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Advantages of a Synthetic Peptide Immunogen Over a Protein Immunogen in the Development of an Anti-Pilus Vaccine for *Pseudomonas aeruginosa*

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Abstract

The type IV pilus is an important adhesin in the establishment of infection by *Pseudomonas aeruginosa*. We have previously reported on a synthetic peptide vaccine targeting the receptor-binding domain of the main structural subunit of the pilus, *PilA*. The receptor-binding domain is a 14-residue disulfide loop at the C-terminal end of the pilin protein. The objective of this study was to compare the immunogenicity of a peptide-conjugate to a protein subunit immunogen to determine which was superior for use in an anti-pilus vaccine. BALB/c mice were immunized with the native PAK strain pilin protein and a synthetic peptide of the receptor-binding domain conjugated to keyhole limpet haemocyanin. A novel pilin protein with a scrambled receptor-binding domain was used to characterize receptor-binding domain-specific antibodies. The titres against the native pilin of the animals immunized with the synthetic peptide-conjugate were higher than the titres of animals immunized with the pilin protein. In addition, the affinities of anti-peptide sera for the intact pilin receptor-binding domain were significantly higher than affinities of anti-pilin protein sera. These results have significant implications for vaccine design and show that there are significant advantages in using a synthetic peptide-conjugate over a subunit pilin protein for an anti-pilus vaccine.

Keywords

anti-adhesin vaccine; antibody; peptide; *Pseudomonas aeruginosa*; synthetic vaccine

Pseudomonas aeruginosa is an opportunistic pathogen and a major cause of nosocomial infections (1). It has been suggested that adhesins play key roles in the initial stages of infection, where they mediate attachment of the bacteria to host epithelial-cell surfaces, allowing for subsequent colonization and potential invasion. The type IV pilus is a multimeric, non-branching, filamentous structure only 6 nm in diameter and up to several micrometers in length and is composed of thousands of identical *PilA* protein monomers (2,3). Because of its length, it has been suggested to mediate initial attachment of the bacteria to host surfaces. Once attached, the coordinated expression of numerous other virulence factors facilitates invasion of the surface by the bacteria. Because of its role early in the pathogenesis of infection, the type IV pilus has been proposed as an attractive vaccine target.

We have previously reported on the design of an anti-adhesin vaccine targeting the receptor-binding domain (RBD) of the type IV pilus of *Pseudomonas aeruginosa*, residues 128–144 (4). Furthermore, we have demonstrated that immunization with intact pili as well as synthetic

peptide analogs of the RBD can improve survival in an ABy/SnJ mouse model of *P. aeruginosa* infection (5). The RBD of *P. aeruginosa* pilin is a good candidate for a peptide vaccine. First, as shown in Figure 1, the 14-residue disulfide loop at the C-terminal of the pilin protein represents a continuous protective epitope (6). Second, synthetic peptides corresponding to the RBD are well-structured in solution (7). RBD peptides are conformationally constrained by a disulfide bridge and structural stability is further increased by the presence of two β -turns within the loop, a type I β -turn in the conserved sequence Asp¹³⁴-X-X-Phe¹³⁷ and a type II β -turn in the conserved sequence Pro¹³⁹-X-Gly-Cys¹⁴². Furthermore, there is conformational similarity between the free peptide and the RBD in the native protein (Figure 1) (8–10) and RBD peptides retain host epithelial cell receptor binding activity (11).

To the best of our knowledge, there is no clear comparison of the polyclonal response of a peptide immunogen and its cognate protein. In this study, we compare mouse polyclonal antisera raised against the PAK strain monomeric pilin protein (29–144) and a synthetic peptide (128–144) of the PAK RBD that is conjugated to keyhole limpet hemocyanin (KLH) with the goal of characterization of the titres and affinities of RBD-specific antibodies. The monomeric pilin protein lacks the N-terminal helix (residues 1–28) that is responsible for multimerization. Use of the monomeric pilin ensures one RBD is exposed for each protein molecule. To characterize RBD-specific antibodies within anti-pilin polyclonal antibodies, we have created a pilin protein with a scrambled RBD region (Figure 2). We show that not only does the synthetic peptide conjugated to KLH generate higher titres of RBD-specific antibodies, but that the anti-peptide antibodies have a higher affinity for the native protein than the anti-pilin antibodies.

Methods and Materials

Peptides

Synthetic peptides derived from the RBD (128–144, KCTSDQDEQFIPKGCSK) of the PAK strain of *P. aeruginosa* were prepared with a three amino acid residue linker (norleucine–Gly–Gly) on the N-terminal to give the peptide nLGGKCTSDQDEQFIPKGCSK for the preparation of peptide-conjugates and without the linker (Ac-KCTSDQDEQFIPKGCSK) for use in competitive ELISA assays. Peptides were manually synthesized by solid-phase peptide chemistry using standard t-BOC chemistry and 4-methyl-benzhydrylamine (MBHA) resin (12). Crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a semi-preparative Agilent Zorbax 300SB-C8 250 mm $\times$ 9.4 mm i.d. column (5 μ m particle size, 300 Å pore size, Agilent Technologies, Palo Alto, CA, USA). Fractions were analysed on a 150 mm $\times$ 2.1 mm i.d. Agilent Zorbax 300SB-C8 narrow bore column. Peptide identity was verified using a Mariner ESI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). The intrachain disulfide bond was formed by dissolving the synthetic peptide in 0.1 M ammonium bicarbonate, pH 9.0 at a peptide concentration of 0.5 mg/mL and overnight incubation at 25 °C. Disulfide formation was confirmed by reaction with *N*-ethylmaleimide (NEM) followed by RP-HPLC (13).

Synthetic peptides to be conjugated to a carrier protein [bovine serum albumin (BSA) or KLH] were selectively iodoacetylated at the N-terminus (14). Briefly, the peptide was dissolved in 100 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6 at 4 °C at 2 mg/mL. A total of two molar equivalents of 0.2 M iodoacetic anhydride in dry 1,4-dioxane in two aliquots was added to the peptide solution with stirring. The extent of the reaction was monitored by RP-HPLC. Upon completion, the reaction mixture was acidified with 0.5 mL glacial acetic acid and applied to a Zorbax 300SB-C8 9.4 $\times$ 150 mm RP-HPLC column and purified. The identity of the product was confirmed by mass spectrometry.

The peptide-KLH conjugate was prepared by first dissolving endotoxin-free KLH (Sigma, St. Louis, MO, USA) at a concentration of 10 mg/mL in freshly prepared 8 M urea. EDTA was added to a concentration of 5 mM. KLH was derivatized with free sulfhydryl groups by adding a 50 molar excess of Traut's reagent (2-iminothiolane) to the KLH solution. The reaction mixture was protected from light and stirred at 25 °C for 1 h. Unreacted Traut's reagent was removed with a PD10 desalting column (Amersham Biosciences, Piscataway, NJ, USA) using a 50 mM sodium phosphate, 5 mM EDTA eluent. The protein containing fractions were concentrated to 15–20 mg/mL using a Ultrafree-15 Centrifugal Filter Device (MWCO 10 000, Millipore, Bedford, MA, USA). The iodoacetyl-peptide was added to the mixture in a 10-fold molar excess to KLH. The reaction mixture was protected from light and incubated at 25 °C for 1 h. Unreacted sulfhydryl groups were capped by addition of 20 molar excess iodoacetamide (Sigma-Aldrich) and incubated for 30 min at 25 °C. Peptide-conjugates were dialysed extensively against phosphate-buffered saline (PBS) with 5 mM EDTA (pH 7.4). The peptide-KLH conjugate was subjected to amino acid analysis. By calculating the number of moles of norleucine, which was incorporated in the N-terminal linker of the synthetic peptide, and using the known amino acid composition of KLH, the total amount of synthetic peptide that had been conjugated to KLH as well as the synthetic peptide:KLH molar ratio were determined (~5:1 per KLH monomer).

Pilin protein expression

The gene encoding the PAK pilin protein (29–144) was cloned into the pRLD vector (15). Pilin proteins were expressed in BL21 (DE3) *E. coli* cells and purified from the periplasmic fraction by an osmotic shock protocol. An overnight culture was used to inoculate a larger culture, which was grown at 37 °C until an OD₆₀₀ of 0.4 was reached. The culture was transferred to 25 °C and allowed to equilibrate for 30 min before inducing protein expression with 0.5 mM isopropyl- β -D-thiogalactopyranoside. Induction proceeded overnight at 25 °C. Protein expression was monitored by SDS-PAGE and/or Western blot. Cells were harvested by centrifugation at 3000 $\times$ g for 10 min at 25 °C. The periplasmic fraction was isolated by an osmotic shock protocol. Briefly, cells were resuspended in TES buffer (100 mM Tris-HCl, 5 mM EDTA, 20% sucrose) at 80 mL/g wet cell mass, shaken at 25 °C for 10 min then centrifuged at 3500 $\times$ g for 20 min. The supernatant was discarded and cells were resuspended in ice cold 5 mM Mg₂SO₄ at 80 mL/g wet cell mass, shaken on ice for 30 min then centrifuged at 4000 $\times$ g for 20 min. The supernatant was decanted and passed through a 0.45 μ m filter. The sample was then lyophilized prior to purification. The lyophilized periplasmic fraction was resuspended in 1/10th of the original culture volume in 0.2% aqueous trifluoroacetic acid (TFA). Insoluble material was removed by centrifugation at 20 000 $\times$ g and passed through a 0.2 μ m filter. Analytical RP-HPLC was performed using a Zorbax 300SB-C8 column (2.1 mm $\times$ 15 cm, 5 μ m particle size, 300 Å pore size) (Agilent Technologies) to determine retention time using a linear AB gradient (Linear 2% B/min gradient to 80% at a flow rate of 0.3 mL/min, where eluent A is 0.2% aq. TFA and eluent B is 0.18% TFA in acetonitrile). The peak in the elution profile corresponding to the PAK monomeric pilin protein was identified by the analysis of fractions using SDS-PAGE. Retention time was 22.5 min, corresponding to approximately 45% acetonitrile for elution. A preparative RP-HPLC at a flow rate of 2 mL/min on a semi-preparative column described above was used (a linear 2% B/min gradient to 30% B, followed by a linear gradient of 0.1% B per minute up to 50% B where eluent A is 0.2% aq. TFA and eluent B is 0.18% TFA in acetonitrile). Fractions were collected over 1 min intervals. The presence of protein was determined by analytical HPLC using a Zorbax 300SB-C8 column (15 cm $\times$ 2.1 mm i.d., 5 μ m particle size, 300 Å pore size) (Agilent Technologies). Pure fractions were analysed by SDS-PAGE by taking 5 μ L of the fraction and adding 5 μ L 0.5 M sodium phosphate, pH 8.0 and 3 μ L of 5 $\times$ SDS-PAGE sample buffer. Fractions found to be pure by analytical RP-HPLC and SDS-PAGE were pooled and the identity of the protein was confirmed by electrospray-TOF mass spectrometry. The pooled fractions were lyophilized

repeatedly to remove acetonitrile and TFA. Protein was resuspended in water and extensively dialysed against PBS to ensure all TFA was removed.

PAK pilin DNA construct with a scrambled receptor binding domain

The scrambled PAK pilin gene was constructed by sequential PCR reactions using overlapping downstream oligonucleotides (Oligo1, 5'-CTGTTTAAATCATCCGCTTTCCAGAGACCATCAGCTGC; Oligo2, 5'-GCTGCTTTTTTCCTGGGTGCCCTGTTTAAATCATCCGC; Oligo3, 5'-ACAAAATTAAATCGGCGCGCTGCTGCTTTTTTCCTGGGT; Oligo4, 5'-ATTGGCCAAGCTTCTTACAAAATTAAATCGGCGC) to build the scrambled RBD sequence from the PAK/pRLD plasmid. An upstream primer corresponding to the DNA sequence of the OmpA (5'-GCT ATC GCG ATT GCA GTG GCA) for each of the four PCR reactions. *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) was used according to the manufacturer's standard PCR protocol. PCR products were purified on a 2% agarose gel and extracted with a QIAEX II gel extraction kit (Qiagen, Valencia, CA, USA). The PCR product was then digested with *Bam*HI and *Hind*III restriction endonucleases (New England Biolabs, Beverly, MA, USA) and ligated into predigested pGEX-2T vector (GE Healthcare, Piscataway, NJ, USA) and transformed into chemically competent BL21(DE3) *E. coli* cells. The construct was confirmed by DNA sequencing.

Scrambled PAK pilin was expressed with an N-terminal glutathione-S-transferase (GST) tag in the same manner as described for the monomeric PAK pilin protein using the pRLD vector. Cells were resuspended in 20 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, pH 7.5 and disrupted by sonication. Cellular debris was removed by centrifugation for 20 min at 20 000 × g. The supernatant was removed and passed through at 0.2 μm filter. Glutathione-sepharose (GE Health Sciences) was prepared and purification was performed according to the manufacturer's instructions. Purified protein was dialysed against PBS overnight at 4 °C.

Amino acid analysis

Peptide and protein concentrations were determined by amino acid analysis by acid hydrolysis of the peptide or protein in 6 N HCl for 24 h at 110 °C and dried under vacuum. Samples were resuspended in sodium diluent, pH 2.2 (Pickering Laboratories, Mountain View, CA, USA) and were analysed on a Beckman 6300 amino acid analyzer (Beckman-Coulter, Fullerton, CA, USA).

Immunization protocols

Animal work was carried out at the Center for Comparative Medicine at the University of Colorado Health Sciences Center, now the University of Colorado Denver, School of Medicine, according to approved Institutional Animal Care and Use Committee (IACUC) protocols. Groups of ten BALB/c mice, 3–4 weeks of age (Jackson Laboratories, West Grove, PA, USA) were used for each immunogen. Peptide-conjugate injections were prepared with conjugate containing 50 μg of peptide-conjugate in 0.1 mL PBS. Pilin protein injections were prepared with 50 μg of protein in 0.1 mL PBS. Based on our analysis of the peptide-KLH conjugate, this resulted in immunization with equal molar amounts of either synthetic peptide or pilin protein. The immunogen solutions were emulsified with an equal volume of adjuvant. Initial immunizations were prepared in Freund's complete adjuvant, followed by Freund's incomplete adjuvant for subsequent injections and alternated between subcutaneous and intraperitoneal sites (16). Mice were injected with 0.2 mL of the emulsion in 2 week intervals for a total of four injections. Preimmune serum was collected prior to immunization and serum was collected following the third and four injections.

Indirect ELISA

High-binding polystyrene 96-well immunoassay plates (Costar 3590, Corning, NY, USA) were coated with 100 μ L of a 0.1 μ M solution of pilin protein or synthetic peptide-BSA conjugate in 100 mM sodium carbonate/sodium bicarbonate buffer, pH 9.5 overnight at 4 °C. Plates were washed three times with PBS-T (pH 7.4, 0.05% Tween-20). Excess binding sites were blocked with 5% BSA in PBS-T for 1 h at 37 °C. Plates were washed again three times before adding 100 μ L of serially diluted mouse serum in PBS-T to each well. The dilutions ranged from 1/1000 to 1/1000 000. Plates were incubated for 1 h at 37 °C. Plates were washed three times before adding 100 μ L/well of a 1/5000 dilution of anti-mouse IgG horseradish peroxidase (HRP) conjugate (Jackson Immunolaboratory). The plates were incubated for 1 h at 37 °C. Plates were washed three times with PBS-T. 100 μ L of a 1 mM solution of 2,2'-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma-Aldrich) in 10 mM sodium citrate pH 4.2 with 0.03% H₂O₂ was incubated for 30 min with shaking. Plates were read on a SpectraMax 386 Plus plate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Binding curves were fit to a four-parameter logistic curve using SigmaPlot 7.0 (SPSS Inc., Chicago, IL, USA) to determine curve mid-points.

Competitive ELISA

High-binding polystyrene 96-well immunoassay plates (Costar 3590, Corning) were coated with 100 μ L/well of a 20 ng/mL solution of PAK pilin protein in 100 mM sodium carbonate/sodium bicarbonate buffer, pH 9.5 overnight at 4 °C. Plates were washed three times with PBS-T (pH 7.4, 0.05% Tween-20). Excess binding sites were blocked with 5% BSA in PBS-T for 1 h at 37 °C. Based on the indirect ELISA, the serum dilution that resulted 50–70% saturation of the pilin-coated surface was identified for each mouse. These fixed dilutions were used in the competition experiments. For the competition experiments, the primary antibody at the dilution determined above was preincubated with serial dilutions of the competitor (peptide or PAK pilin protein) in PBS-T with 0.5% BSA for 2 h at 37 °C. Then, 50 μ L of each antibody/competitor solution was added to the pilin-coated ELISA plates and incubated for 1 h at 37 °C. Plates were washed three times before adding 100 μ L/well of a 1/10 000 dilution of anti-mouse IgG (HRP) conjugate. After thorough washing, 50 μ L of Ultra-TMB (3,3',5,5'-tetramethylbenzidine, Pierce, Rockford, IL, USA) was added to each plate for quantitation and developed according to the manufacturer's instructions. The ELISA plates were read on a plate reader at 450 nm. Data were normalized by dividing the raw absorbance data by the absorbance of wells without competitors. The resulting curves were fit to a modified four parameter logistic binding model using SigmaPlot 7.0 (SPSS Inc.).

The PAK pilin construct with the scrambled RBD was used in indirect ELISA and competitive ELISA experiments to characterize RBD-specific antibodies within the anti-pilin sera. The dilution of scrambled PAK pilin for use in the assay was determined by titration of scrambled pilin protein in an indirect ELISA experiment to determine the dilution of scrambled pilin that was required to block all non-RBD-specific binding to a pilin protein-coated plate. Serial dilutions of a stock scrambled PAK pilin solution (~2 mg/mL) ranging from 1:2 to 1:500 were preincubated with mouse serum dilutions that had been prepared as for a normal indirect ELISA titer. These solutions were added to PAK pilin-coated ELISA plates that had been blocked with BSA to produce a series of titer curves, each corresponding to a fixed dilution of scrambled PAK pilin. As the dilution increased (lower concentrations of the scrambled pilin), the scrambled pilin construct in solution could not completely block binding of the non-RBD-specific antibodies. The maximum dilution (lowest concentration) of scrambled pilin at which there was no increase in binding to the coated plate was identified and then a 8-fold higher concentration (~80 μ g/mL) of scrambled pilin was used as the fixed dilution for the remaining experiments.

For the indirect ELISA experiments using the scrambled RBD pilin, serum dilutions were preincubated with the scrambled pilin protein in 0.5% BSA/PBS-T for 30 min at 25 °C. These preincubated solutions were then added to the antigen coated and blocked plates and the indirect ELISA was carried out as described previously. In the competitive ELISA, the appropriate antibody dilution (as determined by the indirect ELISA using the scrambled protein) was preincubated with the scrambled pilin protein and 0.5% BSA-PBS-T for 30 min at 25 °C. The pilin or peptide competitor was then added to this preincubated solution at appropriate concentrations and incubated for 1 h at 25 °C. The competitive ELISA was then carried out as described previously.

Results

Immunization

Active immunization studies were designed to examine polyclonal sera generated in response to a synthetic peptide-conjugate and to its cognate pilin protein. The two immunogens used in this study were derived from the native *P. aeruginosa* PAK strain (Figure 2). The 17-residue disulfide-bridged peptide immunogen was conjugated to the carrier protein, KLH, as described. The truncated pilin protein immunogen was expressed as a soluble monomer in *E. coli* (9). Two groups of ten BALB/c mice were used in experiments. In the peptide-conjugate group, one mouse died during the test bleed after the second injection. All other mice tolerated injections well with no evidence of adverse local or systemic reactions.

Response

Mice immunized with the PAK pilin protein (29–144) and PAK peptide (128–144)-KLH conjugate showed strong responses to both immunogens. Preimmune sera showed no binding to the peptide-conjugate or pilin protein by indirect ELISA and dot blot in all mice (data not shown). Mice immunized with pilin protein generated an IgG response with a geometric mean mid-point titer against the PAK pilin protein of 1.4×10^4 (Figure 3, panel C). Eight of 10 mice generated an IgG response that recognized the PAK peptide (128–144)-BSA conjugate (Figure 3, panel A). The two remaining mice showed essentially no titer against the peptide-conjugate, suggesting few RBD-specific antibodies were present. In the eight mice that did have a measurable titer against the PAK peptide (128–144)-BSA conjugate, there was considerable variability, with mid-point titers ranging from 1.6×10^3 to 8.5×10^3 (Figure 3, panel A). This was in contrast to the mice immunized with the peptide-conjugate, in which the titers against both the PAK pilin protein (29–144) and the PAK peptide (128–144)-BSA conjugate were consistently high for all mice, as shown in Figure 3, panels B and D. In this group, the geometric mean mid-point titer was 8.8×10^4 and 1.0×10^5 against the PAK peptide-BSA conjugate and the PAK pilin protein, respectively.

These mid-point titer results show that while the PAK pilin protein generates an IgG response, a large proportion of these antibodies are directed against regions other than the RBD (residues 128–144, Table 1). On the other hand, animals immunized with the PAK peptide (128–144)-conjugate only generate antibodies against the RBD and these antibodies are able to recognize the peptide epitope as well as the pilin protein. These results suggest that since antisera raised against the peptide-conjugate are able to bind the native pilin protein, there is a clear advantage in using a synthetic peptide-conjugate as an immunogen over the monomeric pilin protein.

An important focus of this study was analysis of RBD-specific antibodies. To characterize RBD-specific antibodies within the anti-pilin sera, we created a pilin protein construct that lacked a functional RBD. Interestingly, the PAK pilin protein with a C-terminal truncation at residue 128 could not be expressed in *E. coli* alone or as a fusion with the E-coil tag, maltose binding protein, or glutathione-S-transferase (GST) at the N-terminal of the pilin sequence

(data not shown). Instead of a C-terminal truncation, the amino acid sequence of the RBD of the PAK pilin protein was scrambled (Figure 2) and expressed as a fusion protein with GST at the N-terminal of the pilin sequence. In this scrambled pilin protein, the cysteine residues (129 and 142) of the PAK RBD were replaced by alanine residues to prevent possible intra-chain disulfide bond formation and the sequence of the remaining residues (130–144) were shuffled. In this way, the overall amino acid composition of the RBD was maintained; however, it should not be recognized by antibodies raised against the RBD native sequence. Western blot analysis (Figure 4) shows that the pilin protein with scrambled RBD is recognized by an antibody that has specificity for the pilin protein, but a monoclonal antibody specific for the PAK RBD does not bind the PAK protein with scrambled RBD.

The pilin protein with scrambled RBD was used in excess in indirect ELISA assays as well as in competition ELISA assays to block binding of pilin-specific, but not RBD-specific antibodies to pilin protein coating the ELISA plate. Table 1 shows that in the presence of excess scrambled pilin protein, the mid-point titers of pilin antisera on a pilin-coated plate are dramatically reduced. Panel D of Figure 5 shows that at the dilution of scrambled PAK pilin protein used in these experiments, the remaining binding to the pilin protein on the ELISA plates can be accounted for by binding to the RBD. In the presence of 20 μ M free native sequence peptide (128–144) (open diamonds, panel D), the binding of serum from mouse 8 is reduced by approximately 25%. In the presence of the scrambled PAK pilin protein, binding of the serum is reduced by approximately two thirds (open triangles, panel D). When both the free PAK peptide and the scrambled PAK pilin protein are included with the serum dilutions, almost all of the binding to the PAK pilin on the ELISA plate is competitively inhibited (closed squares, panel D). When the scrambled PAK pilin protein is in excess, it does not inhibit binding of anti-peptide sera to PAK pilin protein, indicating that it does not block binding of RBD-specific sera to the pilin protein (not shown). These results demonstrate that at the concentration of scrambled PAK pilin protein used in these experiments, only binding of RBD-specific antibodies to the PAK pilin on the ELISA plate is being measured.

In most cases, the titer of anti-pilin sera that were preincubated with the scrambled pilin protein correlated with the titer against the PAK peptide-BSA conjugate, suggesting that experiments using either preincubation with the scrambled pilin or the peptide-conjugate surface reflected binding of RBD-specific antibodies. For example, the titer of serum from mouse number 12 was not measurable for the PAK peptide BSA-conjugate. Likewise, the titer of serum from mouse 12 (Figure 5, panel B) was not measurable when the scrambled PAK pilin protein is included in the ELISA buffer against PAK pilin protein on the plate showing that there were no antibodies generated to the RBD in this animal (this situation occurred in 1 out of 10 animals, Table 1). The average ratio of the other animals was 0.30 (8 out of 10 mice, excluding mice 7 and 12, Table 1), indicating that for these animals, the majority of the antibodies in the polyclonal sera were not RBD-specific. However, in one of the 10 mice, RBD-specific antibodies dominated the response to the pilin protein, where the ratio of the titers was 0.79 (Mouse 7, Figure 5, Table 1). Interestingly, mouse 15, which did not bind the PAK peptide-BSA conjugate (Figure 3), did bind PAK pilin in the presence of the scrambled pilin protein (Figure 5). Binding to PAK pilin in the presence of the scrambled pilin suggests that the antibody has specificity for the RBD in pilin even though it does not bind the PAK peptide-BSA conjugate. It is possible that antibodies in the serum from mouse 15 specifically recognize a conformation of the RBD that is distinct from the peptide conformation or that these antibodies also have specificity for a portion of the pilin that is spatially near the RBD in the intact pilin.

Affinity of antisera raised against peptide-conjugates compared with pilin proteins

The relative affinity of the antibodies for pilin was determined using a competitive ELISA assay. In this assay, pilin protein was coated on microtiter plates and a dilution series of pilin protein was used to inhibit binding of antibodies to the pilin surface in a concentration-dependent manner. For the antisera raised by immunization with pilin protein, the scrambled pilin construct was included in the dilutions at a fixed concentration to block binding of antibodies that had no specificity for the RBD. As shown in Figure 6, panel A in the presence of the scrambled pilin, the native pilin protein in solution completely inhibits binding of anti-pilin serum to the pilin surface in a concentration-dependent manner. The IC_{50} values for these PAK pilin antisera range from 80 to 720 nM. The geometric mean IC_{50} for PAK pilin antibodies is 220 nM (Table 2). The variance of the IC_{50} values of antibodies raised against PAK pilin is less than the variance of antibodies raised against the PAK peptide KLH conjugate, as shown in Figure 6 (compare panels A and B). For the PAK peptide antisera, the IC_{50} values ranged from 13 to 330 nM. The geometric mean IC_{50} for the PAK peptide mouse antisera was 64 nM (Table 2). The IC_{50} of the mice immunized with PAK pilin was statistically greater than the IC_{50} of mice immunized with the PAK peptide KLH conjugate ($t = 2.51$, $df = 15$, $p = 0.024$).

The relative affinities of the anti-pilin and anti-peptide mouse sera for the free PAK peptide were also assessed by the competitive ELISA assay using PAK pilin-coated ELISA plates. Figure 6 shows the inhibition curves for the mice immunized with PAK pilin (panel C) and the PAK peptide-conjugate (panel D). The PAK peptide was able to competitively inhibit binding of the anti-pilin antisera in the presence of the scrambled PAK pilin protein (panel C). The geometric mean IC_{50} value for the anti-pilin sera was 2.2 nM (Table 2). The PAK peptide was also able to competitively inhibit binding of the anti-peptide sera with very high affinity (panel D). The geometric mean IC_{50} value for the anti-peptide sera was 0.46 nM (Table 2). As with the IC_{50} values for the PAK pilin competitor (Table 2), these data show that the anti-peptide sera have significantly higher relative affinity for the PAK epitope than the anti-pilin sera ($t = 4.51$, $df = 15$, $p = 0.001$). These data also show that the relative affinities of both the anti-pilin and anti-peptide antisera for the peptide are much higher than the affinities of both groups of antisera for the pilin protein Table 2. For the peptide antisera, the IC_{50} for the pilin is nearly 140 times greater than the IC_{50} for PAK peptide. For the pilin antisera, the IC_{50} for pilin is approximately 100 times greater than the IC_{50} for PAK peptide. This trend was expected for anti-peptide sera, but not expected for the anti-pilin sera.

Discussion

We have shown that there are major advantages of using a synthetic peptide rather than protein immunogen for an anti-pilus vaccine for *P. aeruginosa*. While we were able to achieve high anti-pilin titers with both immunogens in mice, the majority of antibodies raised against the pilin protein immunogen were not specific for the RBD (Table 1). In mice immunized with the pilin protein, titers against the peptide-BSA conjugate were 60–400 times lower than the titers against the pilin protein. Including the pilin protein with a scrambled RBD in the ELISA resulted in an average of 70% inhibition of antibody binding to the pilin protein. While the ELISA used to measure the titers reflects a combination of concentration and affinity of the antibodies in the polyclonal antisera, together these results suggest that the majority of the antibodies generated are not RBD-specific (Table 1). Previous studies have shown that binding of the type IV pilus can be inhibited by synthetic peptides corresponding to the C-terminal disulfide loop, but not by peptides corresponding to other regions of the pilus. Taken together, these results suggest that while the pilin protein is immunogenic, it is not an ideal vaccine candidate because relatively few of the antibodies raised against it are RBD-specific.

Animals immunized with the synthetic peptide-conjugate also had high titers against purified PAK pilin protein and these titers correlated with the titers against the peptide BSA-conjugate

(not shown). This shows that peptides corresponding to the RBD can be used as immunogens and the resulting antibodies recognize the epitope in the intact pilin protein. This is significant because antibodies raised against the RBD are known to block pilus-mediated adhesion. Our results clearly show that animals immunized with the synthetic peptide-KLH conjugate have significantly more RBD-specific antibodies than the animals immunized with the pilin protein.

Affinity of antibodies raised against PAK peptide KLH-conjugate and PAK pilin protein

As discussed previously, serum titers can reflect both the affinity and concentration of antibodies present in whole serum. Another focus of this study was to determine whether antibodies raised against the pilin protein differed from antibodies raised against the peptide-conjugate. It is reasonable to assume that the peptide may present a conformationally different epitope to the immune system in comparison to the RBD of the intact pilin protein. Thus, antibodies raised against the peptide may have a lower affinity for the RBD of the intact pilin protein than antibodies raised against the pilin protein itself. In order to determine whether peptide antibodies bind differently than pilin protein antibodies, we developed a method to measure binding to the RBD of the intact pilin protein. A pilin protein with a scrambled RBD was generated to block antibodies that were specific to regions of the pilin protein other than the RBD.

A competitive ELISA assay was used to determine the relative affinities of the antisera for the intact RBD where the anti-pilin antisera were preincubated with an excess of scrambled pilin protein. To our surprise, we found that the anti-peptide antisera had a higher affinity for the intact RBD of the pilin protein than anti-pilin antisera. As shown in Figure 6 and Table 2, antisera from mice immunized with the peptide-conjugate had statistically significant lower geometric mean IC_{50} than mice immunized with the pilin protein. The IC_{50} is inversely related to affinity, which indicates that the peptide antisera have a higher affinity for pilin protein than animals immunized with pilin protein. This is a significant finding because we expect the higher affinity of the anti-peptide antibodies will make them more effective at neutralization of pilus-mediated adhesion.

Other studies of anti-peptide antibodies have raised criticisms of peptides as immunogens. Some studies have shown that the conformation of peptides in complex with anti-peptide antibodies do not resemble the conformation of the same epitope in the cognate, intact protein (17–20). The discrepancy between the native protein conformation and the conformation of the peptide in complex with the antibody is attributed to peptide flexibility. Whereas the equivalent epitope is stabilized by secondary and tertiary interactions in an intact protein, a peptide immunogen has relatively high flexibility. This can give rise to an anti-peptide antibody that recognizes a non-native conformation in two ways. First, the peptide can exist, in solution, in one or many different conformations other than a native-like conformation. If the peptide has a stable conformation in solution that is different than the native conformation, antibodies may be raised against this conformation. Alternatively, high peptide flexibility can allow for an induced-fit mode of antibody recognition, in which the peptide adopts a non-native conformation upon binding an antibody. Either of these scenarios can give rise to the observation of a non-native peptide conformation in an anti-peptide antibody. This can also give rise to the observation that anti-peptide antibodies have significantly higher affinities for the peptide immunogen than the cognate intact protein. In these instances, the peptides were not constrained, which increases the chance that they exist in a non-native conformation. In the case described here, the synthetic peptide immunogen is constrained by the disulfide bridge and the two β -turns which maintain the conformation of the RBD observed in the pilin protein.

We have shown that while the anti-peptide sera have a much higher relative affinity for the peptide than the pilin protein, the same is true for the anti-pilin sera (Table 2). The IC_{50} of the anti-peptide sera from mice for PAK pilin was 140 times greater than the IC_{50} of the same sera

for PAK peptide. Likewise, the IC_{50} of the anti-pilin sera for PAK pilin was 100 times greater than the IC_{50} of the same sera for PAK peptide. While the difference in affinity of antibodies for peptide versus protein has been considered a drawback of using peptide immunogens, our data shows that this difference in affinity is also seen in anti-pilin protein antibodies. Thus, this peptide vaccine produces antibodies with desirable properties.

This problem of anti-peptide antibodies recognizing non-native conformations is minimal in our peptide immunogen for two main reasons. The observation of non-native conformation is attributed to peptide flexibility. In the RBD peptide immunogen, conformational entropy has been drastically reduced as a result of cyclization through the disulfide bond. This conformational constraint eliminates the possibility that the peptide exists in an extended conformation. In addition to cyclization, the RBD peptides are known to be well-structured in solution. NMR studies of PAK, PAO, KB7 and P1 peptides demonstrated the formation of two β -turns (8,21). In contrast, many protein epitopes, when made as peptides, adopt random coil conformations. The presence of β -turns in the synthetic peptide immunogen of the RBD is also advantageous for immunogenicity because β -turns are the most frequently observed structural feature at the interface of an antibody-antigen complex. Based on these features of this peptide immunogen, it is understandable why we were able to successfully generate anti-peptide antibodies that recognize the native pilin protein.

Conclusions and future directions

In this study, we have demonstrated that a synthetic peptide vaccine is superior to a pilin protein-based vaccine. This conclusion is based on our findings that immunization with the peptide KLH-conjugate can generate high titer antisera that recognize the RBD region of the native PAK pilin protein. On the other hand, immunization with the pilin protein generates antisera with much weaker titers against the RBD region of the pilin protein. Remarkably, antibodies raised in response to the peptide-conjugate appear to have equal or higher affinity for the RBD region than antibodies raised in response to the PAK pilin protein. We expect that the higher affinity anti-peptide antibodies will be more effective at neutralization of pilus-mediated adhesion. These findings show that our disulfide-bridged synthetic peptide vaccine targeting the RBD region of the type IV pilus of *P. aeruginosa* should be more effective than a pilin protein-based vaccine.

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Abbreviations

RBD, Receptor binding domain; KLH, Keyhole limpet hemocyanin; BSA, Bovine serum albumin.

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Figure 1.

Panel (A) Ribbon diagram showing the structure of PAK monomeric pilin (residues 29–144, PDB ID: 1DZO). The receptor binding domain is highlighted in magenta, showing the disulfide bond between residues 129 and 142 in green. Panel (B) Comparison of the conformation of the PAK strain receptor binding domain (RBD, residues 128–144) as part of the pilin protein (cyan) and as a free peptide in solution (magenta, PDB ID: 1NIL). The two chains are aligned to the type II beta-turn (residues 139–142). The disulfide bond is yellow.

	29		67
Scramble PAK (29–144)	ARSEGASALASVNPLKTTVEEALSRGWSVKSGTGTEDAT		
PAK pilin (29–144)	ARSEGASALASVNPLKTTVEEALSRGWSVKSGTGTEDAT		
PAK peptide (128–144)	-----		
	68		106
Scramble PAK (29–144)	KKEVPLGVAADANKLGTIALKPDPADGTADITLTFTMGG		
PAK pilin (29–144)	KKEVPLGVAADANKLGTIALKPDPADGTADITLTFTMGG		
PAK peptide (128–144)	-----		
	107	128	144
Scramble PAK (29–144)	AGPKNKGKIIITLTRTAADGLW KADDFKQGTQEKSSAPI		
PAK pilin (29–144)	AGPKNKGKIIITLTRTAADGLW KCTSDQDEQFIPKGCSK		
PAK peptide (128–144)	----- KCTSDQDEQFIPKGCSK		

Figure 2.

Amino acid sequence alignment of truncated PAK pilin (29–144), the scrambled PAK pilin construct (29–144) with a scrambled receptor-binding domain, and PAK peptide (128–144) used in immunizations and binding studies. A disulfide bond is present between cysteine residues 129 and 142.

Figure 3. Indirect ELISA using antisera from mice immunized with either PAK pilin (residues 29–144) (A and C) or PAK peptide (residues 128–144)-KLH conjugate (B and D) binding to a microtiter plate coated with PAK peptide-BSA conjugate (A and B) or PAK pilin (C and D). Each curve corresponds to the titration curve from one mouse.

Figure 4.

Western blot demonstrating recognition of PAK pilin with a scrambled receptor binding domain 128–144 (RBD) protein by pilin-specific polyclonal sera, but not by a RBD-specific antibody. Purified GST, PAK pilin with a scrambled RBD, and PAK pilin were blotted with either polyclonal mouse anti-PAK pilin antisera or an anti-PAK (128–144) monoclonal antibody. The presence of GST was demonstrated by SDS-PAGE (not shown). The two bands observed in the scrambled pilin-GST and PAK pilin blots represent the monomer and dimer.

Figure 5.

Indirect ELISA binding curves of antisera raised to the PAK pilin protein demonstrating inhibition of binding by the PAK pilin protein with a scrambled RBD. Panel (A) Mouse 7. Panel (B) Mouse 12. Panel (C) Mouse 15. In panels (A–C), closed circles show serum dilutions without addition of PAK pilin protein with a scrambled RBD and open triangles show serum dilution PAK pilin with a scrambled RBD binding to PAK pilin. Panel (D) Mouse 8, closed circles show serum dilution only, open diamonds show serum with 20 μM PAK peptide (128–144), triangles show serum plus PAK pilin with scrambled RBD, closed squares show serum with PAK pilin with scrambled RBD and 20 μM PAK peptide.

Figure 6.

Competitive ELISA inhibition curves showing competition between PAK pilin (A and B) or PAK peptide (C and D) in solution and a PAK pilin-coated surface using anti-pilin sera (A and C) and anti-peptide sera (B and D). A fixed dilution of scrambled pilin was included in the competition experiments using anti-pilin sera (A and C) at a concentration high enough to eliminate all antibody binding to pilin other than the receptor binding domain. The N^α-acetylated synthetic peptide was used in the competition experiments.

Table 1

Anti-pilin (29–144) ELISA mid-point titers with and without preincubation with a fixed concentration of the pilin protein with a scrambled RBD

Immunogen	Mouse	Titer against PAK pilin (29–144)		Ratio ^a
		RBD-specific ^b	Whole pilin-specific ^c	
PAK pilin (29–144)	6	4400	20600	0.21
PAK pilin (29–144)	7	13400	16900	0.79
PAK pilin (29–144)	8	8900	48200	0.18
PAK pilin (29–144)	9	2900	6000	0.49
PAK pilin (29–144)	10	16800	36000	0.43
PAK pilin (29–144)	11	1300	8400	0.15
PAK pilin (29–144)	12	-	4100	0
PAK pilin (29–144)	13	8000	24400	0.33
PAK pilin (29–144)	14	7500	23200	0.32
PAK pilin (29–144)	15	7000	23500	0.3

^a Determined by calculating $\text{Titer}_{\text{RBD-specific}} / \text{Titer}_{\text{Whole pilin-specific}}$.

^b Antisera were preincubated with scrambled PAK pilin/BSA to block all non-RBD-specific antibodies prior to ELISA using the PAK pilin protein-coated plate.

^c Antisera were preincubated with BSA prior to ELISA using PAK pilin protein-coated plate.

Table 2

Summary of geometric mean IC_{50} values of competitors PAK pilin (29–144) and PAK peptide (128–144) for anti-peptide and anti-pilin polyclonal sera as determined by competitive ELISA using PAK pilin-coated plates

Antisera	Competitor	
	PAK pilin (29–144) (nM)	PAK peptide (128–144) (nM)
PAK pilin (29–144)	220	2.23
PAK peptide (128–144)-KLH	64	0.46