Immune response in mice and swine to DNA vaccines derived from the Pasteurella multocida toxin gene

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Abstract

DNA vaccines were constructed with either a 5′-truncated or full-length, genetically detoxified toxin gene from Pasteurella multocida and two different DNA vaccine vectors, distinguished by the presence or absence of a secretion signal sequence. Optimal PMT-specific antibody responses and spleen cell secretion of interferon-γ following immunization of mice were achieved with pMM4, the construct containing a signal sequence and encoding the entire toxin. Antibody responses were also induced in pigs immunized with pMM4 and levels increased significantly following booster injections and experimental infection with P. multocida. Significantly increased expression of interferon-γ was detected in only a small subset of pMM4-immunized pigs. This report documents, for the first time, the ability of a DNA vaccine to elicit immune responses to the P. multocida toxin in both mice and swine.

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1. Introduction

Pasteurella multocida is a widespread and costly pathogen responsible for both progressive atrophic rhinitis and pneumonia in swine [1]. The P. multocida toxin (PMT) is a primary virulence factor in atrophic rhinitis and toxin-specific antibody is known to play a major role in protection against the disease [1,2]. However, the toxin-specific antibody response induced by whole-cell vaccines is not optimal due to the low levels of PMT produced by P. multocida during growth. The toxicity of PMT and the difficulty and expense of large-scale purification and inactivation prevent the widespread use of naturally derived toxoid as a stand-alone vaccine or as an additive to existing vaccines.

Investigations characterizing the antigenic and functional domains of PMT provide information useful for rational design of recombinant vaccines that may offer enhanced protection against disease. The C-terminal portion of the toxin contains the catalytically active moiety [3–5] and also appears to be essential for maximal immunogenicity [6,7]. Nontoxic deletion derivatives have been obtained by eliminating sequence required for cell binding from the N-terminal region [3,4,6–8], some of which have been demonstrated to elicit protective antibody responses [6–8]. More recent data indicates epitopes within the N-terminal 487 amino acids may also contribute to protective immunity [8], suggesting that optimal approaches to the derivation of genetically detoxified PMT likely depend upon maximal preservation of native sequence. A major advance in this area was the discovery that mutation of a single amino acid, a cysteine at position 1165, eliminates biological activity in vitro, and toxicity in vivo, without apparent effect on other protein characteristics, including structure, cell binding, and antigenic epitopes [3–5]. Administration of recombinant nontoxicogenic PMT, created through substitution of both amino acids 1164

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and 1165, protected pigs against clinical signs and turbinate atrophy following challenge with native PMT [9]. Nonetheless, production of genetically detoxified PMT for use as a vaccine requires large-scale protein purification and concentration, a means for stabilization of the recombinant protein, and the use of adjuvants. DNA vaccine technology provides a practical alternative means by which vaccine efficacy may be improved. DNA vaccines are relatively easy and cheap to produce in large amounts, are capable of eliciting a broad range of responses, possess intrinsic adjuvant properties, do not require a cold chain or other special conditions for storage and distribution, and have shown promise experimentally in a variety of animal species, including those of agricultural importance [10,11]. The goal of the present study was to evaluate the ability of recombinant, genetically detoxified PMT DNA vaccines to elicit PMT-specific antibody and cell-mediated immune responses in the absence of toxicity. Both a 5′-truncated and a full-length PMT construct were compared, in two different eukaryotic expression vectors, in order to identify an optimal approach.

2. Materials and methods

2.1. DNA purification and sequencing

Chromosomal DNA from P. multocida used as the source of the gene coding for PMT, toxA, was prepared with a commercially available kit (Genta Systems, Minneapolis, MN). Column-purified plasmid DNA used for sequencing and subsequent in vitro manipulations was obtained as specified by the product insert (Wizard Plus Miniprep, Promega, Madison, WI). DNA for immunization was purified using EndoFree Plasmid Giga kits (Qiagen, Valencia, CA), as instructed by the manufacturer.

Cloned PCR amplicons were sequenced using fluorescence-based cycle sequencing with AmpliTaq and BigDye™ Terminators on an ABI 3700 sequencer, at the National Animal Disease Center Genomics Unit. Multiple clones were evaluated from which consensus sequences were derived, with a minimum of three reads and at least one in each direction, in order to rule out clones potentially containing rare polymerase misincorporations. Sequences were analyzed using Vector NTI Suite software (Invitrogen, Carlsbad, CA). The toxA DNA sequence for P. multocida strain 4533 was deposited in GenBank under accession number EF441531.

2.2. Bacterial strains, vectors, and plasmid construction

The eukaryotic expression vectors VR1020 [12] and VR1028 were obtained from Vical, Inc. (San Diego, CA). Both contain a kanamycin resistance selection marker and a cloning region for gene insertion located upstream of a polyadenylation termination sequence. Heterologous gene expression is under control of a highly efficient cytomegalovirus promoter. VR1020 additionally contains sequence immediately 5′ of the cloning region encoding the human tissue plasminogen activator (TPA) signal sequence. Insertion of a heterologous gene into the adjacent BamHI site creates an in-frame fusion with the TPA sequence.

PCR with the high fidelity Fji”Turbo polymerase (Stratagene, La Jolla, CA) was used to generate two amplicons from the toxigenic P. multocida swine isolate 4533 [13]: (1) a fragment of the toxA gene encoding amino acids 150–1285 (5′ΔtoxA) and (2) the entire PMT coding region with the exception of the start codon (which is provided by the expression vectors), comprising amino acids 2–1285 (wt, toxA). To facilitate subsequent cloning steps primers contained the recognition sequence for BamHI at their 5′ ends. Amplicons were purified using spin columns (Qiagen) and digested with BamHI.

The wild type toxA amplicon was ligated into pBlue-script II SK* (Stratagene) which had previously been digested with BamHI and treated with calf intestinal phosphatase. Following transformation into competent Escherichia coli XL1-Blue (Stratagene), insert-containing transformants were identified by growth of white colonies on LB agar containing 100 μg/ml ampicillin and previously spread with 100 μl each 2% X-gal in dimethylformamide and 10 mM IPTG. Agarose gel electrophoresis of BamHI-digested plasmid DNA demonstrated the presence of appropriately sized inserts. Following sequence analysis a single clone, designated pMM1, was utilized for mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s recommendations. Primers were designed to replace the G at base position 3495 (relative to the first position of the start codon) with a C, thereby altering the codon from TGT (cysteine) to TCT (serine). In keeping with the original report this mutation is referred to as C1165S [5]. A transformant with wild type strain 4533 toxA sequence containing the C1165S mutation was identified by sequencing and designated pMM3. The BamHI insert from pMM3 (toxA-C1165S) was gel-puriﬁed using SeaPlaque GTG agarose (Cambrex, Walkersville, MD) and a GeneClean Spin Kit (Bio101 Systems, Morgan Irvine, CA).

The 5′ΔtoxA amplicon and toxA-C1165S insert from pMM3 were each independently ligated to VR1020 or VR1028, which had previously been digested with BamHI and treated with calf intestinal phosphatase. Plasmids were transformed into competent E. coli XL1-Blue and transformants were identified by growth on LB agar containing 50 μg/ml kanamycin. Plasmid DNA was puriﬁed from multiple transformants for each construct and the orientation of the inserts was established by restriction enzyme analysis. VR1020 and VR1028 clones containing 5′ΔtoxA in the forward orientation were designated pPR1 and pPB10, respectively. VR1020 and VR1028 containing toxA-C1165S in the forward orientation were designated pMM4 and pMM5, respectively. Plasmid inserts and the adjacent 5′ region of the vectors were sequenced to conﬁrm proper reading frame and gene sequence.
Two experiments were carried out using 4–6-week-old female BALB/c mice. The initial experiment included 78 mice, 12 of which served as unimmunized controls and were euthanized just prior to the first set of injections. The remaining mice were randomly allotted to four groups of 16–17 each, all of which were injected in the right hind quadriceps with 50 μl of endotoxin-free PBS containing 50 μg of pPR1, VR1020 (vector control), pPB10, or VR1028 (vector control). Three weeks later six mice from each group were euthanized and the remainder were boosted with 50 μg of the appropriate plasmid. After 3 additional weeks, five or six of the mice from each group were euthanized and a boost of 50 μg of the appropriate plasmid was administered to those remaining. Three weeks later all remaining mice were euthanized. Tissue samples were aseptically removed from all and blood was collected by cardiac puncture.

A total of 72 mice were used in the second experiment, 10 of which were euthanized prior to the first set of injections. The remainder were grouped into four sets of 15–16 mice each and were immunized and boosted as in the first experiment with either pMM4, VR1020, pMM5, or VR1028. Blood and tissue were collected from all mice as in the first experiment. In both experiments all mice were observed daily for signs of illness and vaccine toxicity.

A single experiment was carried out in pigs. Forty piglets 2–3 weeks of age from a herd with high health status were transferred to an isolation facility at the National Animal Disease Center. Ten pigs each were placed randomly into four isolation rooms. Following a 1-week period of acclimation, five pigs selected at random in each room were injected intradermally with 0.5 ml of endotoxin-free PBS containing 50 μg of pPR1, VR1020 (vector control), pPB10, or VR1028 (vector control). Three weeks later six mice from each group were euthanized and the remainder were boosted with 50 μg of the appropriate plasmid. After 3 additional weeks, five or six of the mice from each group were euthanized and a boost of 50 μg of the appropriate plasmid was administered to those remaining. Three weeks later all remaining mice were euthanized. Tissue samples were aseptically removed from all and blood was collected by cardiac puncture.

The codon adaptation index [14] for the toxA gene was determined using the University of Maryland, Baltimore County, Biological Sciences CAI calculator (www.evolvingcode.net/codon/CAI_Calculator.php).

2.3. Immunization regimen

In the first mouse experiment samples of muscle, liver, and kidney were recovered at the time of euthanasia. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Five micron sections were cut, stained with hematoxylin and eosin, and examined by light microscopy. Sections from the quadriceps muscle chosen as the site of injection were processed for immunohistochemical analysis using a previously generated polyclonal pig anti-PMT antiserum [16] and a biotin/avidin-alkaline phosphatase detection system (Kirkegaard & Perry Laboratories, Inc.).

Only quadriceps muscle used as the site of injection was sampled from mice in the second experiment. Tissue was processed for histopathology and immunohistopathology as in the first mouse experiment.

2.4. Histopathology

In the first mouse experiment samples of muscle, liver, and kidney were recovered at the time of euthanasia. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Five micron sections were cut, stained with hematoxylin and eosin, and examined by light microscopy. Sections from the quadriceps muscle chosen as the site of injection were processed for immunohistochemical analysis using a previously generated polyclonal pig anti-PMT antiserum [16] and a biotin/avidin-alkaline phosphatase detection system (Kirkegaard & Perry Laboratories, Inc.).

Only quadriceps muscle used as the site of injection was sampled from mice in the second experiment. Tissue was processed for histopathology and immunohistopathology as in the first mouse experiment.

2.5. Measurement of PMT-specific antibody

Sera obtained at the time of bleeding were stored at −20°C until evaluation. Sera from all unimmunized mice in a single experiment were pooled and treated as a single sample, as were sera from mice injected with the same plasmid at each time point in each of the two experiments. Pig sera were evaluated individually. Sera were tested in triplicate at least twice, using a minimum of two dilutions ranging from 10−1 to 10−3.

A commercially obtained reagent kit (Kirkegaard & Perry Laboratories, Inc.) was used for detection of anti-PMT antibody by ELISA. Immulon 2 microtiter plates were coated with PMT purified from strain 4533 (List Biological Labs, Inc., 0.75 μg/well), diluted in kit Coating Buffer, at 4°C overnight. Remaining steps in the assay were carried out as recommended by the manufacturer. The secondary antibodies used were either anti-mouse IgG conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories Inc.; 1:10,000) or anti-pig IgG-Fc conjugated with horseradish peroxidase (Bethyl Laboratories, Inc.; 1:1000), as appropriate. Following addition of the kit substrate solution, plates were read every 15 min for 1 h at an absorbance of 405 nm. Data reported here were derived from readings after 30 min of development using sera diluted to 10−5. Results are depicted as the difference in the average A405 of the test group indicated as compared to the control group indicated (ΔA405). Student's two-tailed t-test was used to evaluate the statistical significance of differences in absorbance values between groups. Sera were defined as anti-PMT positive if a p-value < 0.05 was obtained as compared to the negative control group indicated. In other comparisons, a p-value < 0.05
was considered to indicate a statistically significant difference.

2.6. Effect of immunization on expression of interferon (IFN)-γ

PMT-specific secretion of IFN-γ from spleen mononuclear cells was used as a marker for cellular immune responses in mice. Mouse spleens were homogenized and mononuclear cells were isolated by density-gradient centrifugation [17]. Cells from all mice in the same experimental group were pooled for subsequent analysis. Mononuclear cells were cultured in vitro with heat-inactivated PMT (List Biological Labs, Inc.). Serial 2-fold dilutions of cell culture supernatants were tested in duplicate for secretion of interferon-γ by ELISA (Endogen), according to the manufacturer’s recommendations. Units of IFN-γ were calculated relative to levels in vector-immunized IFN-γ standard provided (Endogen). Differences in values between treatment groups were evaluated using a two-way ANOVA. Multiple means comparisons were conducted using Bonferroni’s test. For both methods of analysis, statistically significant differences were defined as comparisons with p-values < 0.05.

In pigs, the effect of immunization on induction of IFN-γ expression from peripheral blood mononuclear cells was used as a marker for cellular immune responses, as measured by real-time reverse transcriptase PCR. This approach provides a highly sensitive method suitable for serially obtained samples without the need for euthanasia [18,19]. Cells were isolated by density-gradient centrifugation, as previously described [20], from blood collected 26 days after primary immunization and 2 weeks after booster immunization. A commercially obtained kit (Qiagen) was used for purification of RNA. cDNA was synthesized with random primers (Invitrogen) and real-time PCR was carried out for detection of IFN-γ expression by SYBR Green, as described by the manufacturer (Applied Biosystems). Briefly, cDNA, SYBR green master mix, and primers (at a final concentration of 600 nM each) were combined in a volume of 20 μl and subjected to the following cycling conditions: 95 °C for 15 min, 45 cycles of 95 °C for 15 s followed by 50 °C for 1 min, and a final dissociation step. All samples were run in duplicate and were normalized based on expression of β-actin. Primer sequences were derived using Primer Express (Integrated DNA Technologies) and primer pair efficiency was confirmed using the method described by Livak and Schmittgen [21]. Primers used for detection of IFN-γ were: 5′-GCCATCGTGAACCTCATC-3′ (forward) and 5′-TCTGGGCTTTGGAACATAG-3′ (reverse). β-Actin primers were: 5′-CTCCTTCTTGGCGATGGA (forward) and 5′-CGACCTCTCATGATCGTTGGA-3′ (reverse). Levels of IFN-γ mRNA in cells obtained from pigs immunized with pMM4 were calculated relative to levels in vector-immunized controls using the 2^−ΔΔCT method [21]. Data were analyzed using a paired t-test; statistically significant differences were defined as comparisons with p-values < 0.05.

2.7. Detection of plasmid DNA in mouse muscle

PCR was used in an attempt to demonstrate the presence of vector or recombinant plasmids in mouse muscle tissue recovered from the site of injection. Extraction and purification of DNA from fixed, paraffin-embedded tissue sections was accomplished as reported previously [22]. PCR primer sets were designed to amplify either a 238 base pair fragment common to both VR1020 and VR1028 (forward: 5′-ATTCATTGTTGATTTGGCCTAGC-3′; reverse: 5′-TATGCCTCTTCCGACCATCAAGCA-3′) or a 161 base pair fragment of toxA (forward: 5′-TGTTGGAGTCAGGCGAATA-3′; reverse, 5′-GCGATGTCAGTAAACACGAGGC-3′). Primers specific for a 200 base pair segment of the mouse β-actin gene (forward, 5′-TGTTAGGGTTGGGAATGGTCAGA-3′; reverse, 5′-TGTTGGGCGCAGATCTTCATGAT-3′) were used in positive control PCRs. Cycling conditions were as described [22] except that the optimal annealing temperature for each primer set was chosen based on the results of preliminary temperature gradient PCRs with purified vector or recombinant plasmid DNA. PCR products were analyzed by agarose gel electrophoresis in 3:1 NuSieve (Cambrex BioScience Rockland, Inc., Rockland, ME, USA) containing 0.5 μg/ml ethidium bromide.

2.8. Analysis of swabs and tissue specimens

Nasal swabs were plated on 10% sheep’s blood agar plates containing either 20 μg/ml penicillin, 10 μg/ml amphotericin B, 10 μg/ml streptomycin, and 10 μg/ml spectinomycin for selective isolation of B. bronchiseptica or 2 μg/ml amikacin, 4 μg/ml vancomycin, and 4 μg/ml amphotericin B for isolation of P. multocida.

At the time of euthanasia snouts were transversely sectioned at the first premolar tooth and examined visually for evidence of turbinate atrophy and nasal septum deviation. Samples of tonsil and turbinate retrieved from each pig were weighed and homogenized in a volume of sterile PBS sufficient to generate a 10% slurry (w/v). One hundred microliters of each homogenate were cultured in duplicate on plates selective for B. bronchiseptica and P. multocida, as described above. The dilution and volume used permits detection at the level of ≥100 cfu/g of tissue.

The identity of representative suspect colonies arising from swabs and tissues was confirmed by PCR analysis [23].

2.9. Sera from bacterin-immunized swine

Sera from three pregnant sows used in an unrelated study (designated 8366, 8532, and 8638), were obtained for comparison with sera of pigs in the study reported here using the PMT ELISA described above. The pregnant sows were previously immunized with a commercially available atrophic rhinitis vaccine containing a toxigenic P. multocida bacterin (Boehringer Ingelheim Vetmedica), according to the manufacturer’s recommendations. In that study, it was...
demonstrated that the sows provided protection to their offspring against atrophic rhinitis following challenge with *B. bronchiseptica* and toxigenic *P. multocida* based on piglet turbinate scores from snouts transversely sectioned at the first premolar tooth (personal communication, K. Lechtenberg, Midwest Veterinary Research). As described previously [24], the scoring system assigns a value ranging from 0 (no abnormalities) to 18 (complete atrophy of the turbinates with severe septal deviation) to each snout section. Average turbinate scores of challenged piglets from sows 8366, 8532, and 8638 were 1.7, 5.0, and 2.0, respectively, as compared to an average turbinate score of 10.0 in challenged piglets of unimmunized control sows.

3. Results

3.1. Sequence of wild type toxA gene from *P. multocida* strain 4533

Prior to constructing the DNA vaccines, the toxA sequence of *P. multocida* strain 4533 was derived to establish whether it is representative of other known toxA sequences and to determine the overall frequency of polymorphisms. At present, the GenBank database contains seven complete toxA DNA sequences for six different isolates of *P. multocida* (two nonidentical sequences have been submitted from different clones of the isolate LFB3). Sequence alignment reveals 100% identity between 4533 and one of the two LFB3 sequences. All remaining sequences are unique and comprise, together with the 4533/LFB3 polymorph, seven DNA variants (data not shown). As compared to the first sequence to be deposited in GenBank, for isolate DVI NCTC 12178 [25], the variants exhibit 4–11 base pair substitutions. At base pair positions 910 and 2323 (numbering the first position of the start codon as 1) all isolates have a two-base inversion relative to DVI NCTC 12178; all other substitutions are unique to individual isolates. The deduced amino acid sequences predict six variants of PMT, with two to seven amino acid substitutions relative to DVI NCTC 12178 (Fig. 1). The toxA gene of 4533 has a high degree of sequence identity with other isolates and is suitable for use as a DNA vaccine.

3.2. Immunogenicity of DNA vaccines in mice

In the first mouse experiment, the serum pools from mice receiving a single injection of either pPR1 or pPB10 were positive for PMT-specific antibody (*p* = 0.004 versus the VR1020 group and *p* = 0.045 versus the VR1028 group, respectively). Unexpectedly, for both vaccine groups the ΔA405 of serum pools of mice boosted once or twice were indistinguishable from those boosted with vector plasmids (Fig. 2A). Sim-

![Fig. 1. Alignment of the deduced amino acid sequences derived from the complete *P. multocida* toxA sequences currently available. Dashes represent conserved amino acids; substitutions are indicated by the appropriate letter. Intervening sequence omitted from the alignment is identical for all isolates. The sequence shown for LFB3 is derived from the 2003 submission to GenBank. Sequence shown for 4533 is identical to that of the 1993 submission for LFB3.](image-url)
Fig. 2. Anti-PMT response of mice in the first (A) or second (B) experiment as measured by ELISA. Average $\Delta A_{405}$ values of the group indicated were calculated based on comparison to average values of a reagent-only negative control. Groups: (A) ( ) VR1020; ( ) pPR1; ( ) VR1028; ( ) pPB10; (B) ( ) VR1020; ( ) pMM4; ( ) VR1028; ( ) pMM5. Time points: (1) 3 weeks after the first injection, (2) 3 weeks after the second injection, (3) 3 weeks after the third injection.

ilarly, a significant increase in PMT-specific secretion of IFN-γ could be demonstrated following the first injection of either pPR1 or pPB10, but not after subsequent booster injections (Fig. 3A). Values obtained using PMT-stimulated cells from mice immunized with vector plasmids were not distinguishable from those obtained with unstimulated cells.

One explanation for the inability to sustain an immune response to PMT could be the occurrence in toxA of codons rarely or poorly used in the mouse. To compare codon usage in P. multocida with codon distribution in the BALB/c mouse, the codon adaptation index (CAI) was determined based on the genome-derived codon usage template of the BALB/c’s closest genetic match, Mus musculus domesticus (personal communication, The Jackson Laboratory, Bar Harbor, ME). A CAI of $\geq 0.5$ is indicative of acceptable codon distribution between the gene of interest and the host species (www.evolvingcode.net/codon/CAI_Calculator.php). Based on the obtained value of 0.641, expression of native toxA sequence in the mouse is not predicted to be problematic.

Alternatively, sequence missing from the amino terminus of the truncated PMT encoded by pPR1 and pPB10 may result in poor immunogenicity in vivo. Therefore, an additional DNA vaccine encoding the entire toxA open reading frame, but with a point mutation previously shown to inactivate enzymatic activity [3,5], was constructed in each of the plasmid vector backgrounds and tested in a second mouse experiment. The serum pools from mice injected one to three times with the VR1020-derived vaccine pMM4 were positive for PMT-specific antibody ($p \leq 0.046$ versus the VR1020 serum pool) and the response increased significantly after each booster injection ($p \leq 0.023$; Fig. 2B). The serum pools from mice injected with the VR1028-derived vaccine pMM5 were negative for antibody to PMT regardless of the number of injections given.

PMT-specific IFN-γ secretion from cells of mice immunized once with either pMM4 or pMM5 was higher than from unstimulated controls (Fig. 3B, time point 1), but the differences are not statistically significant. However, significant differences were apparent following both the first and second booster injections for the group receiving pMM4 (Fig. 3B, time points 2 and 3; $p \leq 0.01$) and after the second booster injection for the group receiving pMM5 (time point 3; $p < 0.001$). Values obtained using PMT-stimulated cells from mice immunized with vector plasmids were not distinguishable from those obtained with unstimulated cells. For both pMM4 and pMM5, only the second of the two booster injections elicited a significant increase in the ability to secrete IFN-γ as compared to levels detectable after the primary injections ($p < 0.01$). There was no difference between the amount of IFN-γ secreted by cells from mice immunized once with pMM4 versus pMM5, although significantly higher levels were detected in cells from mice immunized with pMM4.

Fig. 3. PMT-specific spleen cell secretion of IFN-γ following immunization of mice. Groups: (A) unstimulated ( ) or PMT-stimulated ( ) spleen cells of mice injected with pPR1; unstimulated ( ) or PMT-stimulated ( ) spleen cells of mice injected with pPB10 and (B) unstimulated ( ) or PMT-stimulated ( ) spleen cells of mice injected with pMM4; unstimulated ( ) or PMT-stimulated ( ) spleen cells of mice injected with pMM5. Time points: (1) 3 weeks after the first injection, (2) 3 weeks after the second injection, (3) 3 weeks after the third injection.
following both booster injections (Fig. 3B, time points 2 and 3; \( p < 0.049 \)).

3.3. Analysis of muscle tissue for PMT antigen and plasmid DNA

Expression of PMT antigen in muscle tissue from injection sites was not detected in any group from either the first or second mouse experiment following immunohistochemical analysis. Although an amplicon of the predicted size was obtained following PCR with primers specific for mouse \( \beta \)-actin using DNA extracted from paraffin-embedded tissues of all groups in both experiments, no amplicons were observed from any group when using toxA-specific or vector-specific primers (data not shown).

3.4. Immunogenicity in pigs

Based on comparison with pooled sera from all vector-immunized pigs, individual sera from 12/20 pigs given a single dose of pMM4 were positive for PMT antibody 26 days post-injection; 18/20 were positive 2 weeks after receiving a booster dose \(( p \leq 0.0007, \text{data not shown})\). However, absorbance values of pre-immune sera displayed considerable, and repeatable, pig-to-pig variation. Therefore, it was concluded that a comparison of absorbance values derived from pre-immunization versus post-immunization serum samples from a single pig was a more appropriate indicator of the response to immunization with pMM4. Using this approach, 10/20 pigs were defined as seropositive 26 days post-injection; 18/20 were positive 2 weeks after receiving a booster dose \(( p \leq 0.0002)\). Two groups could be distinguished based on the increase in \( \Delta A_{405} \) of serum obtained 2 weeks after the booster dose as compared to pre-immune serum: (1) a low-responder group with \( \Delta A_{405} \) values \( \leq 0.367 \) \((n=9, \text{average value } = 0.229)\) and (2) a high-responder group with \( \Delta A_{405} \) values \( \geq 0.478 \) \((n=11, \text{average value } = 1.013; \text{average increase } = 2.6 \times 10^{-3} \text{ versus low responders})\).

An effective DNA vaccine based on PMT should prime animals for a vigorous antibody response upon exposure to toxigenic \( P. \) multocida. To simulate such an event, all high responders and four of the low responders were inoculated with \( P. \) multocida strain 4533 approximately 3 weeks after the pMM4 boost. These pigs were also infected with \( B. \) bronchiseptica. There was a 1-fold increase in expression of IFN-\( \gamma \) mRNA from peripheral blood mononuclear cells of pigs immunized with pMM4 compared to pigs immunized with VR1020, both after primary and booster immunizations, but the difference is not statistically significant. However, significant increases were detectable in cells from 3/20 individuals in the pMM4 group following primary immunization (ranging from 2- to 3.5-fold). Significant increases were also observed in cells of the same three pigs, as well as a fourth, after booster immunizations (ranging from 4- to 15-fold increases). There was no apparent correlation between the level of anti-PMT elicited and the level of expression of IFN-\( \gamma \) mRNA elicited by immunization with pMM4.

3.5. Evaluation of DNA vaccine toxicity

All animals in all experiments remained outwardly healthy and vigorous with no evidence of PMT toxicity. No gross
pathological abnormalities were apparent in any animal at necropsy. Histopathological evaluation of tissues from mice in the first experiment revealed normal pathology in both the liver and kidney, two organs highly sensitive to the toxic effects of PMT.

3.6. Isolation of B. bronchiseptica and P. multocida from nasal swabs and tissues

Ten or fewer colonies of B. bronchiseptica were obtained from nasal swabs of several pigs prior to the first set of plasmid injections, including at least one in each isolation room, despite having procured animals from a high health status herd. As the experiment proceeded, infection spread to other pigs and only 3 of 40 remained culture negative at the time originally planned to inoculate pigs with B. bronchiseptica. A decision to continue the study was made, since all animals appeared vigorous and healthy, no clinical signs of disease or distress were noted at any time, and the presence of B. bronchiseptica is not expected to significantly affect the ability of pigs to mount an immune response to PMT. To ensure that all pigs were exposed to a level of B. bronchiseptica known to facilitate colonization by P. multocida, inoculation was carried out as originally planned. At no time prior to experimental infection was any pig culture positive for P. multocida. All animals experimentally infected with only B. bronchiseptica remained culture negative for P. multocida throughout the study.

At the time of euthanasia P. multocida was detectable in the turbinate and/or tonsil of 10/15 PMT seropositive, challenged pigs at levels ranging from 0.1 to \(5.477 \times 10^3\) cfu/g, with relatively low levels found in most. Since the quantity of P. multocida present in many pigs approaches the limit of detection, negative results for the remaining pigs in the group may reflect either an actual absence of the bacterium in the tissue at the time of sampling or the presence of low levels not detectable by our methods. P. multocida was isolated from the turbinate and/or tonsil of 15/15 challenged, vector-immunized pigs at levels ranging from 0.1 to \(9.35 \times 10^3\) cfu/g. The average cfu/g in both the tonsil and turbinate of vector-immunized pigs was higher than for PMT seropositive pigs, but the differences are not statistically significant. B. bronchiseptica was present in the tonsil and/or turbinate of only six pigs in each of the two groups at the close of the experiment (0.2–7.425 \(\times 10^3\) cfu/g), with no statistically significant difference between the groups. Since pigs in the study were inadvertently naturally infected with B. bronchiseptica, the low incidence and degree of colonization found at the time of euthanasia may reflect rapid clearance of the organisms following the subsequent, intentional infection.

As expected, given the age of the pigs at the time of P. multocida challenge and the relatively low level of colonization, no significant degree of turbinate atrophy was observed in any pig from any experimental group (data not shown).

3.7. Comparison of serum anti-PMT levels in bacterin-immunized and pMM4-immunized pigs

Although anti-PMT antibody responses were readily apparent in all pMM4-immunized pigs 2 weeks after the booster injections, it is unclear from our investigation whether the level of antibody present might be sufficient to provide protection against atrophic rhinitis. A comparison of anti-PMT levels between sera from three conventionally vaccinated pregnant sows, whose piglets were afforded protection against atrophic rhinitis, and sera from three pigs immunized with pMM4 was carried out to address this question. The pMM4-immunized pigs with the highest (pig 10), lowest (pig 20), and an intermediate level (pig 36) of anti-PMT 2 weeks after booster injections were selected for this comparison.

Only one of three sera from conventionally immunized pigs displayed a reproducible increase in absorbance as compared to matched pre-immunization samples, and the difference was not statistically significant (average \(\Delta A_{405} = 0.077\), \(p = 0.24\)). In contrast, all three sera from pigs immunized with pMM4 exhibited statistically significant increases in absorbance versus pre-immunization sera, with an average \(\Delta A_{405}\) value of 1.847, 0.832, and 0.263 for pigs 10, 36, and 20, respectively (\(p \leq 7.8 \times 10^{-6}\)).

4. Discussion

This report documents, for the first time, the ability of a DNA vaccine to elicit immune responses to PMT in both mice and swine. The demonstrated importance of antibody to PMT in protection against atrophic rhinitis suggests vaccines capable of inducing high titers in serum will provide maximum efficacy [1,2]. In fact, several investigators have previously reported that a vaccine containing a truncated PMT, purified from recombinant E. coli, induced high titers of toxin-specific antibody and provided protection against clinical signs and turbinate atrophy in pigs [26–28]. A limitation of conventional, whole-cell vaccines for P. multocida is the relatively low level of PMT-specific antibody induced by immunization, as observed in the present study with independently obtained sera. Similarly, infection of pigs with toxigenic P. multocida is accompanied by no, or a minimal, anti-PMT response [2,29]. These observations may be explained, at least in part, by the small amount of PMT produced during bacterial growth, estimated to comprise no more than 0.1–1% of total protein [2]. However, intranasal administration of higher levels of native PMT also elicits, at best, a poor antibody response [30–32]. In contrast, PMT-specific antibody is readily detectable following intramuscular injection of either native PMT [30] or genetically detoxified PMT containing a C1165S substitution [9], as encoded by the DNA vaccine used here. Therefore, the perception that PMT is a poor immunogen may have more to do with antigen load and route of administration than a true lack of antigenicity per se.
Currently available vaccines for atrophic rhinitis offer moderately good protection against disease but the ability to induce a more robust response to PMT is desirable. Bording et al. found a single-component vaccine containing only truncated toxin induced higher anti-PMT titers and was more efficacious than a conventional, multicomponent vaccine [26]. Based on direct comparison by ELISA, intradermal immunization with pMM4 DNA resulted in significantly higher levels of PMT-specific serum antibody than those associated with intramuscular immunization using a commercial bacterin. Although it is reasonable to hypothesize the serum antibody levels observed predict superior protection against challenge, our experiments do not address levels of colostral antibody provided to offspring, a critical component of protection. Only a challenge experiment which tests the ability of pMM4-immunized sows to protect offspring against atrophic rhinitis will provide direct evidence of the efficacy offered by this approach. Other investigators have proposed the use of purified, recombinant PMT or PMT fragments produced in E. coli as subunit vaccines and were able to demonstrate in pigs anti-PMT responses capable of protecting against the effects of subsequent administration of purified toxin [8,9] or challenge with toxigenic P. multocida [6,26–28]. The culture systems used permit production of the recombinant products at levels much higher than for naturally occurring PMT. Nonetheless, purification of the toxin from bacterial cultures is still required. DNA vaccine technology overcomes this limitation and eliminates the need for a cold chain, providing a more economical and practical means by which vaccine efficacy might be improved. Additionally, in contrast to previously proposed PMT subunit vaccines, the DNA vaccines described here elicited responses in both mice and pigs without the inclusion of an adjuvant.

As previously noted, considerable variation was observed in absorbance values of some pre-immune piglet sera. Consequently, the possibility that piglets with higher pre-immune serum absorbance values were previously infected with toxigenic P. multocida at a level insufficient for detection by culture, thereby priming them for secondary responses following subsequent administration of pMM4, must be considered. Nonetheless, several pieces of evidence suggest this to be highly unlikely. Piglets and sows used for this study were obtained from high health status herds, regularly monitored for atrophic rhinitis, and had no evidence of that or any other respiratory disease. We and other research groups routinely obtain animals for infection experiments from these herds and, over a period of many years, have never encountered any evidence of atrophic rhinitis and/or P. multocida in pigs or piglets. Additionally, if toxigenic P. multocida was present in numbers insufficient for detection by culture it seems unlikely that the very small amount of PMT to which piglets might have been exposed would be sufficient to induce an immune response amenable to boosting. In fact, as demonstrated here and in the work of others [2,29], it is difficult to detect any anti-PMT response in pigs exposed to or immunized with toxigenic P. multocida. Examination of the distribution of pMM4-immunized pigs within the groups defined as high and low responders shows that four of five pigs with the highest pre-immune ELISA values fall into the low-responder group. Additionally, the six pigs with the lowest pre-immune ELISA values are distributed evenly between low- and high-responder groups. The absence of an obvious correlation between pre-immune ELISA values and the anti-PMT response following injection with pMM4 strongly suggests that all pigs were immunologically naive with respect to PMT prior to immunization.

Our experiments in mice were designed to compare the responses obtained using two different vectors with identical toxA-derived inserts. The vector VR1020, used for vaccines pPR1 and pMM4, contains a signal sequence missing from VR1028 which promotes secretion of the gene product. Accordingly, vaccines utilizing VR1020 as a backbone are predicted to favor a humoral response. Results of the first mouse experiment are not consistent with this prediction, since an anti-PMT response was detected in mice immunized with either pPR1 or pPB10 and there was no statistically significant difference in ELISA absorbance values obtained from each group. However, the relatively low level of antibody elicited and the failure to maintain a response, despite booster injections, indicates the toxA sequence contained within these two vaccines does not code for a strongly immunogenic toxin fragment. When the entire toxA coding sequence was included in either the VR1020 or VR1028 vectors, an antibody response was detectable only in the group receiving the VR1020-based vaccine pMM4, even after repeated injections. Interestingly, although PMT-specific secretion of IFN-γ could be demonstrated in mice following multiple injections of either pMM4 or pMM5, significantly higher levels were obtained from cells of those receiving pMM4. Therefore, secretion of PMT appears advantageous for development of maximal immune responses to a DNA vaccine, at least in mice. It is unclear what role, if any, cell-mediated responses might play in protection against disease due to toxigenic P. multocida. In mice, it is known that specific cytokine responses are associated with the induction of particular classes and subclasses of antibodies. For example, the type I cytokine IFN-γ is associated with the production of IgG2a whereas type II cytokines, such as IL-4 and IL-13, are associated with IgG1 and IgE. In pigs, such an association is less clear since few reagents useful for identification of immunoglobulin classes and subclasses are available.

There is no obvious explanation for the inability to sustain an anti-PMT response in mice boosted with pPR1 or pPB10. Mapping of functional domains and antigenic epitopes predicts the truncated PMT encoded by these vaccines is likely to be enzymatically inactive yet retain immunogenicity [6–8]. Nonetheless, the absence of 149 amino acids from the amino terminus may negatively affect the formation of critical antigenic epitopes or protein stability. Efforts to demonstrate the presence of PMT antigen in the tissue of muscle serving as the injection site were unsuccessful. However, tissues were only collected for evaluation 3 weeks after each injection. Based
on the antibody and cell-mediated PMT-specific responses observed after the initial injection, at least some antigen production must have occurred in vivo. It may be that expression was not optimal, resulting in very low levels and/or transient production of antigen not detectable at the time of tissue sampling.

Although DNA vaccine approaches showing promise in rodent models do not always provide similar results in large animal models, we chose to evaluate only pMM4 in pigs given its clear superiority in mice. Our data do not rule out the possibility that the VR1028-based vaccine, pMM5, might also induce an antibody response in swine, but do establish the immunogenicity of pMM4 and provide a foundation for further studies designed to evaluate its ability to protect against disease.

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