dose and the time at which the specific MAb was administered. Significant survival of animals challenged with 1,000 LD_{50}s of *P. aeruginosa* strains was observed when the specific MAb at doses of 10 and 50 μg per animal was administered as late as 7 h after burn and challenge. Delaying administration of MAb until 20 h after burn and challenge, when the mice were septic, resulted in prolonged survival of animals receiving the higher concentrations of MAb but could not prevent death of the animals. As with any anti-infective therapy, early intervention with MAbs appeared critical to a favorable outcome.

Host defense against *P. aeruginosa* infections is believed to rely on phagocytosis of the organisms by host polymorphonuclear leukocytes in the presence of heat-labile opsonins and heat-stable antibodies (53), primarily type-specific LPS antibodies (52). These observations raise interesting questions regarding the mechanism of protection by antiflagellum antibodies, since binding of antiflagellum antibodies to a bacterium conceivably would not result in sufficient opsonization of the organism such that phagocytosis could be induced. Anderson and Montie (2, 3) have shown that phagocytosis of *P. aeruginosa* by mouse peritoneal polymorphonuclear leukocytes was greater in vitro in the presence of antiflagellum rabbit antisera than in the presence of normal rabbit sera. Preliminary experiments in our laboratory indicated that opsonophagocytosis by the antiflagellum MAbs did occur, but the efficiency of opsonization varied according to the bacterial isolate used in the assay. Furthermore, opsonophagocytic activity of the antiflagellum MAbs was far inferior to that of specific anti-LPS MAbs (unpublished observation), which are very efficient opsonins (48, 49). The fact that antiflagellum MAbs were less effective opsonins than anti-LPS MAbs does not preclude the possibility that opsonization of bacteria by antiflagellum antibodies is involved in prevention of lethal infection. Additional in vitro and in vivo studies may clarify the role that opsonization by antiflagellum antibodies plays in protection.

The possibility that antiflagellum antibodies protected infected animals by bactericidal or bacteriostatic activity was discounted by results of in vitro studies. Omission of polymorphonuclear leukocytes in opsonization assay controls demonstrated that the MAbs did not have bactericidal or bacteriostatic activity in the presence or absence of complement (unpublished observations). Drake and Montie (12) also demonstrated lack of bactericidal or bacteriostatic activity of antiflagellum antisera.

The primary mechanism proposed by which antiflagellum antibodies protect animals in the burn wound sepsis model is by inhibiting the motility of bacteria, thereby sequestering the infection to the burn site (13, 25, 26). This conclusion was suggested in part by the importance of motility to the pathogenesis of *P. aeruginosa* in burn wound sepsis models (13, 36, 38). The role of motility inhibition of *P. aeruginosa* in burn wound infections was more directly addressed by Drake and Montie (13), who examined dissemination of *P. aeruginosa* in a mouse burn wound sepsis model. They found that a mutant lacking flagella was unable to disseminate from the burn wound site. Moreover, dissemination of the parent flagellated strain to the liver or blood of animals treated with antiflagellum antisera before challenge was also significantly reduced. Since the only known difference between the mutant strain and the parent was the loss of flagella, and since dissemination of the parent strain in the presence of antiflagellum sera was like that observed with the mutant in untreated animals, the loss of virulence of the parent flagellated strain was attributed to inhibition of motility by the antiflagellum antisera.

The phenomenon of motility inhibition of *P. aeruginosa* by antiflagellum antibodies has been observed in vitro in soft agar assays with antiflagellum antisera (12, 23) and with antiflagellum MAbs (Fig. 3). *P. aeruginosa* strains that were used in the therapeutic protection studies were inhibited in a dose-dependent manner by the specific MAb in the soft agar assay. The minimum concentration of IIG5 required to provide maximum inhibition of migration of the type *a* flagellated strain was 10 μg/ml. Less IVE8, 2.5 μg/ml, was required to provide maximum inhibition of the type *b* flagellated strain. Additional studies are required to determine whether a correlation exists between in vitro motility inhibition and in vivo protective efficacy.

Agglutination of bacteria by antiflagellum antisera has been observed (12, 23) and suggested as the means of motility inhibition (12). We have shown in this study that binding of specific antiflagellum MAb at concentrations of 10 and 100 μg/ml to bacteria (1.0 OD_{660} unit) resulted in agglutination of a portion of the population. Moreover, organisms not incorporated into clumps were nonmotile, devoid of any directed motion. At the lower concentration of bacteria (0.5 OD_{660} unit) agglutination was rare, but loss of motility was still observed. This finding suggests that binding of antiflagellum antibodies to bacteria inhibits motility, not only by agglutination of several bacteria but also by directly affecting the motility of a single organism.

The usefulness of antiflagellum MAbs for immunotherapy depends on the potential of the MAbs for treating infection by a majority of *P. aeruginosa* strains. Analysis of the cross-reaction of the anti-type *a* MAb, IIG5, in particular, with a large sample of strains was important because type *a* flagella have been defined as serologically complex (4). Binding of IIG5 and IVE8 to 257 flagellated *P. aeruginosa* clinical isolates in an ELISA showed that more than 98% of the clinical isolates were identified by the MAbs as either type *a* or type *b*.

The extensive cross-reactivity of IIG5 suggested that it recognized an epitope on the a_0 antigen, which was characterized in serological studies as the subtype *a* antigen present on all type *a* flagellated *P. aeruginosa* strains (4). Of the 257 clinical isolates, 4 were not identified as type *a* or type *b* by IIG5 or IVE8. Two of the four isolates, however, were recognized by another antiflagellum type *a* MAb, designated IF4, prepared in our laboratory (unpublished observations). IF4 bound to a less commonly expressed type *a* antigen, since it reacted with only 38% of the clinical isolates identified as type *a* strains by IIG5. Whether binding of IF4 to two clinical isolates not recognized by IIG5 indicates that the a_0 antigen is not expressed by all type *a* flagella or that IIG5 recognizes a subgroup of the a_0 subtype antigen is not known. The flagellum types of the two isolates not recognized by IIG5, IVE8, or IF4 are unknown at present.

In summary, two murine antiflagellum MAbs have been developed that collectively recognize >98% of flagellated

clinical isolates of *P. aeruginosa*. Both MAbs provided specific and significant prophylactic and therapeutic protection against lethal challenge with *P. aeruginosa* strains. These findings plus the observation that more than 95% of all *P. aeruginosa* clinical isolates are flagellated (4, 5, 31) suggest that two antiflagellum MAbs could be used to treat infection caused by the vast majority of *P. aeruginosa* strains. Even though other anti-*P. aeruginosa* MAbs, in particular anti-LPS MAbs, have been prepared and shown to be protective in animal models (10, 42, 46), to approach the clinical coverage that two antiflagellum MAbs offer could require as many as 17 anti-LPS MAbs. The preparation of such a cocktail for clinical administration would be a formidable task. Hence, current efforts in the laboratory are directed toward development of human anti-*P. aeruginosa* flagellum MAbs.

ACKNOWLEDGMENTS

We thank R. Darveau and H. Raff for critical review of the manuscript. Special recognition is given to Gary Keizur, Cara Watkins, and Grace Maloney for performance of the animal protection studies. We thank Dennis Hanson and Glenna Lynch for their technical assistance. We are indebted to the Infectious Disease Therapeutics Biological Resources group for their bacteriology expertise and assistance.

LITERATURE CITED

1. **Allison, J. S., M. Dawson, D. Drake, and T. C. Montie.** 1985. Electrophoretic separation and molecular weight characterization of *Pseudomonas aeruginosa* H-antigen flagellins. Infect. Immun. **49**:770–774.
2. **Anderson, T. R., and T. C. Montie.** 1987. Opsonophagocytosis of *Pseudomonas aeruginosa* treated with antiflagellar serum. Infect. Immun. **55**:3204–3206.
3. **Anderson, T. R., and T. C. Montie.** 1989. Flagellar antibody stimulated opsonophagocytosis of *Pseudomonas aeruginosa* associated with response to either *a*- or *b*-type flagellar antigen. Can. J. Microbiol. **35**:890–894.
4. **Ansorg, R.** 1978. Flagella specific H antigenic schema of *Pseudomonas aeruginosa*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **242**:228–238.
5. **Ansorg, R. A., M. E. Knoche, A. F. Spies, and C. J. Kraus.** 1984. Differentiation of the major flagellar antigens of *Pseudomonas aeruginosa* by the slide coagglutination technique. J. Clin. Microbiol. **20**:84–88.
6. **Batteiger, B., W. J. Newhall V, and R. B. Jones.** 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55**:297–307.
7. **Bodey, G. P., R. Bolivar, V. Fainstein, and L. Jadeja.** 1983. Infections caused by *Pseudomonas aeruginosa*. Rev. Infect. Dis. **5**:279–313.
8. **Collins, M. S., and R. E. Roby.** 1983. Anti-*Pseudomonas aeruginosa* activity of an intravenous human IgG preparation in burned mice. J. Trauma **23**:530–534.
9. **Cryz, S. J., Jr.** 1984. *Pseudomonas aeruginosa* infections, p. 317–351. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
10. **Cryz, S. J., Jr., E. Fürer, and R. Germanier.** 1983. Protection against *Pseudomonas aeruginosa* infection in a murine burn wound sepsis model by passive transfer of antitoxin A, antielastase, and antilipopolysaccharide. Infect. Immun. **39**:1067–1071.
11. **Cryz, S. J., Jr., E. Fürer, and R. Germanier.** 1984. Protection against fatal *Pseudomonas aeruginosa* burn wound sepsis by immunization with lipopolysaccharide and high-molecular-weight polysaccharide. Infect. Immun. **43**:795–799.
12. **Drake, D., and T. C. Montie.** 1987. Protection against *Pseudomonas aeruginosa* infection by passive transfer of anti-flagellar serum. Can. J. Microbiol. **33**:755–763.
13. **Drake, D., and T. C. Montie.** 1988. Flagella, motility and invasive virulence of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **134**:43–52.
14. **Engvall, E.** 1977. Quantitative enzyme immunoassay (ELISA) in microbiology. Med. Biol. **55**:193–200.
15. **Ey, P. L., S. J. Prowse, and C. R. Jenkin.** 1978. Isolation of pure IgG_1, IgG_{2a}, IgG_{2b} immunoglobulins from mouse serum using protein A Sepharose. Immunochemistry **15**:429–436.
16. **Finney, D. J.** 1971. Probit analysis, p. 20–49. Cambridge University Press, London.
17. **Fisher, M. W., H. B. Devlin, and F. J. Gnabasik.** 1969. New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. J. Bacteriol. **98**:835–836.
18. **Flick, M. R., and L. E. Cluff.** 1976. Pseudomonas bacteremia, review of 108 cases. Am. J. Med. **60**:501–508.
19. **Forbes, L.** 1981. Rapid flagella stain. J. Clin. Microbiol. **13**:807–809.
20. **Freirich, E. J., E. A. Gehan, D. P. Rall, L. H. Schmidt, and H. E. Skipper.** 1966. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother. Rep. **50**:219–244.
21. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of permeability barrier. J. Bacteriol. **136**:381–390.
22. **Hendrickson, D. A.** 1985. Reagents and stains, p. 1096. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
23. **Holder, I. A.** 1985. The pathogenesis of infections owing to *Pseudomonas aeruginosa* using the burned mouse model: experimental studies from the Shriners Burns Institute, Cincinnati. Can. J. Microbiol. **31**:393–402.
24. **Holder, I. A.** 1988. Pseudomonas immunotherapy. Serodiagn. Immunother. **2**:7–16.
25. **Holder, I. A., and J. G. Naglich.** 1986. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: immunization using divalent flagella preparations. J. Trauma **26**:118–122.
26. **Holder, I. A., R. Wheeler, and T. C. Montie.** 1982. Flagellar preparations from *Pseudomonas aeruginosa*: animal protection studies. Infect. Immun. **35**:276–280.
27. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder.** 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1**:3–8.
28. **Johnson, G. D., R. S. Davidson, K. D. McNamee, G. Russell, D. Goodwin, and E. J. Holborow.** 1982. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J. Immunol. Methods **55**:231–242.
29. **Kennett, R. H.** 1980. Enzyme-linked antibody assay with cells attached to polyvinyl chloride plates, p. 376–377. *In* R. H. Kennett, T. J. McKearn, and K. B. Bechtol (ed.), Monoclonal antibodies. Hybridomas: a new dimension in biological analyses. Plenum Publishing Corp., New York.
30. **Laemmli, U. K.** 1970. Cleavage of structural protein during the assembly of the head of the bacteriophage T4. Nature (London) **227**:680–685.
31. **Lanyi, B.** 1970. Serological properties of *Pseudomonas aeruginosa*. II. Type-specific thermolabile (flagellar) antigens. Acta Microbiol. Acad. Sci. Hung. **17**:35–48.
32. **Lanyi, B., and T. Bergan.** 1978. Serological characterization of *Pseudomonas aeruginosa*, p. 93–168. *In* T. Bergan and H. R. Norris (ed.), Methods in microbiology. Academic Press, Inc. (London), Ltd., London.
33. **Leary, J. J., D. J. Brigati, and D. C. Ward.** 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc. Natl. Acad. Sci. USA **80**:4045–4049.
34. **Liu, P. V., H. Matsumoto, H. Kusama, and T. Bergan.** 1983. Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. **33**:256–264.

35. **Matthews, D. E., and V. T. Farewell.** 1985. Using and understanding medical statistics, p. 56, 79–87. S. Karger, Basel.
36. **McManus, A. T., E. E. Moody, and A. D. Mason.** 1980. Bacterial motility: a component in experimental *Pseudomonas aeruginosa* burn wound sepsis. Burns **6**:235–239.
37. **Montie, T. C., R. C. Craven, and I. A. Holder.** 1982. Flagellar preparations from *Pseudomonas aeruginosa*: isolation and characterization. Infect. Immun. **35**:281–288.
38. **Montie, T. C., D. Doyle-Huntzinger, R. C. Craven, and I. A. Holder.** 1982. Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned-mouse model. Infect. Immun. **38**:1296–1298.
39. **Pavlovskis, O. R., D. C. Edman, S. H. Leppla, B. Wretland, L. R. Lewis, and K. E. Martin.** 1981. Protection against experimental *Pseudomonas aeruginosa* infection in mice by active immunization with exotoxin A toxoids. Infect. Immun. **32**:681–689.
40. **Pavlovskis, O. R., M. Pollack, L. T. Callahan III, and B. H. Iglewski.** 1977. Passive protection by antitoxin in experimental *Pseudomonas aeruginosa* burn infections. Infect. Immun. **18**: 596–602.
41. **Pennington, J. E., H. Y. Reynolds, and P. P. Carbone.** 1973. Pseudomonas pneumonia: a retrospective study of 36 cases. Am. J. Med. **55**:155–160.
42. **Pennington, J. E., G. J. Small, M. E. Lostrom, and G. B. Pier.** 1986. Polyclonal and monoclonal antibody therapy for experimental *Pseudomonas aeruginosa* pneumonia. Infect. Immun. **54**:239–244.
43. **Pitt, T. L., and D. E. Bradley.** 1975. The antibody response to the flagella of *Pseudomonas aeruginosa*. J. Med. Microbiol. **8**:97–106.
44. **Reynolds, H. Y., A. S. Levine, R. E. Wood, C. H. Zierdt, D. C. Dale, and J. E. Pennington.** 1975. *Pseudomonas aeruginosa* infections: persisting problems and current research to find new therapies. Ann. Intern. Med. **82**:819–831.
45. **Sastry, P. A., J. R. Pearlstone, L. B. Smillie, and W. Paranchych.** 1985. Studies on the primary structure and antigenic determinants of pilin isolated from *Pseudomonas aeruginosa* K. Can. J. Biochem. Cell Biol. **63**:284–291.
46. **Sawada, S., M. Suzuki, T. Kawamura, S. Fujinaga, Y. Masuho, and K. Tomibe.** 1984. Protection against infection with *Pseudomonas aeruginosa* by passive transfer of monoclonal antibodies to lipopolysaccharides and outer membrane proteins. J. Infect. Dis. **150**:570–576.
47. **Seppala, I., H. Sarvas, F. Peterfy, and O. Makela.** 1981. The four subclasses of IgG can be isolated from mouse serum by using protein A-sepharose. Scand. J. Immunol. **14**:335–342.
48. **Siadak, A. W., and M. E. Lostrom.** 1984. Cell-driven viral transformation, p. 167–185. *In* E. G. Engleman, S. K. H. Foung, J. Larrick, and A. Raubitschek (ed.), Human hybridomas and monoclonal antibodies. Plenum Publishing Corp., New York.
49. **Stoll, B. J., M. Pollack, L. S. Young, N. Koles, R. Gascon, and G. B. Pier.** 1986. Functionally active monoclonal antibody that recognizes an epitope on the O side chain of *Pseudomonas aeruginosa* immunotype-1 lipopolysaccharide. Infect. Immun. **53**:656–662.
50. **Tam, M. R., T. M. Buchanan, E. G. Sandstrom, K. K. Holmes, J. S. Knapp, A. W. Siadak, and R. C. Nowinski.** 1982. Serological classification of *Neisseria gonorrhoeae* with monoclonal antibodies. Infect. Immun. **36**:1042–1053.
51. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
52. **Young, L. S.** 1972. Human immunity to *Pseudomonas aeruginosa*. II. Relationship between heat-stable opsonins and type-specific lipopolysaccharides. J. Infect. Dis. **126**:277–287.
53. **Young, L. S., and D. Armstrong.** 1972. Human immunity to *Pseudomonas aeruginosa*. I. In-vitro interaction of bacteria, polymorphonuclear leukocytes, and serum factors. J. Infect. Dis. **126**:257–276.