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An Engineered Human Antibody Fab Fragment Specific for *Pseudomonas aeruginosa* PcrV Antigen Has Potent Antibacterial Activity▽

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Pseudomonas aeruginosa **is an opportunistic pathogen that can cause acute lung injury and mortality through the delivery of exotoxins by the type III secretion system (TTSS). PcrV is an important structural protein of the TTSS. An engineered human antibody Fab fragment that binds to the *P. aeruginosa* PcrV protein with high affinity has been identified and has potent in vitro neutralization activity against the TTSS. The instillation of a single dose of Fab into the lungs of mice provided protection against lethal pulmonary challenge of *P. aeruginosa* and led to a substantial reduction of viable bacterial counts in the lungs. These results demonstrate that blocking of the TTSS by a Fab lacking antibody Fc-mediated effector functions can be sufficient for the effective clearance of pulmonary *P. aeruginosa* infection.**

Pseudomonas aeruginosa is an opportunistic pathogen that causes both acute and chronic infections in compromised individuals. It is a frequent causative agent of bacteremia in burn victims (32) and immunocompromised patients (18). It is also the most common cause of nosocomial gram-negative pneumonia (7, 25), especially in mechanically ventilated patients (25), and is the most prevalent pathogen in the lungs of individuals with cystic fibrosis (CF) (10, 17, 20). In CF, *P. aeruginosa* infection follows a well-established pattern of recurrent pulmonary infection in early childhood leading to the establishment of chronic infection in older CF patients, where it is a major contributing factor in the progressive decline in lung function and disease exacerbations leading to respiratory failure (10, 17).

Morbidity and mortality associated with *P. aeruginosa* infections remain high despite the availability of antibiotics to which the bacterium is sensitive, and antibiotic resistance is an increasingly common problem in nosocomial infections (6).

The type III secretion system (TTSS) is an important virulence determinant of *P. aeruginosa* in animal models of infection (15) and is required for the systemic spread of *P. aeruginosa* in a mouse pulmonary challenge model (31). The expression of a functional TTSS also correlates with poor prognosis in clinical infections (26, 27). This needle-like structure comprises a complex secretion and translocation machinery to inject a set of up to four different exotoxins (ExoS, ExoT, ExoU, and ExoY) directly into the cytoplasm of eukaryotic cells (9, 33, 34). Various strains of *P. aeruginosa* secrete different exotoxins. In addition, the TTSS can mediate direct cytotoxicity toward macrophages and neutrophils in the absence of exotoxins, a process called "oncosis," requiring bacterial swarming in response to macrophage factors and leading to a direct perforation of the cell membranes (3, 4). In all of these functions of the TTSS, the needle tip protein, PcrV, is an essential component of the translocation apparatus.

Antisera raised against PcrV in rabbits have been shown to block the translocation of *Pseudomonas* exotoxins into mammalian cells (12, 28) and to protect against lethality in a mouse model of acute pulmonary *Pseudomonas* infection (28, 29). Polyclonal anti-PcrV antibodies have also been shown to reduce lung damage and protect against bacteremia and septic shock in rat and rabbit pulmonary infection models (29) and to protect burned mice from infection (22). A mouse monoclonal anti-PcrV antibody, monoclonal antibody (MAb) 166, with potent neutralizing activity in mouse and rat models of *Pseudomonas* infection has also been described (11). This antibody inhibits the function of the TTSS in cell-based assays (11, 12). The MAb acts to prevent sepsis and mortality in an acute pulmonary infection model in mice when delivered either systemically or by intratracheal administration (11) and reduces lung damage due to *Pseudomonas* in a rat model (8). The antibody has activity when dosed either prophylactically or therapeutically in these models both as whole immunoglobulin G (IgG) and as a Fab fragment, indicating that the inhibition of TTSS function is sufficient to inhibit lung damage in pulmonary *Pseudomonas* infections.

Here, we identify an engineered human antibody Fab fragment specific for the *P. aeruginosa* PcrV protein which competes with MAb 166 for binding to the same epitope on PcrV. The human Fab shows potent TTSS-neutralizing activity,

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equivalent to the activity of MAb 166, in cellular cytotoxicity assays. This Fab shows potent in vivo activity in protecting mice from potentially lethal doses of *P. aeruginosa*. In addition, the Fab mediates a substantial clearance of bacteria from the lungs of infected mice, suggesting that antagonism of the TTSS is sufficient not only to prevent damage to the pulmonary epithelium but also to restore normal immunological clearance mechanisms for the effective resolution of *Pseudomonas* infection in the complete absence of antibody effector functions.

TABLE 1. Kinetics of binding of anti-PcrV Fab 1A8 and MAb 166 Fab fragment to purified PcrV protein[a]

Fab	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)
MAb 166 Fab	5.9×10^5	6.2×10^{-4}	1.1
Fab 1A8	7.8×10^5	4.7×10^{-4}	0.6

[a] Binding kinetics were determined by surface plasmon resonance analysis at 37°C (using a Biacore T100 instrument). The association constant (K_a), dissociation constant (K_d), and overall affinity (K_D) are shown.

MATERIALS AND METHODS

Antibodies and antigen. Mouse monoclonal anti-PcrV antibodies MAb 166 and MAb 3.7.5 were described previously (11). MAb 166 is a neutralizing antibody, and MAb 3.7.5 is nonneutralizing. MAb 166 Fab is a Fab fragment derived from a papain digest of MAb 166.

Recombinant PcrV, cloned as a fusion protein in frame with an amino-terminal glutathione *S*-transferase (GST) purification tag, was prepared as described previously (23).

Generation of Fab 1A8. Fab 1A8, a human Fab fragment with the specificity of mouse MAb 166, was isolated from an epitope-focused library of human Fab fragments by a process of antibody "humaneering" (our unpublished data).

Briefly, minimal sequence information was obtained from the CDR3 regions of the heavy and light chains of MAb 166 and was transferred to a human library of partial V-region gene sequences. Reconstructed V regions containing human germ line J-segment sequences and human kappa and human gamma CH1 constant region sequences were expressed as a combinatorial library of Fab fragments by secretion from *Escherichia coli* cells to generate an epitope-focused library of human antibodies. Fab fragments specific for PcrV were identified in a filter-binding assay using nitrocellulose filters coated with 5 μg/ml GST-PcrV.

Fab 1A8 and irrelevant control Fab were expressed in *E. coli* cells and purified from the periplasmic fraction by protein G affinity chromatography using HiTrap protein G HP columns (Amersham Biosciences). Fabs were eluted in low pH, neutralized in 1 M Tris base, and desalted into phosphate-buffered saline (PBS) (pH 7.4).

Bacterial strains and cell lines. PA103 (ATCC 29260) is a strain of *P. aeruginosa* originally isolated from human sputum (19). It secretes the ExoU and ExoT exotoxins, is highly cytotoxic in vitro, and causes damage to the pulmonary epithelium in vivo. PA103ΔUT (11) is an isogenic strain of PA103 that does not secrete either ExoU or ExoT. PA103ΔPcrV is an isogenic strain of PA103 that lacks PcrV expression (30). Strains were routinely cultured in Min S medium (13).

P3-X63-Ag8 (X63) mouse myeloma cells (ATCC) were cultured in RPMI 1640 medium (Media Tech) with 10% fetal bovine serum (HyClone). J774 murine macrophage cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Media Tech) with 10% fetal bovine serum. Cells used in cytotoxicity studies were detached by treatment with 0.02% EDTA for 15 min.

Binding kinetic analysis. The kinetics of Fab binding to the GST-PcrV fusion protein were determined by surface plasmon resonance analysis at 37°C using a Biacore T100 instrument at Biosensor Tools, Inc., as described previously (23). The GST-PcrV protein was immobilized on sensors at three different densities, and data were globally fitted to a simple 1:1 interactive model.

Cytotoxicity studies. An exotoxin-dependent cytotoxicity assay was established using X63 cells as the target. A total of 2×10^5 cells were infected with *P. aeruginosa* strain PA103 at a multiplicity of infection (MOI) of 10 in a volume of 0.1 ml culture medium in wells of a 96-well plate in the presence of antibody or Fab. After incubation for up to 3 h at 37°C with 5% CO_2, cells were transferred into 12- by 75-mm flow cytometry tubes and stained with propidium iodide (PI) (Sigma) according to the manufacturer's instructions. The proportion of permeabilized cells was quantified by flow cytometry using a Coulter (Excel-MCL) flow cytometer. Data were analyzed using Prism4 software (Graphpad). (Cytotoxicity was normalized to dead cells in untreated samples.) For comparison of the potencies of different antibodies, mean concentrations required for 50% inhibition (IC_{50}) were obtained from three independent assays and compared using a Student's *t* test. *P* values of <0.05 were considered to be significant.

An exotoxin-independent cytotoxicity assay was established using the J774 murine macrophage cell line as the target. J774 cells were incubated with exotoxin-deficient *P. aeruginosa* strain PA103ΔUT at an MOI of 10 for up to 5 h, stained with PI, and analyzed as described above.

Competitive ELISA. Ninety-six-well enzyme-linked immunosorbent assay (ELISA) plates (Costar) were coated with 100 ng/well of GST-PcrV in 50 μl PBS for 1h at 37°C. Wells were washed once with PBS containing 0.1% Tween 20 (PBST), blocked with 5% powdered skim milk (Marvel) in PBST for 1 h at 37°C, and washed three times with PBST. Primary anti-PcrV antibodies were added to the wells at a concentration of 50 nM in the presence of various concentrations of competitor and incubated for 1 h at room temperature. Wells were washed three times with PBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-human kappa antibody (Sigma), for human Fab detection, or HRP-conjugated goat anti-mouse light chain (Dako), for detection of mouse MAbs, for 1 h at room temperature to reveal the binding of the corresponding primary antibody. Wells were washed three times with PBST and once with PBS, and binding was assessed by adding TMB reagent (Sigma). Reactions were stopped by the addition of 2 M H_2SO_4 to the medium and quantified by the absorbance at 450 nm using a Spectramax-plus model 384 spectrophotometer. Data were analyzed using Prism4 software (Graphpad).

Mouse studies. Male BALB/c mice, 8 to 12 weeks of age, were inoculated with 1.5×10^6 CFU of *P. aeruginosa* by intratracheal administration. Strains of *P. aeruginosa* were grown overnight in deferrated dialysate of Trypticase soy broth supplemented with 10 mM nitrilotriacetic acid (Sigma Chemical Co., St. Louis, MO) to induce TTSS expression (27) and were diluted and grown to exponential phase prior to administration. Bacteria were quantified by the absorbance at 600 nm and verified by colony growth on 2× yeast extract-tryptone agar plates. Antibody or Fab was premixed with bacteria immediately prior to instillation. Mice were monitored for body temperature (rectal temperatures) and survival. Rectal temperatures were measured hourly for the first 12 h and at 24, 32, and 48 h in surviving mice. Lungs of mice surviving for 48 h were harvested and tested for *P. aeruginosa* CFU. All experiments were done in compliance with Animal Care Committee rules of the University of California at San Francisco, and all protocols were approved prior to the start of the experiments. Survival curves for different Fabs were compared using the Mantel-Cox log-rank test. Differences in rectal body temperatures between treatment groups and bacterial counts at different doses of Fab were compared by a *t* test. In each case, *P* values of <0.05 were considered to be significant.

RESULTS

Antigen binding. 1A8 is an engineered human Fab isolated from an epitope-focused library in which minimal sequences from the CDR3 regions of mouse anti-PcrV antibody MAb 166 were transferred to a library of human V segments and expressed by secretion from *E. coli* cells. 1A8 has a human VH3 subclass heavy chain, containing the first constant domain of human IgG1, and a human VK1 subclass kappa light chain.

Kinetic constants for the binding of Fab 1A8 to the recombinant *P. aeruginosa* PcrV protein were determined by surface plasmon resonance analysis in comparison with the Fab fragment of MAb 166 (Table 1). Fab 1A8 binds to PcrV with approximately a twofold-higher affinity than MAb 166 Fab.

The epitope-focused library from which 1A8 was cloned is expected to contain antibodies of restricted specificity determined by the CDR3 sequences from MAb 166. In order to confirm the specificity of Fab 1A8, competition ELISAs were carried out. Purified MAb 166 and Fab 1A8 were added to a recombinant PcrV-coated ELISA plate in various concentrations (Fig. 1A). The binding of Fab 1A8 was inhibited by the addition of increasing concentrations of MAb 166 competitor

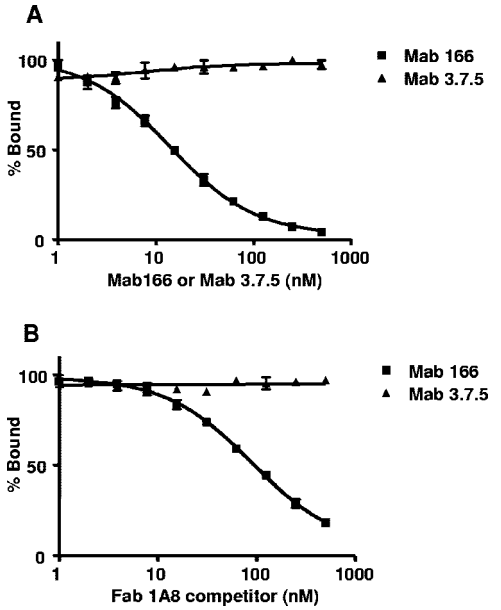

FIG. 1. Competition ELISA between MAb 166 and Fab 1A8. (A) Fab 1A8 (50 nM) was mixed with various concentrations of either MAb 166 or MAb 3.7.5, and residual binding of Fab 1A8 to GST-PcrV was detected using anti-human kappa-HRP conjugate. (B) Either MAb 166 or MAb 3.7.5 (20 nM) was mixed with various concentrations of 1A8, added to GST-PcrV, and detected with anti-mouse-HRP conjugate. Data, in triplicate, are from a single representative experiment. Means and standard errors (SE) are shown.

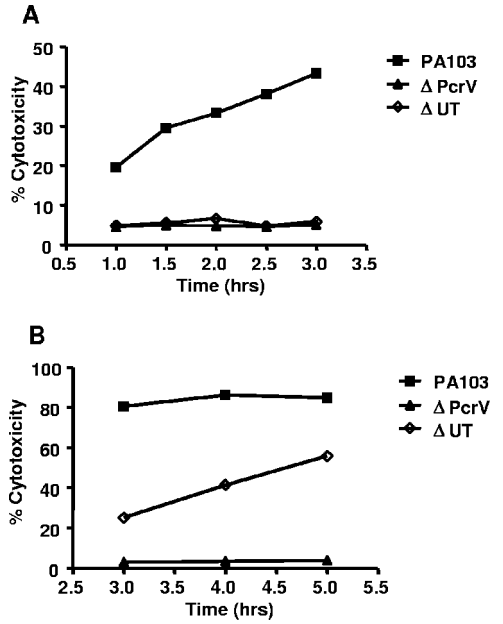

FIG. 2. Cytotoxicity of *P. aeruginosa* strains to mouse cell lines. Data for strain PA103 and mutants defective in PcrV expression (ΔPcrV) or defective for exotoxins U and T (ΔUT) directed against a myeloma cell line (X63) (A) or a macrophage cell line (J774) (B) were determined by PI staining and flow cytometry. Cells were incubated with bacteria (MOI = 10) for the times indicated. Data shown are from a single representative experiment.

but was unaffected by another anti-PcrV antibody, MAb 3.7.5, directed against a different epitope. The converse experiment, in which anti-MAb 166 binding was competed with increasing amounts of Fab 1A8, showed similar blocking (Fig. 1B), indicating that Fab 1A8 and MAb 166 compete for binding to the same epitope on the PcrV protein.

Antagonism of TTSS-mediated cytotoxicity. The TTSS mediates the direct translocation of *Pseudomonas* exotoxins from the bacteria to host cells with which it comes into contact. Hence, *Pseudomonas* strains expressing exotoxins show potent cytotoxic activity against all mammalian cell types. In addition, the TTSS has been shown to mediate the direct killing of macrophages by perforation of the cell membrane, a process termed "oncosis." This mechanism of cell killing does not require exotoxins but is dependent on PcrV (4).

An exotoxin-dependent cytotoxicity assay was established using the mouse myeloma cell line X63 as the target. A total of 2×10^5 cells were infected with *P. aeruginosa* strain PA103, which expresses both the ExoU and ExoT toxins, at an MOI of 10, and the numbers of dead cells were determined at various time points by PI staining and flow cytometry. Figure 2A shows the killing of X63 cells by PA103 and the lack of killing by a mutant isogenic strain lacking PcrV (PA103ΔPcrV) or a strain which retains the TTSS but lacks exotoxins ExoU and ExoT (PA103ΔUT).

The J774 murine macrophage cell line was used to establish a similar assay for TTSS-mediated exotoxin-independent oncosis (Fig. 2B). The exotoxin-defective mutant PA103ΔUT caused significant toxin-independent killing. The macrophage line is resistant to the PcrV-deficient strain (PA103ΔPcrV), confirming that oncosis is PcrV dependent. The highest level of cytotoxicity was seen when the cells were incubated with PA103 due to the combined effects of exotoxin-mediated cytotoxicity and oncosis. Although cell killing by oncosis proceeds with a slower time course than cytotoxicity due to PA103, significant oncosis caused by PA103ΔUT was observed by 5 h, and this time point was used for subsequent oncosis assays.

The inhibition of *P. aeruginosa* strain PA103 cytotoxicity for X63 myeloma cells mediated by Fab 1A8 or MAb 166 is shown in Fig. 3. In this assay, the human Fab fragment is comparable to the murine MAb 166 IgG when equivalent molar concentrations of antibody binding sites are compared, consistent with the relative affinity of human Fab for PcrV.

Fab 1A8 is also capable of inhibiting exotoxin-independent macrophage cytotoxicity (oncosis) using the strain defective for ExoU and ExoT expression (Fig. 4). Human Fab 1A8 has a higher potency than MAb 166 in this assay, although the difference did not reach statistical significance. Hence, Fab 1A8 is a potent inhibitor of both exotoxin-dependent and exotoxin-independent cytotoxicity mediated by the TTSS.

In vivo activity of Fab 1A8 in a mouse model. An acute lethality model of *Pseudomonas* pneumonia was used to assess the in vivo efficacy of Fab 1A8 in comparison with that of MAb 166. The ability of *P. aeruginosa* PA103 to establish pneumonia, sepsis due to the systemic spread of bacteria, and ultimate lethality in this model has been shown to be PcrV dependent (28), and the model has been used to characterize the activities of anti-PcrV antibodies including MAb 166 (11, 28). MAb 166 has potent activity when coinstilled with bacteria or delivered systemically and can be administered prior to infection or

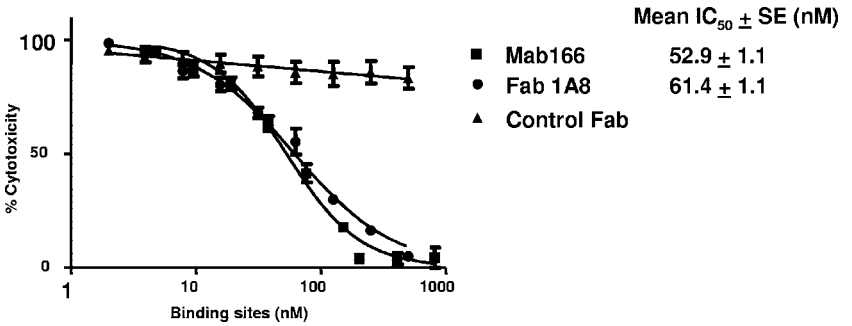

FIG. 3. Inhibition of *Pseudomonas* exotoxin-dependent cytotoxicity by Fab 1A8. X63 cells were incubated with PA103 (MOI = 10) for 3 h in the presence of various concentrations of Fab 1A8, MAb 166, or control Fab with no binding to PcrV or other *P. aeruginosa* proteins. Percent cytotoxicity was determined by PI staining and flow cytometry. Data are from three independent assays. Mean IC_{50} values ± SE are shown. A *t* test analysis showed no significant difference between MAb 166 IgG and Fab 1A8 ($P > 0.05$).

several hours postinfection with activity in the prevention of lethality (11).

P. aeruginosa strain PA103 was instilled directly into the lungs of mice at a dose of 1.5×10^6 CFU/mouse by intratracheal administration, an inoculum shown previously to be sufficient to lead to lethality in 100% of the animals (three times the 90% lethal dose) (28). Survival and body temperature were monitored for 48 h, and surviving mice at this time point were sacrificed for determinations of bacterial counts in the lungs.

The survival data (Fig. 5) indicate that both human Fab 1A8 and murine Fab can prevent lethality caused by the highly cytotoxic strain PA103. Control mice infected with PA103 and treated with an irrelevant control Fab were all dead within 24 h of inoculation. Treatment of mice with 10 μg MAb 166 or Fab 1A8 led to the survival of 100% of the mice at 48 h. Since Fab 1A8 lacks the antibody Fc region, antibody effector functions are not required for the prevention of lethality. Fab 1A8 is significantly more potent than MAb 166 Fab in the prevention of lethality. Fab 1A8 provides significant protection from lethality at doses of 1.25 μg and 0.625 μg/mouse, doses at which mouse MAb 166 Fab-treated animals showed 100% mortality ($P < 0.05$ for differences between Fab 1A8 and MAb 166 Fab at 2.5-μg, 1.25-μg, and 0.625-μg doses). The activity of Fab 1A8 is comparable to that of MAb 166 IgG in the prevention of lethality.

Fab 1A8 is also effective in inducing the recovery of body temperature (Fig. 6), which is indicative of protection from sepsis. Untreated mice infected with PA103 showed a rapid drop in body temperature within the first 5 h of infection. Treatment with MAb 166 IgG, Fab 1A8, or MAb 166 Fab provided a dose-dependent reduction in the decline in body temperature. The maximal decrease in body temperature (at 5 h) was significantly less in each of the treated groups at 10 or 5 μg antibody or Fab than that of the control group ($P < 0.05$). Lower doses led to less significant protection. The recovery of body temperature within 12 to 24 h in the antibody-treated groups correlates with subsequent survival. Doses of Fab 1A8 or MAb 166 as low as 1.25 μg/mouse led to a rapid recovery of body temperature and prevented lethality in at least 80% of mice. However, this dose of the mouse MAb 166 Fab fragment was insufficient to allow body temperature recovery, and all mice in this group were dead at 48 h postinfection.

Surviving mice at 48 h postchallenge were also analyzed for the presence of residual *Pseudomonas* CFU in the lungs. Remarkably, both MAb 166 and the Fab fragments analyzed stimulated the clearance of bacteria (Fig. 7). After 48 h, the bacterial counts were reduced at least 1,000-fold from the infectious dose of 1.5×10^6 CFU/mouse in all mice treated with 10 μg Fab 1A8. Eighty percent of mice treated with this dose of Fab 1A8 showed no detectable *Pseudomonas* cells in the lungs after 48 h. Bacterial clearance in response to treatment with Fab 1A8 was dose dependent; the reduction in the

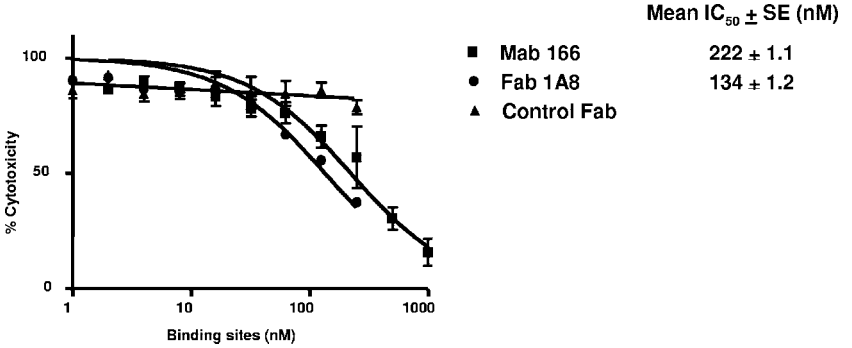

FIG. 4. Inhibition of *Pseudomonas* exotoxin-independent oncosis by Fab 1A8. J774 cells were incubated with the exotoxin-defective mutant PA103ΔUT (MOI = 10) for 5 h in the presence of various concentrations of Fab 1A8, MAb 166, or control Fab. Cytotoxicity was determined by PI staining and flow cytometry. Data are from three independent experiments. Mean IC_{50} values ± SE are shown. A *t* test showed no significant difference between MAb 166 IgG and Fab 1A8 ($P > 0.05$).

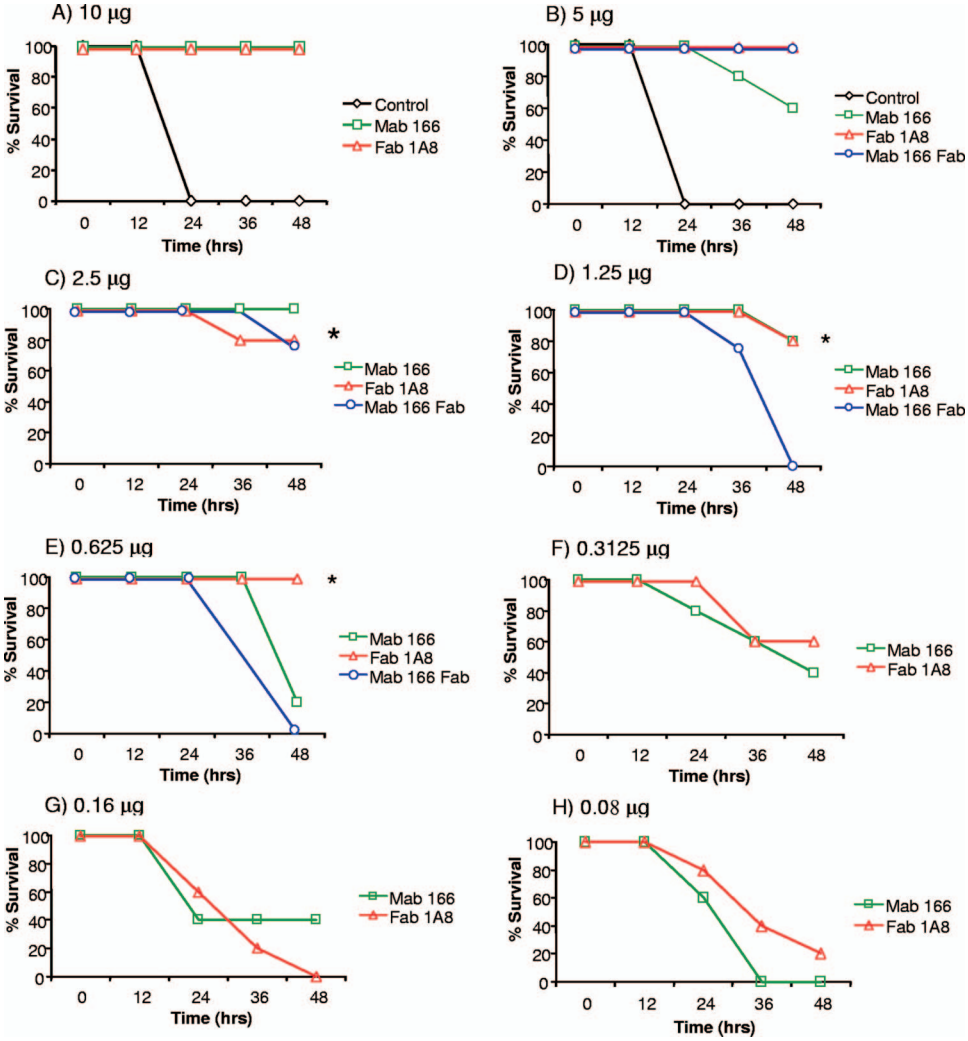

FIG. 5. Time course of survival of mice treated with various doses of antibodies to PcrV at the time of challenge with a lethal dose of PA103. MAb 166 and Fab fragments were coinstilled with 1.5×10^6 bacteria via the intratracheal route (five mice per group, with four mice for MAb 166 Fab groups). The control was a nonspecific Fab with no binding to PcrV or any *P. aeruginosa* protein. Mice were treated with antibody doses of 10 μg (A), 5 μg (B), 2.5 μg (C) 1.25 μg (*, $P = 0.01$ for Fab 1A8 versus MAb 166 Fab) (D), 0.625 μg (*, $P = 0.002$ for 1A8 versus MAb 166 Fab) (E), 0.3125 μg (F), 0.16 μg (G), and 0.08 μg (H). *P* values for differences between treatment groups were determined by a Mantel-Cox log-rank test.

number of bacteria in the lungs of surviving mice achieved statistical significance in both the 10-μg and 5-μg treatment groups compared to mice treated with 0.3125 μg ($P < 0.05$) (Fig. 7). Fab 1A8 has potency comparable to that of whole-IgG MAb 166 in this analysis, indicating that Fc effector functions do not contribute significantly to the ability of the antibody to stimulate bacterial clearance.

DISCUSSION

An engineered human antibody Fab fragment that binds to an epitope on the *P. aeruginosa* PcrV protein defined by the previously characterized MAb 166 has been identified. Fab 1A8 has potent TTSS neutralization activity as a monovalent Fab fragment and protects cells in culture from both exotoxin-mediated cytotoxicity and exotoxin-independent TTSS-mediated oncosis of macrophages.

Fab 1A8 also has potent activity in vivo in an acute pulmonary infection model in mice, showing the prevention of lethality, recovery of body temperature (an indicator of prevention of sepsis), and enhanced clearance of bacteria from the lungs. Mice infected with a potentially lethal dose of *P. aeruginosa* (three times the 90% lethal dose) survived when a single 10-μg dose of Fab 1A8 was coinstilled into the lungs, and surviving mice showed a substantial clearance of bacteria from the lungs. Indeed, 80% of mice treated with 10 μg Fab 1A8 showed no detectable viable bacteria in the lungs within 48 h of treatment.

The in vivo activity of Fab 1A8 was compared with those of mouse MAb 166, which was previously shown to have efficacy in this model (11), and the Fab fragment of MAb 166. All three molecules showed dose-dependent activity in the prevention of lethality, recovery of body temperature, and clearance of bacteria. MAb 166 IgG was more potent than its Fab fragment, although a direct comparison of the two should be treated with

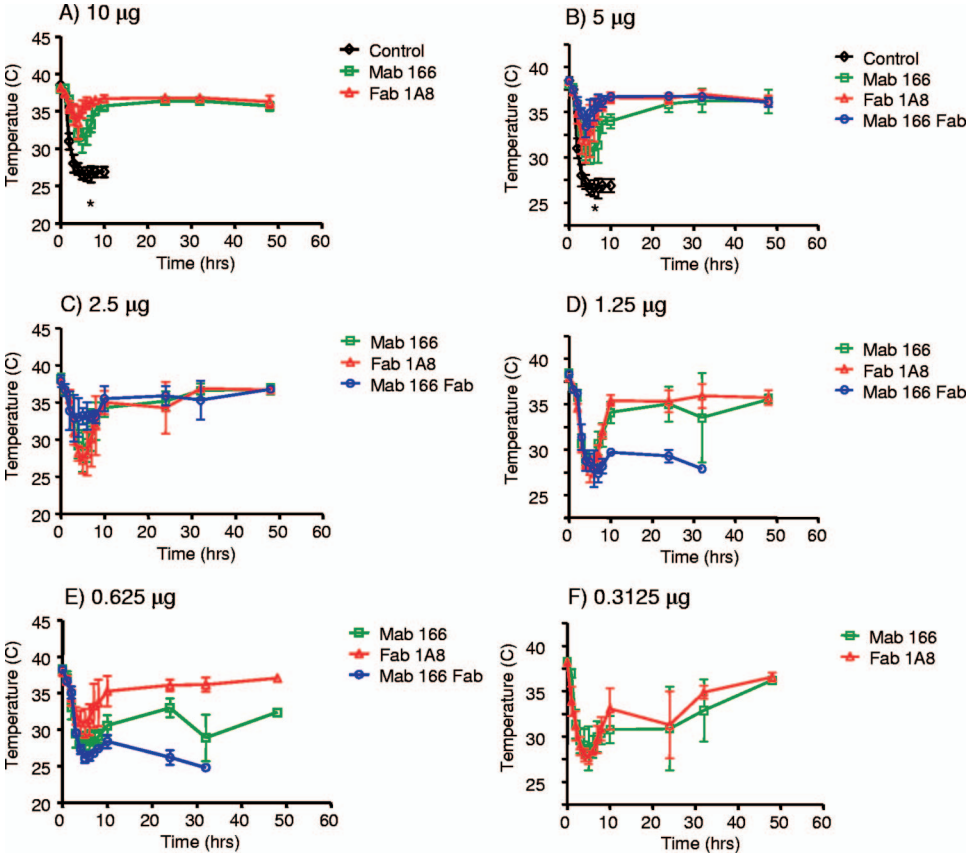

FIG. 6. Body temperature analysis of mice treated with anti-PcrV antibodies. Rectal temperatures are shown for 48 h or until mortality (means ± SE). Antibody doses were 10 μg (A), 5 μg (B), 2.5 μg (C), 1.25 μg (D), 0.625 μg (E), and 0.3125 μg (F). *, $P < 0.05$ for differences in body temperature between control mice and mice treated with MAb 166 IgG, MAb 166 Fab, or human Fab 1A8 at 10-μg or 5-μg doses (unpaired *t* test).

caution due to the multiple differences between the molecules, including differences in molecular weights, effector functions, and pharmacokinetic properties. The IgG has approximately 30% fewer antigen-binding sites per microgram of protein due to the difference in molecular weight, but more importantly, IgGs have long in vivo half-lives (in the order of 2 to 3 days in mice), whereas Fab fragments have been shown to be cleared from the circulation with half-lives of a few minutes (1, 2). MAb 166 is an IgG2b antibody expected to have potent Fc-mediated effector functions. Nevertheless, the activity of the MAb 166 Fab fragment indicates that Fc effector functions are not essential for in vivo activity, either in protection from sepsis and mortality or in stimulating bacterial clearance.

When human Fab 1A8 is compared directly with murine Fab, 1A8 shows higher potency in the reduction of mortality, with a statistically significant enhancement of survival at low doses of Fab (1.25 μg and 0.625 μg/mouse). The efficacy of Fab 1A8 at low doses again indicates that potent activity can be achieved by an anti-PcrV antibody in the absence of Fc effector functions. We speculate that the higher affinity of Fab 1A8 for PcrV (600 pM) than MAb 166 Fab (1.1 nM) may have contributed to the increased potency of human Fab 1A8 in vivo compared with that of murine Fab by contributing to a greater retention of antibody in the infected tissues.

Effector functions such as opsonization and complement activation have been shown to play a critical role in the activity of other antibodies to bacterial cell surface components, including antibodies to *Pseudomonas* lipopolysaccharide (14) and several antibodies to *Staphylococcus aureus* antigens (5, 16). There have been comparatively few published examples of antibody blocking activity in the absence of effector function to prevent bacterially mediated lethality. A single-chain antibody to *Bacillus anthracis* exotoxin has shown protective activity against *B. anthracis* spores by impairing host colonization (21). Antibodies to *S. aureus* quorum-sensing molecules that similarly interfere with colonization have been described (24). Fab 1A8 provides an additional example of an antibody targeting a key virulence factor essential for bacterial pathogenicity. The rapid and efficient bacterial clearance stimulated by Fab 1A8 demonstrates the critical role for the TTSS in the pathogenicity of *P. aeruginosa*. The activity of Fab 1A8 is consistent with a role for anti-PcrV antibodies in the neutralization of the TTSS to protect cells of the immune system such as alveolar macrophages and neutrophils in order to allow the clearance of bacteria by normal immunological mechanisms, as was proposed previously (11, 28). Fab 1A8 may also prevent damage to the pulmonary epithelium, as demonstrated for MAb 166 in a rat model (8), thereby restricting the dissemination of bacteria via the bloodstream and thus preventing sepsis.

The efficacy of Fab 1A8 in the mouse pneumonia model

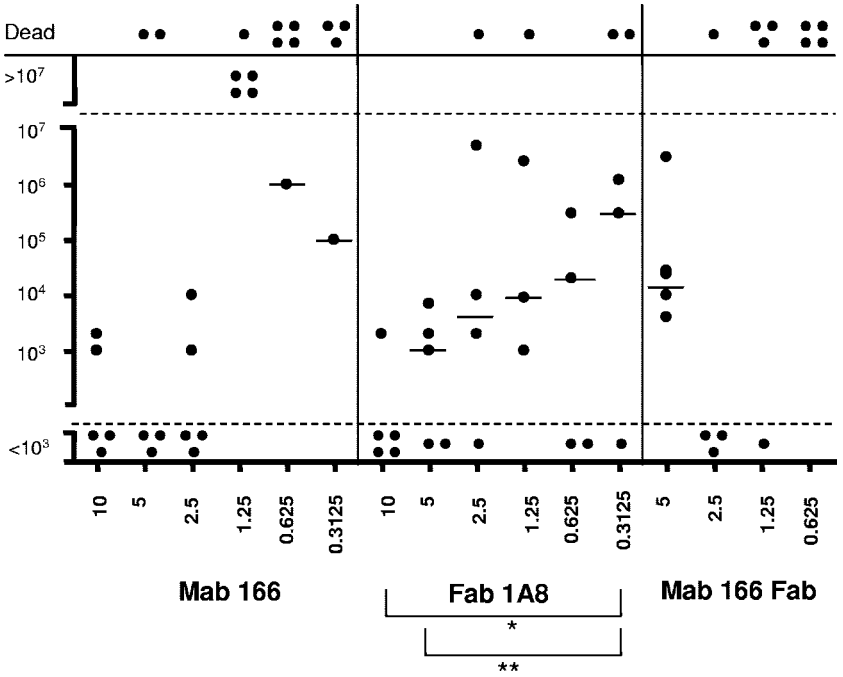

FIG. 7. Clearance of *P. aeruginosa* from the lungs of infected mice by anti-PcrV antibodies. Mice were infected with 1.5×10^6 CFU PA103 coinstilled with MAb 166 IgG, MAb 166 Fab, or human Fab 1A8 at the doses shown (in μg). The graph shows CFU/lung isolated from individual mice surviving at 48 h. The number of dead mice at this time point is shown at the top. The median CFU/lung for surviving mice in each group is shown with a bar. *, $P < 0.05$ compared with the 0.3125-μg/mouse dose (unpaired *t* test); **, $P < 0.05$ compared with the 0.3125-μg/mouse dose (unpaired *t* test).

establishes an attractive target for therapeutic intervention in human disease. The PcrV protein is highly conserved among strains of *P. aeruginosa*, and Fab 1A8 binds to PcrV from multiple clinical isolates tested (our unpublished results). The ability to use a Fab fragment for the treatment of *P. aeruginosa* infection in patients with ventilator-associated pneumonia or in chronically infected CF patients minimizes the risk of excess inflammation or complement deposition in the lungs due to treatment with the antibody. It is likely that a further extension of the in vivo half-life by conjugation of Fab to polyethylene glycol, as described previously for other antibody Fab fragments (1, 2), will allow systemic administration and provide prolonged protection against this important pathogen with potential for human therapeutic use.

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