Immunization with a *Pseudomonas aeruginosa* Elastase Peptide Reduces Severity of Experimental Lung Infections Due to *P. aeruginosa* or *Burkholderia cepacia*

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*Pseudomonas aeruginosa* and *Burkholderia cepacia* produce metalloproteases that effect lung injury. Two epitopes (peptides 15 and 42) previously identified on *P. aeruginosa* elastase induce the production of antibodies that neutralize protease activity. The effects of immunization with synthetic peptides based on these epitopes on experimental lung infections due to *P. aeruginosa* or *B. cepacia* were examined. Rats were immunized with peptides conjugated to keyhole limpet hemocyanin or tetanus toxoid before infection. Immunization with peptide 15 (pep15) resulted in a decrease in total cells and polymorphonuclear leukocytes in bronchoalveolar lavage (BAL) fluid and a 50%–70% decrease in lung histopathologic changes, compared with findings in controls. Immunization with peptide 42 decreased cells in BAL fluid but did not decrease lung pathologic changes. Immunization with pep15 alone was just as effective in protecting against lung injury as immunization with a combination of both peptides. These studies suggest that immunization with pep15 can reduce the severity of lung infections due to *P. aeruginosa* or *B. cepacia*.

Chronic airway infection with *Pseudomonas aeruginosa* or *Burkholderia cepacia* is a major cause of mortality in patients with cystic fibrosis (CF) [1, 2]. Despite advances in antimicrobial therapy, the treatment and prevention of infections caused by these organisms remain a clinical challenge [1, 2]. *P. aeruginosa* is present in >60% of respiratory tract cultures from CF patients, and once colonization occurs, *P. aeruginosa* is difficult or impossible to eradicate [2, 3]. Acquisition of *B. cepacia* is particularly problematic because of its resistance to most antibiotics [4]. Patients colonized with certain *B. cepacia* strains present with “cepacia syndrome,” which is characterized by a rapid decline in pulmonary function and is frequently associated with bacteremia and a high mortality rate [5].

Although *P. aeruginosa* and *B. cepacia* produce a number of potential virulence factors, considerable evidence suggests that extracellular zinc metalloproteases play a major role in lung disease. *P. aeruginosa* produces 2 zinc metalloproteases, elastase (LasB) and alkaline protease (AP). Two proteases have been described in *B. cepacia*, a 36-kDa protease (PSCP) and an immunologically related 40-kDa protease [6, 7]. *P. aeruginosa* elastase degrades several biologically important substrates, including elastin [8], collagen [9], immunoglobulins [10–12], transferrin and lactoferrin [13–15], complement components [16, 17], and α1-antiprotease inhibitor [18]. PSCP does not degrade elastin; however, it does degrade collagen [6], human IgG and IgA, transferrin, and lactoferrin (C.K. and P.A.S., unpublished data).

In addition to its direct role in tissue destruction and lung injury, *P. aeruginosa* LasB can function in the modulation of the host immune system and can help the organism evade host defenses. Supernatants of *P. aeruginosa* isolates from CF patients have been shown to digest IgG into Fab gamma, Fab(α)2 fragments, and a free Fc gamma fragment, consistent with those fragments found in the respiratory secretions of CF patients [10]. This activity is inhibited by EDTA, which inhibits LasB by chelating the zinc ion. Purified LasB has been shown to cleave IgG from CF patients into 2 major fragments that significantly inhibit bacterial uptake by neutrophils in reactions with *P. aeruginosa* lipopolysaccharide (LPS)–reactive Fab [11].

Iron in vivo is normally bound to transferrin and lactoferrin and is unable to catalyze the formation of hydroxyl radicals from superoxide and hydrogen peroxide via the Haber-Weiss reaction. LasB has been shown to cleave transferrin and release iron, which can then generate hydroxyl radicals at the site of *P. aeruginosa* infections and contribute to tissue injury [13–15]. Addition of LasB-treated transferrin to stimulated neutrophils also resulted in hydroxyl radical formation [15]. Britigan et al. [13] have determined that the same cleavage products of transferrin and lactoferrin generated in vitro by *P. aeruginosa* LasB...
are detectable in bronchoalveolar lavage (BAL) samples from CF patients. The same investigators have shown that diferric transferrin cleaved with 

\( P. \) aeruginosa LasB enhances pulmonary artery endothelial cell monolayer injury resulting from exposure to hydrogen peroxide, products of the xanthine/xanthine oxidase reaction, or phorbol myristic acid (PMA)–stimulated neutrophils [15]. Iron associated with transferrin appeared to be responsible for the cell injury. These studies indicate that LasB alteration of transferrin, and possibly lactoferrin, may serve as a mechanism of tissue injury in Pseudomonas infections.

We have previously described monoclonal antibodies generated against PSCP which react with neutralize the proteolytic activity of LasB as well as that of other zinc metalloproteases of the thermolysin class [7]. The epitopes recognized on \( P. \) aeruginosa LasB by these monoclonal antibodies were mapped by using overlapping, 9-mer synthetic peptides, spanning a 13.9-kDa fragment containing the active site [19]. The antibodies reacted strongly with 2 distinct peptides. Peptide \( \text{HGFTEQNSG}_{342} \) (designated pep15) overlaps the motif \( \text{HEXXH}_{337} \text{HEXXH}_{342} \), which is conserved in the active site of many zinc-dependent endopeptidases. Peptide \( \text{RYMDQPSRD}_{403} \) (designated pep42) lies between \( \text{E}_{361} \), which binds a zinc atom, and \( \text{H}_{392} \), which acts as a proton donor at the active site. The sequences corresponding to pep15 and pep42 were found to be conserved among bacterial metalloproteases. Polyclonal rabbit antibodies reacted strongly with 2 distinct peptides. Peptide \( \text{ALR YMDQPSRD}_{403} \) was also shown to react with the thermolysin class of metalloproteases, including LasB and PSCP, and to neutralize their proteolytic activity [19].

Because there is considerable evidence that \( P. \) aeruginosa LasB plays a significant role in lung injury that occurs during \( P. \) aeruginosa chronic lung infections, it is possible that antibodies that neutralize the activity of this protease would provide protection against the damage that occurs during \( P. \) aeruginosa lung infections. Evidence to date also suggests that PSCP may play a role in lung injury during \( B. \) cepacia infections [6]. The objective of the current study was to determine the efficacy of immunization with synthetic peptides corresponding to the neutralizing epitopes previously identified on \( P. \) aeruginosa (represented by pep15 and pep42) in decreasing lung injury during \( P. \) aeruginosa or \( B. \) cepacia experimental infections.

Materials and Methods

**Bacterial strains and culture conditions.** \( P. \) aeruginosa strain PAO and \( B. \) cepacia strain Pc715j were used in this study. PAO is a wild-type strain that produces elastase and AP [20]. Pc715j was originally isolated from a patient with CF and produces both PSCP and the 40-kDa protease [7]. Pc715j belongs to genomovar III [21] (P.A. Sokol, unpublished observations). Cultures were grown overnight at 32°C in dialysed trypsinase soy broth treated with Chelex-100 (Bio-Rad, Hercules, CA) [22]. These growth conditions are optimal for maximum yields of most virulence factors in these strains.

**Peptide synthesis and conjugation.** Peptides HGFTEQNSG (pep15) and GALRYMDQPSRD (pep42) were prepared by following the general procedure for solid-phase peptide synthesis as described by Erickson and Merrifield [23]. Pep42 was cross-linked to the protein without a spacer because the native sequence contained GA at the N-terminus. Each peptide contained alpha-N-benzoylbenzoyl ornithine as the N-terminal group for the purposes of photocross-linking and the quantitation of peptide : protein ratios. The synthesis, purification, and characterization of the peptides were performed as described elsewhere [24].

The preparation of peptide-protein conjugates was performed as described by Lee et al. [25]. Peptides containing the photoreactive group, benzoyl benzoic acid, that were attached to the N-terminal end were conjugated to keyhole limpet hemocyanin (KLH) or tetanus toxoid (TT). Unconjugated peptides were removed by successive dialysis against 8 M urea, 1 M urea, and PBS at pH 7.2. The product was lyophilized, and peptide incorporation was determined by amino acid analysis. In the PAO-infection experiments, the pep15-KLH ratio was 100 nmol/mg (5 : 1), and the pep42-KLH ratio was 140 nmol/mg (7 : 1). In the Pc715j-infection experiments, the pep15 and pep42-KLH ratios were 200 nmol/mg (10 : 1). The pep15-TT and pep42-TT conjugates had an average conjugation ratio of 135 nmol/mg (19 : 1) and 124 nmol/mg (17 : 1), respectively.

**Experimental animals.** Immunization and protection studies were performed in the agar bead model of chronic lung infection in rats [26]. Sprague-Dawley male rats (100–125 g; Charles River, Canada) were used for each infection experiment. In the experiments with the KLH-peptide conjugates, rats were randomly separated into 3 groups and immunized intramuscularly with 30 \( \mu \)g of each pep15-KLH, pep42-KLH, or KLH prepared in complete Freund’s adjuvant. Animals were given booster injections subcutaneously twice at 14-day intervals with the same antigen prepared in incomplete Freund’s adjuvant. Four days after the last immunization, using serum obtained from 0.1 mL of blood obtained from the tail vein, we determined the antibody titers of 4 rats from each group. Seven days after the last immunization, the rats underwent tracheostomy under anesthesia and were inoculated with 0.05 mL of a suspension of 105 cfu of PAO or Pc715j embedded in agar beads, as described elsewhere [26].

At 3 and 7 days after inoculation, 5 animals from each group were lavaged with 0.9% saline. A portion of the lavage fluid was used for neutrophil assays and the remainder aliquoted and stored at −70°C. All animals were killed by cardiac puncture under anesthesia on either day 3 or day 7 after inoculation. Sera were collected and stored at −20°C and examined for the presence of antibody to the peptides by ELISA. Lungs from 7–9 animals in each group were removed aseptically on day 7, placed in 3 mL of PBS, and homogenized (Polytron homogenizer; Brinkman Instruments, Westbury, NY). Serial dilutions of the homogenates were plated on trypticase soy agar. The colony-forming units were counted following incubation at 37°C overnight. Lungs from 8 animals in each group were fixed in 10% formalin in PBS. Sagittal slices of the left lobes of these lungs were mounted and stained with hemotoxylin and eosin. The percentage of infiltration was determined by the method of Dunnill [27] as described elsewhere [28]. We used a Zeiss integrating eyepiece to record the number of points overlying the sur-
Table 1. Reciprocal antibody titters to peptides 15 and 42.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Immunogen</th>
<th>Serum IgG</th>
<th>Serum IgA</th>
<th>BAL IgG</th>
<th>BAL IgA</th>
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<tbody>
<tr>
<td>1&lt;sup&gt;c&lt;/sup&gt; Pep15-KLH</td>
<td>221,714 ± 159,411</td>
<td>7860 ± 7653</td>
<td>625 ± 570</td>
<td>450 ± 132</td>
<td></td>
</tr>
<tr>
<td>Pep42-KLH</td>
<td>160,000 ± 135,765</td>
<td>15,364 ± 13,690</td>
<td>1125 ± 509</td>
<td>100 ± 122</td>
<td></td>
</tr>
<tr>
<td>Pep15-KLH</td>
<td>264,626 ± 310,134</td>
<td>14,680 ± 17,320</td>
<td>300 ± 100</td>
<td>950 ± 958</td>
<td></td>
</tr>
<tr>
<td>Pep42-KLH</td>
<td>293,818 ± 173,305</td>
<td>11,818 ± 10,581</td>
<td>1025 ± 992</td>
<td>4200 ± 1778</td>
<td></td>
</tr>
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NOTE. BAL, bronchoalveolar lavage; KLH, keyhole limpet hemocyanin. In KLH-immunized control animals, all antibody titters were <100.

<sup>a</sup> Trim mean ± SD of 25 samples, 2 highest and 2 lowest excluded.

<sup>b</sup> Trim mean ± SD of 10 samples, highest and lowest excluded.

<sup>c</sup> Animals were subsequently infected with *Pseudomonas aeruginosa* strain PAO.

<sup>d</sup> Animals were subsequently infected with *Burkholderia cepacia* strain Pc715j.

face area of the infiltrate and divided this value by the total number of points counted over the entire surface area.

In the experiments with the TT conjugates, the rats were divided into 3 groups and immunized with 30 μg of pep15-TT, 30 μg of pep15-TT plus 30 μg of pep42-TT, or PBS mixed with adjuvant. The animals were given a booster injection once with the same antigen 14 days later. Five days later test bleeds were performed on 3 animals from each group. Animals were infected with agar beads containing PAO 7 days after the second immunization. BAL fluid was obtained from 5 animals in each group on postinfection (PI) days 4 and 28. Quantitative bacteriologic and histopathologic analyses were performed on PI days 7 and 28. Cardiac bleeds were performed on all animals just before killing them to monitor antibody titers.

**ELISA.** Antibody titers in rat sera were determined by ELISA, with pep15 or pep42 conjugated to BSA (60 nmol/mg and 90 nmol/mg, respectively) used as the coating antigen (2 μg/mL), essentially as described elsewhere [7]. Horseradish peroxidase (HRP)-labeled anti-rat IgG (1 : 2000; Kirkegaard-Perry, Gaithersburg, MD) was used to determine IgG titers. To determine IgA titers, we used goat anti-rat IgA (1 : 500; Sigma, St. Louis) followed by HRP-conjugated anti-goat IgG (1 : 1000; Miles Scientific, Recksdale, Ontario) for antibody detection.

**BAL.** BAL fluid was obtained from animals anesthetized with inhaled ether and killed by cardiac bleeds. The trachea was exposed and cannulated with a blunt-tipped 18-gauge needle. The lungs were lavaged with 2.5 mL aliquots of warm (37°C) 10 mM sodium phosphate, 150 mM NaCl, pH 7.5. Protein concentration was estimated by using the Bio-Rad protein assay dye reagent.

**Neutrophil assays.** The total number of cells present in the BAL fluid (~8 mL) was counted with a Neubauer hemocytometer (American Optical Corp., Buffalo, NY). The cellular fraction was recovered by centrifugation at 400 g for 10 min at 4°C. The resulting pellets were gently resuspended in 0.2 mL of 10 mM sodium phosphate and 150 mM NaCl, pH 7.5, containing 0.01% human albumin (Miles Scientific). Differential counts were made on the first 200 intact cells visualized on Wright-Giemsa-stained smears of the cytosol preparations.

**Polymorphonuclear leukocyte (PMNL) elastase assays.** Neutrophil elastase (NE) activity was determined by using the chromogenic peptide substrate succinyl-l-alanyl-l-alanine-p-nitroanilide (SLAPN; Sigma) [29, 30]. A 50-mM stock solution of SLAPN was prepared by dissolving the solid in saline, diluting to 500 μM with saline, and 10 μL was mixed with 990 μL of 0.25% Triton X-100. The reaction was initiated by addition of 10 μL of BAL fluid to a final concentration of 2 mg/mL SLAPN. The reaction was stopped after 5 minutes by addition of 200 μL of 0.1 N HCl. The absorbance at 405 nm was determined using a standard curve prepared for each experiment.

**Ethanol-induced neutrophil emigration.** The ability of ethanol to increase neutrophil emigration was assessed by measuring the total cell counts in BAL fluid obtained 4 and 28 days after immunization. Animals were anesthetized with inhaled ether and the trachea was exposed. A 10-mL aliquot of 95% ethanol in 0.9% saline was instilled into the trachea to a final concentration of 15% ethanol. The lungs were lavaged with 5 mL aliquots of saline at 10 minutes and the cell counts performed as described above.

**Figure 1.** Total no. of cells in bronchoalveolar lavage fluid from animals infected with *Pseudomonas aeruginosa* strain PAO (A) or *Burkholderia cepacia* strain Pc715j (B). Groups of 5 animals were lavaged with 10 mL of 0.9% PBS. The cells were counted by use of a Neubauer hemocytometer (American Optical Corp., Buffalo, NY). A, Total cell counts were significantly different between animals immunized with either peptide 15 (pep15)-keyhole limpet hemocyanin (KLH) or pep42-KLH and those immunized with KLH on both postinfection (PI) days 3 and 7 (P < .05, analysis of variance [ANOVA] with Dunnett multiple comparisons test). B, Total cell counts were significantly lower in animals immunized with pep15-KLH on PI day 7 and pep42-KLH on PI days 3 and 7 (P < .05, ANOVA with Dunnett multiple comparisons test).
was prepared in dimethyl sulfoxide (Fisher Scientific, Ottawa, Ontario). To assay for NE activity, we mixed 147 μL of BAL fluid with 1 mM SLAPN in a final volume of 150 μL and incubated the mixture at 37°C with shaking. Assays were performed in the presence and absence of 5 mM phenylmethylsulfonyl fluoride (PMSF), which inhibits serine proteases, to distinguish between NE activity or metalloprotease activity due to either \( P. aeruginosa \) LasB or macrophage metalloproteases.

**Western blotting of NE.** Human NE (3 μg; Elastin Products, Owensville, MO) was separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore, New Bedford, MA), and reacted to rabbit anti-pep15, rabbit anti-pep42, or normal rabbit serum at a dilution of 1 : 200 for 1 h at 37°C [19]. The blots were reacted to protein A-HRP (1 : 2000; Sigma), and positive reactions were detected with HRP substrate (Bio-Rad).

**Statistical analyses.** Analysis of variance (ANOVA) and Student’s \( t \) tests were performed with INSTAT software (GraphPad Software, San Diego). \( P < .05 \) was considered statistically significant.

### Results

**Immune response to peptide-KLH conjugates.** The rats were immunized with pep15-KLH, pep42-KLH, or KLH. Blood samples were obtained from 4 rats from each group 4 days after each of the second and third immunizations. Antibody titers were determined by ELISA, with the corresponding peptide conjugated to BSA used as the coating antigen. Four days after the second immunization, the antibody titers to pep15-KLH had a range of 200–51,000, with a median titer of 6400. The titers to pep42-KLH ranged from 100 to \( >1 \times 10^4 \), with a median titer of 32,000. After the third immunization, the titers to either peptide were \( >1 \times 10^4 \). Animals were infected with either \( P. aeruginosa \) PAO or \( B. cepacia \) Pc715j 7 days after the third immunization.

At 3 or 7 days after infection, the rats were killed and either were subjected to BAL to examine their inflammatory cell response or had their lungs removed for quantitative pathologic or bacteriologic analyses. All rats were also bled by cardiac puncture before they were killed. The serum IgG and IgA titers were determined by ELISA. The IgA and IgG titers of the BAL fluid were also determined. The mean titers are shown in table 1. The mean serum IgG and IgA titers to pep15 and pep42 were similar in the 2 experiments, and there was no significant difference between the antibody titers obtained, regardless of which peptide was the immunizing antigen. IgG and IgA antibodies to pep15 and pep42 were also detected in the BAL fluid. With the exception of a higher IgA response to pep42 in the BAL fluid from animals in the \( B. cepacia \) experiment, the BAL antibody titers were similar in the 2 experiments, and the responses were similar for the 2 peptides.

**Effect of immunization with peptide-KLH conjugates on inflammatory cell response during infections with \( P. aeruginosa \) or \( B. cepacia \).** Pulmonary infection by \( P. aeruginosa \) in CF patients is followed by an inflammatory response characterized by an influx of PMNL into the lung [31]. To determine the effects of immunization with the peptide epitopes on lung inflammation, we performed lavage on groups of animals on PI days 3 and 7, and the total cell number, neutrophil number, protein concentration, and NE-like activity were determined in the BAL fluid.

The total cell counts in the BAL fluid were lower on both PI days 3 and 7 in animals immunized with either pep15-KLH or pep42-KLH than in animals immunized with KLH. In the

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**Figure 2.** No. of polymorphonuclear leukocytes (PMNL) in bronchoalveolar lavage (BAL) fluid from animals infected with \( Pseudomonas aeruginosa \) strain PAO (A) or \( Burkholderia cepacia \) strain Pc715j (B). The no. of PMNL was determined from the differential cell count in BAL fluid from the same animals shown in figure 1. A and B, Nos. of PMNL were significantly different between animals immunized with peptide 15–keyhole limpet hemocyanin (KLH) and those immunized with KLH on both postinfection days 3 and 7 (\( P < .05 \), analysis of variance with Dunnett multiple comparisons test). The no. of PMNL in BAL fluid from animals immunized with peptide 42-KLH was not significantly different from that in BAL fluid from KLH-immunized animals.
animals subsequently infected with *P. aeruginosa* (figure 1A), the cell counts were ~65% lower on day 3 and 45% lower on day 7 in animals immunized with the peptide conjugates than in the control animals immunized with KLH (*P* < .05, ANOVA with Dunnett multiple comparisons test). In the animals subsequently infected with *B. cepacia*, there was a decrease in cell numbers of ~40%–50% on day 3 and 33%–43% on day 7, compared with cell counts in the control group (figure 1B). This difference in total cell counts was significantly lower than that in controls for animals immunized with pep15-KLH on day 7 and pep42-KLH on both days 3 and 7 (*P* < .05, ANOVA with Dunnett multiple comparisons test) but not quite significant in the animals immunized with pep15-KLH examined on PI day 3 (*P* = .06).

The number of PMNL was also determined in the BAL fluid from the same groups of animals. In animals subsequently infected with *P. aeruginosa*, the numbers of PMNL in the BAL fluid in rats immunized with pep15-KLH were 75% and 80% lower on days 3 and 7, respectively, than they were in the control group (figure 2A; *P* < .05, ANOVA with Dunnett multiple comparisons test). Although there was an ~50%–60% decrease in the number of PMNL in the animals immunized with pep42-KLH compared with controls, this difference was not significant (*P* > .05, ANOVA). Similar results were obtained in the experiment when rats were subsequently infected with *B. cepacia* (figure 2B). There was a significant decrease in the number of PMNL in the BAL on days 3 and 7 in the animals immunized with pep15-KLH compared with that seen in the controls (*P* < .05, ANOVA with Dunnett multiple comparisons test). The difference in the number of PMNL in the BAL fluid from animals immunized with pep42-KLH compared with that seen in controls was not significant.

In CF patients, neutrophils release a variety of proteases. The combination of bacterial proteases and neutrophil proteases, particularly NE, is thought to cause a considerable portion of the damage to connective tissue proteins that form the lung matrix [32]. Immunization with pep15-KLH or pep42-KLH had no significant effect on either total elastase activity measured in the BAL fluid or NE-like activity inhibitable by PMSF in the animals subsequently infected with either *P. aeruginosa* or *B. cepacia* (data not shown). The lack of effect on NE-like activity is not surprising, because this is a serine protease and not a metalloprotease. Antibodies to pep15 or pep42 did not cross-react with human NE on immunoblots of SDS-PAGE (data not shown).

**Effect of immunization with peptide-KLH conjugates on lung pathologic changes during infections with *P. aeruginosa* or *B. cepacia**. On PI day 7, the lungs were removed from groups of 8 animals and examined for quantitative and qualitative histopathologic changes. The percentage of the lungs infiltrated with inflammatory exudate was determined by a point-counting method [27, 28]. In the *P. aeruginosa* experiment, there was 61% less pathologic change in the lungs of animals immunized with pep15-KLH than in those of rats immunized with KLH (figure 3A; *P* < .001, *t* test for unpaired observations). In the *B. cepacia* experiment, there was 53% less pathologic change in the lungs of animals immunized with pep15-KLH than in the KLH control group (figure 3B; *P* < .05, *t* test for unpaired observations). Immunization with pep42-KLH did not significantly alter the progression of lung pathologic change compared with that seen in controls in animals that were subsequently infected with either *P. aeruginosa* or *B. cepacia* (figures 3A and 3B). The histopathologic changes in animals infected with *P. aeruginosa* were characterized by multiple microabscesses and a significant PMNL response. The animals infected with *B. cepacia* presented a histopathologic picture characterized by a mixed cellular infiltrate composed primarily of mononuclear phagocytes and lymphocytes as described elsewhere [33]. Immunization

![Figure 3](https://academic.oup.com/jid/article-abstract/181/5/1682/2191494)
with pep15-KLH did not result in any apparent qualitative histopathologic change compared with that seen in control animals, although the quantitative histopathologic changes were significant.

Effect of immunization with peptide-KLH conjugates on numbers of bacteria in the lung during infections with P. aeruginosa or B. cepacia. On PI day 7, quantitative bacteriological analyses were performed on lung homogenates from animals infected with either P. aeruginosa or B. cepacia. There were no differences between the number of bacteria recovered from animals immunized with pep15-KLH, pep42-KLH, or controls (data not shown). Therefore, the observed decrease in lung pathologic changes in animals immunized with pep15-KLH is not due to the presence of fewer bacteria in the lungs.

Immunization with peptideTT conjugates. Because immunization with pep15-KLH resulted in a significant decrease in lung injury in experimental infections with P. aeruginosa and B. cepacia, similar experiments were performed with pep15 conjugated to TT, followed by challenge with P. aeruginosa PAO. TT was selected as a carrier protein in these experiments because it has been approved for use in human vaccines. In these experiments, the effect of immunization on disease progression was examined on PI days 4, 7, and 28 to determine if the improvement in lung pathologic changes observed in animals immunized with pep15 persisted during long-term chronic infection. Immunization with pep42-TT alone was not examined because immunization with pep42-KLH did not result in a significant improvement in lung histopathologic features. Immunization with a combination of pep15-TT and pep42-TT, however, was performed to determine if immunization with both epitopes provided a greater benefit than immunization with only pep15 conjugated to a carrier protein.

Animals were immunized with either 30 µg of pep15-TT or 30 µg of pep15-TT plus 30 µg pep42-TT. Control animals were injected with PBS mixed with adjuvant. Five days after the second immunization, blood samples were obtained from 3 animals from each group, and the antibody titers to pep15, pep42, or both were determined by ELISA, with pep15-BSA or pep42-BSA used as the coating antigen. The titers in the animals tested had a range of 16,000–128,000. The reciprocal titers for each group (mean ± SD) were 32,000 ± 27,713 for pep15-TT, 42,666 ± 18,455 for pep15-TT/pep42-TT against pep15, and 69,333 ± 56,190 for pep15-TT/pep42-TT against pep42. The differences in antibody titers among the 3 groups were not significant (P > .05, ANOVA). Because the antibody titers were consistently higher after 2 immunizations in animals immunized with the TT conjugates than titers previously observed with the KLH conjugates, the animals were challenged with P. aeruginosa 7 days after the second immunization rather than after the third immunization.

On PI days 4, 7, and 28, either animals were subjected to BAL to examine their inflammatory cell response or their lungs were removed for quantitative pathologic or bacteriologic analyses. The IgG and IgA titers in serum and BAL fluid were determined by ELISA. The logs of the reciprocal ELISA titers for pep15-TT against pep42. The differences in antibody titers among the 3 groups were not significant (P > .05, ANOVA). Because the antibody titers were consistently higher after 2 immunizations in animals immunized with the TT conjugates than titers previously observed with the KLH conjugates, the animals were challenged with P. aeruginosa 7 days after the second immunization rather than after the third immunization.

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lower in the animals immunized with either pep15-TT or pep15-TT/pep42-TT than that in the control group \((P < .05, \text{ ANOVA with Dunnett multiple comparisons test})\). There was no difference in the total number of cells or number of PMNL in the BAL fluid between the animals immunized with pep15-TT and those immunized with pep15-TT/pep42-TT. Immunization with pep15 resulted in a decreased inflammatory cell response during \(P. \text{ aeruginosa} \) infections, regardless of the carrier protein used for immunization.

On PI day 28, the total number of cells in the BAL fluid was similar to the number on day 4 after infection; however, the number of PMNL in the BAL fluid at this time was \(\sim 1 \log\) lower than it was on day 4. Although there was an apparent decrease in cell numbers in the animals immunized with pep15-TT or pep15-TT/pep42-TT compared with controls (figure 5B), this decrease was not quite significant \((P = .09, \text{ ANOVA})\). Because of the death of 2 animals shortly after infection, presumably due to surgery complications, there were only 4 animals in the pep15-TT–immunized group, compared with 5 animals in the other groups on day 28 for BAL fluid analysis. This resulted in the group immunized with pep15-TT having too few values to pass the normality test for populations with Gaussian distributions. Because no difference was observed between the pep15-TT group and the pep15-TT/pep42-TT group for any of the parameters analyzed, the data from these 2 groups of animals were combined and were reexamined for significant differences from the control group. The total cell counts of the combined peptide-immunized group \((1.77 \times 10^5 \pm 1.5 \times 10^4)\) compared with the control group \((2.41 \times 10^5 \pm 1.9 \times 10^4)\) were determined to be significantly different \((P < .05, \text{ Student’s } t\text{ test for unpaired observations})\).

On day 28 there was an \(~50\%\) decrease in the number of PMNL in the BAL fluid in the animals immunized with pep15-TT and an \(80\%\) decrease in the number of PMNL in the animals immunized with pep15-TT/pep42-TT, compared with the control group (figure 5B). This decrease was not quite statistically significant. When the mean number of PMNL in the immunized group combined as described earlier was compared with that in the control group, however, the difference in the number of PMNL in the BAL fluid between the immunized \((1.0 \times 10^4 \pm 5.6 \times 10^3)\) and the control groups \((3.2 \times 10^4 \pm 1.2 \times 10^3)\) was significant \((P < .05, \text{ Student’s } t\text{ test for unpaired observations})\).

Effect of immunization with peptide-TT conjugates on quantitative bacteriology during infections with \(P. \text{ aeruginosa} \). On PI days 7 and 28, quantitative bacteriological analysis was performed on lung homogenates (data not shown). On PI day 7, there were \(\sim 10^5 \sim 10^6\) cfu/lung, and on day 28, \(\sim 10^4\) cfu remained in the lungs. As previously observed in the immunization experiments with the KLH conjugates, immunization with pep15 did not have any impact on the number of \(P. \text{ aeruginosa} \) persisting in the lungs up to 28 days after infection (data not shown).

Effect of immunization with peptide-TT conjugates on lung pathologic changes during infections with \(P. \text{ aeruginosa} \). On PI days 7 and 28, the lungs were removed from groups of animals and were examined for quantitative and qualitative histopathologic changes, and the percentage of the lungs infiltrated with inflammatory exudate was determined (figure 6). The degree of lung pathologic change that was observed on day 7 in animals immunized with pep15-TT or pep15-TT/pep42-TT was \(59\% \) or \(55\%\) lower, respectively, than that in control animals \((P < .001, \text{ ANOVA})\). This finding is similar to that observed when animals were immunized with pep15-KLH.

**Figure 5.** Total no. of cells and polymorphonuclear leukocytes (PMNL) in bronchoalveolar lavage (BAL) fluid, from animals infected with *Pseudomonas aeruginosa*, on postinfection (PI) days 4 (A) and 28 (B). The scale on the right axes differs by 1 log between panels A and B. Values represent mean ± SE of 5 animals per group with the exception of the peptide 15 (pep15)–tetanus toxoid (TT) immunized group on day 28, which contained 4 animals. A, Total cell counts \((P < .001, \text{ analysis of variance [ANOVA]})\) and nos. of PMNL \((P < .05, \text{ ANOVA})\) were significantly different between the animals immunized with either pep15-TT or pep15-TT/pep42-TT and controls. B, Total cell counts and nos. of PMNL of the combined immunized group (pep15-TT and pep15-TT/pep42-TT) were significantly different from those of the control group \((P < .05, \text{ Student’s } t\text{ test for unpaired observations})\).
On day 28, the degree of pathologic change was 52% and 70% lower in animals immunized with pep15-TT or pep15-TT/pep42-TT, respectively, compared with that in control animals. The difference in the degree of lung pathologic change was significant between either of the 2 immunized groups and the control group ($P < .001$, ANOVA). Although immunization with both peptides appeared to have an added benefit in reducing lung pathologic change in this experiment, this difference was not significant ($P > .05$, ANOVA). These studies indicate that immunization with synthetic peptide-conjugates containing pep15 can significantly reduce lung injury due to infections with either P. aeruginosa or B. cepacia. The reduction in lung pathologic change was maintained during a long-term chronic infection in this animal model.

Discussion

Previously, rabbit antisera raised against either pep15 or pep42 conjugated to BSA were shown to effectively neutralize P. aeruginosa LasB in an in vitro assay [19]. In the present study we have demonstrated that immunization with either pep15-KLH or pep42-KLH resulted in similar serum and BAL fluid antibody titers. Despite the similarity in titers, however, immunization with pep15-KLH before infection with either P. aeruginosa or B. cepacia, resulted in a significant improvement in lung pathologic change, compared with immunization with pep42-KLH or findings seen in control animals. Immunization with either peptide resulted in a decreased total cell count in BAL fluid. Animals immunized with pep15-KLH, however, also had ~70% fewer PMNL in the BAL fluid and a 50%–70% decrease in lung histopathologic features compared with controls.

Pep15 overlaps the last histidine in the $^{33}_{541}$HExxH motif characteristic of the active sites of zinc-dependent metalloproteases. Pep42 is located between $E_{337}$, a zinc-binding site, and $H_{420}$, which acts as a proton donor at the active site. Antibodies to pep42 appear to be able to bind to P. aeruginosa LasB in vitro with sufficient affinity to effectively inhibit proteolytic activity, possibly by blocking the active site cleft. Antibodies to this epitope do not appear to be effective in reducing proteolytic activity in vivo. Antibodies binding to pep15 would directly block the active site of LasB and neutralize proteolytic activity. The results obtained suggest that anti-pep15 antibodies are effective at neutralizing proteolytic activity both in vivo and in vitro. Similar results were obtained regardless of whether pep15 was conjugated to KLH or to TT, indicating that the immunization effect is not dependent on the choice of carrier protein. To investigate the possibility that a combination of antibodies to pep15 and pep42 would bind to both sites and potentially have a synergistic effect in inhibiting proteolytic activity, we immunized some animals with both pep15-TT and pep42-TT. In these experiments, there was no added benefit of immunizing with the combination of peptides, because similar effects on inflammatory cells and lung histopathologic change were observed in animals immunized with only pep15-TT.

Pep15 induces a good antibody response, and after 2 immunizations, the serum IgG titers were in the $10^{2}$–$10^{4}$ range. Three immunizations were performed in the KLH experiments, but this did not generate a better antibody response or a greater reduction in lung pathologic change than did the 2 immunizations performed in the TT experiments. The control animals did not have antibody titers to pep15 $> 100$ even at PI day 28, suggesting that animals do not normally produce an immune response to this epitope during the course of infection. The antibody titers to pep15 were significantly higher on day 28 than on day 4 or day 7, however, suggesting that exposure to...
LasB produced by *P. aeruginosa* during infection can provide a booster effect and enhance the antibody response in animals previously immunized with pep15.

Immunization with pep15-KLH or pep15-TT followed by infection with *P. aeruginosa* or *B. cepacia* resulted in a decrease of 52%–70% in lung pathologic change and a decrease of 75%–90% in the number of PMNL in the BAL fluid, depending on the experiment. This reduction in the severity of lung disease occurred in the absence of a significant difference between the number of colony-forming units recovered from the lungs in the immunized groups and those of the control groups. These data indicate that antibodies to pep15 do not clear the organism and suggest that the reduction in lung pathologic change and inflammation is due to a decrease in proteolytic activity during infection. Because there was no effect on NE-like activity, this suggests that the improvement was effected by bacterial metalloprotease activity, due primarily to *P. aeruginosa* LasB or *B. cepacia* PSCP.

Studies by Homma et al. [34] suggested that neutralizing antibodies to *P. aeruginosa* LasB can be protective against *P. aeruginosa* lung disease. These investigators compared the ability of LasB and AP toxoids and OEP, a common antigen consisting of LPS and protein components of the cell wall, to protect against hemorrhagic pneumonia due to *P. aeruginosa* in mink. Immunization of rabbits with toxoids of either LasB or AP generated antiserum that could neutralize the enzyme activity of the respective enzyme. Although these preparations required 4 or 5 immunizations to generate a passive hemagglutination titer of 10^2–10^3, the serum produced could neutralize LasB or protease activity. This suggests that the treatment with formalin and lysine may have facilitated the exposure of LasB epitopes that could induce the formation of neutralizing antibodies. When mink were immunized with LasB toxoid, followed by challenge with *P. aeruginosa*, the LD_50 increased from ~10^5 to 10^6 bacteria [34]. The results obtained by using the LasB toxoid were similar to those obtained by using protease toxoid alone or a mixture of protease toxoid, LasB toxoid, and OEP but were significantly improved over those obtained by immunization with OEP alone. A multicomponent vaccine, including these 3 components plus a toxoid of exotoxin A, has been reported to have promising results in patients with diffuse panbronchiolitis [35].

A more recent study by Gilleland et al. [36] compared the efficacy of LasB as a vaccine, used alone or in combination with exotoxin A toxoid and outer membrane protein F, in a rat agar-bead model. LasB was the only antigen tested that reduced the degree of inflammation in the lungs. There was significantly less peribronchial inflammation in rats immunized with LasB than in those given the other preparations. Immunization with LasB or exotoxin A toxoid in combination with protein F did not enhance protection beyond what was observed with protein F alone. In these experiments, however, rats were immunized with 100 μg of active LasB, which possibly could have contributed to disease in these animals or could have cleaved the protein F and exotoxin A toxoid used in the combination vaccines. It is not clear why a LasB toxoid preparation was not tested in these studies by using an approach similar to that used with toxoid exotoxin A.

Several other antigens, including LPS components, alginate, and outer membrane proteins, have been investigated as potential vaccine candidates for *P. aeruginosa* lung infections in either animal models or in CF patients with varying degrees of success (for reviews see [37, 38]). While it is not our intention to review all the studies of potential vaccine candidates, some comparisons can be made between pep15 and other potential vaccine antigens.

The effects of immunization with alginate on subsequent *P. aeruginosa* infections was examined by using the same animal model as was used in the current study [39]. The effects of immunization on bacterial clearance were strain dependent, and, interestingly, the nonmucoid strain was cleared more easily than the paired mucoid strain, even when alginate was purified from the homologous strain. With at least one of the alginate preparations used, evidence for immune complex deposition in tissues was observed, suggesting that alginate may not be a good candidate as a protective antigen [39]. Johansen et al. [40] reported that immunization with a depolymerized (*M_60,000*) alginate preparation conjugated to toxin A resulted in a reduction in lung pathologic changes and PMNL infiltration in the absence of bacterial clearance in an agar-bead chronic infection model. Immunization with intact alginate did not provide any protective effect; however, these alginate preparations were from different strains than the low-molecular-weight alginate [40]. Because all rats were challenged with the same PAO strain, differences in the efficacy of these vaccines could be due to either effects of heterologous alginate, differences in molecular weight of the alginate, or the inclusion of toxin A in the vaccine. Pier et al. [41] have reported that a high-molecular-weight preparation of alginate induced opsonic antibodies in humans that were effective against heterologous strains. Alginate from various *P. aeruginosa* strains have both conserved and strain-specific epitopes, and the high-molecular-weight preparations are proposed to be more similar in antigenic composition [42]. The ability to produce cross-protection with various alginate components, either alone or conjugated to exotoxin A, was highly variable in mouse experiments when animals were challenged with heterologous strains [37]. Because no antigenic variation has been detected in *P. aeruginosa* LasB, one would not anticipate such strain variation in efficacy when pep15 is used as a vaccine antigen.

LPS and its components have received a great deal of attention as potential vaccine candidates. The use of LPS as a protective antigen is quite complex, because there are many different serotypes with varying degrees of cross-reactivity [37]. In early studies in CF patients, heptavalent LPS vaccines and cell wall extracts of 16-O antigen serogroups were not shown to
provide clinical benefit, and in at least 1 study, immunization was found to be detrimental [43–45]. There may be potential benefits in using components of LPS that may be less toxic and provide broader cross-reactivity. A lipid A–free octavalent O-polysaccharide vaccine covalently coupled to exotoxin A has been tested in CF patients; however, after 3 years, 39% of the patients were colonized or chronically infected with P. aeruginosa [46]. The O-PS toxin A vaccine has also been investigated in an agar-bead chronic infection model in rats [40]. Immunization with the O-PS toxin A followed by challenge with a mucoid derivative of PAO had no significant effect on either lung pathologic change or bacterial clearance [40]. Core oligosaccharide and A band LPS may contain more cross-protective components than the serogroup-specific polysaccharide; however, it seems unlikely that a single LPS component will cross-protect against all P. aeruginosa strains.

As mentioned earlier, P. aeruginosa outer membrane protein F has also been investigated as a vaccine candidate. Outer membrane proteins are conserved in all P. aeruginosa serotypes, and immunization with protein F was shown to provide some protection against challenge with representative strains from all 7 Fisher-Devlin immunotypes [47]. In the agar-bead model, immunization with protein F reduced both the incidence of pulmonary colonization and the incidence of pulmonary lesions, compared with those seen in control groups. In colonized animals, however, there was no difference in the number of bacteria recovered from lungs between the immunized and the control groups. In another study, a 14-residue synthetic peptide (peptide 10), from a predicted surface-exposed epitope in the C-terminal portion of protein F, was conjugated to KLH and was shown to induce the production of cross-reactive antibodies that mediated the opsonophagocytic uptake of P. aeruginosa by PMNL [48]. Immunization of rats with peptide 10-KLH followed by challenge with PAO in the agar-bead model resulted in a reduction in the number of rats with lesions similar to that observed with protein F [49]. In this study, the effect of immunization on colonization was not examined. More recently, immunization with a chimeric influenza virus containing peptide 10 was also shown to reduce pulmonary lesions and the incidence of bacterial colonization in an agar-bead mouse model of chronic pulmonary infection [50].

Because different scoring systems are used to report lung pathologic changes by Gilleland’s group [47, 49, 50] and ours, it is not possible to directly compare the extent of reduction in lung pathologic change in rats immunized with protein F peptide 10 and elastase pep15. Both of these peptides, however, have the potential to reduce the severity of lung disease due to P. aeruginosa infections in a chronic infection model. It would be interesting to immunize rats with a combination of peptides 10 and 15 to see if there is further reduction in lung pathologic change.

There have been no other reports of immunization studies with potential vaccine candidates for B. cepacia. Pep15 is the first broad-host-range immunogen proposed as a vaccine for CF patients. No other common antigens in P. aeruginosa and B. cepacia have been described. In our experiments, immunization with pep15 was equally effective in reducing lung pathologic change when animals were challenged with either P. aeruginosa or B. cepacia, the 2 major bacterial pathogens that cause chronic respiratory infections in CF patients. Because of its efficacy and broad host-range potential, pep15 has significant potential as a vaccine or therapeutic agent in the treatment of lung infections due to P. aeruginosa or B. cepacia and warrants further investigation.

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