Title: Evaluation of Nucleic Acid Delivery Methods for a Genetic (DNA) Vaccine against PRRS - **NPB# 98-025**

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Abstract.
The injection of recombinant DNA into an animal for vaccination purposes (i.e., naked DNA immunization) has great potential for controling the infectious diseases of swine. However, an issue that needs consideration is the optimal route and method of delivery of naked DNA for immunization in pigs. Therefore the aim of this project was to evaluate the efficacy of two different methods of DNA delivery to immunize pigs against PRRS virus. The two methods of administration were: (i) intradermal utilizing a gene gun and (ii) intramuscular via a needle and syringe. As a vaccine we utilized a mixture of two plasmids containing cDNA encoding for either PRRS virus glycoprotein GP4 or GP5. These were given twice at a 4-week interval. By four weeks after the second administration of the naked DNA only a very weak cellular immune response against PRRS virus had been induced regardless of the method of delivery. Likewise, a humoral immune response was not detectable when using the IDEXX PRRS ELISA. We reasoned that the poor performance of the DNA vaccine could be at least partly due to the death (apoptosis)-inducing effect of GP5, which would result in low expression of the introduced cDNAs. To circumvent this problem and achieve our goal of testing the efficacy of the two proposed methods of DNA immunization, we changed our strategy. Previous studies in our laboratory have shown that porcine interleukin-12 (poIL-12) can enhance the cellular immune response of pigs to a commercial PRRS modified live virus (MLV) vaccine. Thus, we tested the ability of poIL-12 cDNA introduced into pigs by either of the two proposed routes to enhance the cellular immune response to a PRRS MLV vaccine. When the poIL-12 cDNA was administered biolostically (gene gun) in conjunction with the intramuscular injection of PRRS MLV vaccine, the frequency of PRRS virus-specific interferon (IFN)- -secreting cells was three-fold greater than that found in pigs immunized with the PRRS MLV vaccine alone or in combination with poIL-12 cDNA injected into the muscle ($p<0.03$). However, in both cases, the negligible titer of virus-specific neutralizing antibodies induced by the MLV vaccine was not altered by the administration of poIL-12 cDNA. These results indicate that biolistic delivery of naked cDNA to pigs is more effective than intramuscular injection of the same entity. Moreover, the observed divergent humoral and cellular immune responses suggest that the development of virus neutralizing antibodies and of IFN- -secreting cells are independently regulated. In any case, poIL-12 cDNA, when administered via gene gun has the potential to

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be used as an adjuvant to enhance the poor cellular immune response stimulated by PRRS MLV vaccines.

Introduction. The effectiveness of currently available PRRS vaccines is the subject of continuous discussion and concern. Independent evaluation of the most commonly used commercial modified live virus **(MLV)** vaccine indicates that, at least under controlled laboratory conditions, this vaccine provides a suboptimal level of protective immunity (Osorio, 1998). Under field conditions there is controversy regarding the effectiveness of this vaccine, since apparently outbreaks of PRRS have occurred despite immunization (Mengeling et al., 1997). Clearly, a highly effective PRRS vaccine is urgently needed. To address this issue our laboratory has been investigating the characteristics of the immune response of pigs to PRRS virus. Our studies have shown that PRRS vaccines are unable to stimulate a strong cellular immune response or to induce the generation of significant titers of virus neutralizing antibodies (Meier et al., 2000). This phenomenon occurs despite the fact that a strong non-virus neutralizing antibody response against PRRS virus is detected very soon after vaccination. These observations indicate that although the vaccine is immunogenic (i.e., able to induce an immune response), for some reason there is a poor stimulation of an immune response capable of inhibiting virus replication (i.e., virus neutralizing antibodies and/or interferon (IFN)- -secreting T cells). Because of the economic importance of this disease and the apparent shortcomings of currently available vaccines, alternatives to current vaccines are being sought. Vaccine technology has advanced to the point that experimentally it is now possible to immunize pigs against **PRRS virus** by simply injecting a complementary copy of only part of the virus genome (nucleic acid) so that only one of the viral proteins is produced without the need of the viral infection. This so called **genetic or DNA vaccine,** represents a new way to induce immunity against a pathogen (Barry and Johnston, 1997). A single gene is prepared in the form of complementary deoxyribonucleic acid (cDNA) from the viral mRNA and is then inserted into an *eukaryotic expression vector*. This *expression vector* or **"plasmid"** enables the production of the selected viral protein by the cells of an animal without the need for the whole virus. The engineered *expression vector* is initially grown in bacteria, and then the purified plasmid is directly inoculated into the animal. As a result of the administration of the cDNA-containing vector, a protein(s) coded by this engineered nucleic acid is produced, and an immune response is elicited against the expressed protein. Currently, the most common ways of delivering a **DNA vaccine** to an animal is by either physical injection of the material with a syringe and needle or biolistic penetration using a **gene gun.** Interestingly, the route and method of delivery of DNA vaccines have been shown to influence the intensity and nature of the immune responses in mice and non-human primates (McCluskie et al., 1999; reviewed in Gurunathan et al., 2000). It has become evident that at least in mice the intradermal delivery of naked DNA with a gene gun is more efficient than either intramuscular or intradermal injection via a needle and syringe (Barry and Johnston, 1997; Bennett et al., 2000). In view of these differences, it is likely that the relative efficiencies of doses, routes and methods of immunization will vary between all species. Therefore, to optimize the method of DNA delivery, research on the target species has to be conducted. In this regard, a direct comparison between the efficacy of the gene gun technology and the injection of naked DNA with a needle and syringe in pigs has not been performed.

Objective. The aim of this project was to compare the efficacy of two different routes of DNA delivery at inducing an immune response to a PRRS virus DNA vaccine in pigs. The two routes compared were: intradermal utilizing a gene gun and intramuscular via a needle and syringe.

Procedures.. The plasmids comprising the DNA vaccine express either PRRS virus open reading frame 4 and 5, which encode viral glycoproteins GP4 and GP5, respectively. In addition to compare the relative vaccine potential of the two methods of DNA delivery, we

examined the ability of a third plasmid encoding for porcine interleukin-12 (poIL-12) to enhance the swine immune response to a commercial PRRS MLV vaccine.

Generation of PRRS ORF4 and ORF5 and porcine IL-12 expression vectors. Complementary DNA copies of PRRS virus strain VR2332 ORF 4 or 5 were directionally inserted into the mammalian expression vector pcDNA3.1 resulting in the production of pcDNA3.1ORF4 and pcDNA3.1ORF5 constructs (Kwang et al., 1999). Likewise, linked cDNAs encoding the p35 and p40 subunits of poIL-12 (provided by Dr. Dennis Foss, Univ. of Minnesota) were directionally cloned into pcDNA3 to produce the plasmid pcPIL4. Prior to immunization all plasmids were grown in *E. coli* DH5 and isolated using a Qiagen large scale plasmid purification kit. The expression of the recombinant PRRS virus proteins has been previously demonstrated by transfection of the respective plasmids into MARC-145 cells and immunofluorescence staining of the transfected cells with anti-PRRS antisera (Kwang et al., 1999). To verify that active porcine IL-12 could be produced from pcPIL4, Chinese hamster ovary (CHO) cells were transfected with this plasmid and the supernatents obtained at 48 hr post transfection from treated and untreated monolayers were tested in a IL-12 bioassay. Only supernates from CHO cells transfected with the pcPIL4 were found to have bioactive poIL-12.

DNA vaccination of pigs against PRRS virus. Eight week-old crossbred (Yorkshire x Landrace) PRRS seronegative pigs were randomly divided into three groups of 5 animals. Two groups received a 1:1 mixture of pcDNA3.1ORF4 and pcDNA3.1ORF5 twice at a 4 week interval in the following routes and doses: Group 1, a single intramuscular injection in the dorsal neck area (400 µg of DNA/injection); Group 2, gene gun (1 µg of DNA/shot, 10 shots per pig in the inguinal region). The third group served as a negative control and was not vaccinated. Blood samples were drawn just prior to the first vaccination to confirm the PRRS seronegative status of the animals. Then the animals were bled every 2 weeks thereafter to monitor cellular and humoral immune responses to the vaccination. The PRRS virus-specific immune response was evaluated by measuring the frequency of IFN- -secreting cells by ELISPOT (Zuckermann et al., 1998a) and the amount of antibodies by PRRS IDEXX ELISA. Statistical differences in the intensity of the immune response to different modes of vaccination were determined by Fisher's protected least significance difference (PLSD). The data was analyzed with the StatView program (Abacus Concepts).

Enhancing effect of porcine IL-12 cDNA on swine immunity to a PRRS vaccine. Ten week-old cross-bred PRRS seronegative pigs, were randomly assigned into five groups of five pigs each. Animals in four of these groups were vaccinated intramuscularly in the adductor muscle (inner thigh) with a PRRS MLV vaccine (Ingelvac PRRS MLV, Boehringer Ingelheim, Nobl). At the time of vaccination two of these groups also received the pcPIL4 plasmid containing the poIL-12 cDNA either by injection into the muscle or skin. Intramuscular injection was accomplished with a needle and syringe (200 µg of plasmid in a 1 ml volume of phosphate buffered saline, pH 7) at a location proximal to the vaccination site. For dermal delivery of the plasmid the Bio-Rad gene gun was used. Gold beads (average diameter of 5 µm) were loaded with 1 µg of pcPIL4 plasmid/0.5 mg of gold. Cartridges for the gene gun were then prepared with the Bio-Rad Tubing Prep Station by coating the Gold Coat tubing with 0.5 mg per cartridge of the plasmid-loaded gold particles according to the manufacturer's instructions. Each pig received the contents of ten cartridges, which were individually delivered into the skin covering the medial aspect of the pelvic limb at a discharge pressure of 350 PSI. The shots were given in an area closely surrounding the vaccination site. The third group of vaccinated

pigs was also injected with 1 ml of baculovirus-derived recombinant porcine (rpo)IL-12 (ENDOGEN) at an adjacent site. These pigs received 20 µg of rpoIL-12 on days 0, 2, 4 and 6 following vaccination. Only the MLV vaccine was administered to the fourth group, whereas the last group was used as unvaccinated controls. The vaccination and cytokine administration protocol was repeated in its entirety at 4 weeks after the first immunization. Plasma and mononuclear cells were obtained at biweekly intervals from peripheral venous blood of all pigs. These cells were used to monitor the development of the cellular PRRS-virus specific IFN- response as previously described (Meier et al., 2000).

Results and Discussion. This study was undertaken to compare the ability of two different delivery systems to induce an immune response to a PRRS virus DNA vaccine. The DNA vaccine was composed of a 1:1 mixture of plasmids containing cDNA encoding for the PRRS virus glycoproteins GP4 or GP5. The DNA was injected intramuscularly twice at a 4-week interval with a needle and syringe (total dose 400 µg of DNA) or intradermaly utilizing a gene gun (total dose 10 µg of DNA). These amounts were chosen based on our previous experience with intramuscular DNA injection (Kwang et al., 1999) and the known superior efficiency of the gene gun to stimulate an immune response to the protein encoded by the administered cDNA. As a positive control a group of pigs was only immunized with a conventional PRRS MLV vaccine. The development of humoral and cellular immunity was monitored for 8 weeks following the start of the vaccination protocol. As shown in Figure 1 immunization with the DNA vaccine did not induce a humoral immune response detectable by the IDEXX PRRS ELISA (Fig. 1). However, a weak cellular immune response could be detected at two weeks after the second immunization when using the highly sensitive IFN- ELISPOT assay (Fig. 2). No significant differences were observed between the magnitude of the immune response exhibited by the two groups of pigs receiving the DNA vaccine. In contrast, animals injected with the conventional PRRS MLV vaccine developed a strong antibody response within two weeks after immunization (Fig. 1). Interestingly, the second (booster) immunization did not cause the normally expected increase in antibody titer. A cellular immune response was also elicited by the MLV vaccine (Fig. 2) although even at its peak (2 weeks after the boost) it was fairly weak as compared to the intensity of the response normally elicited by immunization with a pseudorabies **(PrV)** MLV vaccine (Zuckermann et al., 1998a; Zuckermann et al., 1998b). This result confirms our observation that the cellular immune response to PRRS MLV vaccines is relatively minor (Meier et al., 1999). The magnitude of the response to the intramuscular injection of the DNA vaccine was similar in strength to that previously reported for intramuscular administration of plasmids encoding for either PRRS ORF 4, 5, 6 or 7 (Kwang et al., 1999). In that study the immune response to the DNA vaccine was also fairly low, however it was clear that cDNA encoding for GP4 and GP5 were promising candidates for a PRRS DNA vaccine. Based on preliminary studies with a PrV naked DNA vaccine (Zuckermann, unpublished observations), we had expected that the biolistic administration of the plasmids would result in a marked increase in the intensity of the immune response to the PRRS DNA vaccine. However, as we show here, this was not the case. Even the co-administration of a plasmid encoding poIL-12 as an adjuvant to the DNA vaccine failed to stimulate a significant immune response (data not shown). We reasoned that the poor performance of the DNA vaccine could be at least partly due to the death (apoptosis)-inducing effect of GP5 which would result in low expression of the introduced cDNA (Suarez et al, 1996). Our results are at odds with those of Pirzadeh and Dea (1998), where some success at immunizing pigs against PRRS with a DNA vaccine was reported. The reason for this discrepancy is unclear, although it can likely be attributed to differences in the plasmid constructs and their expression efficiency.

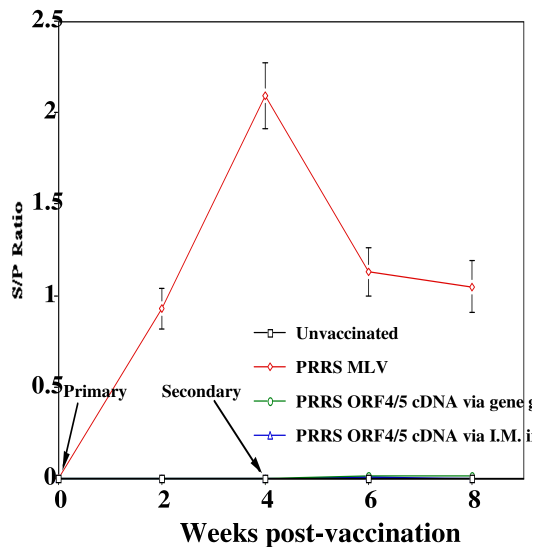

Figure 1. Humoral immune response following immunization with a conventional PRRS MLV vaccine or a DNA vaccine delivered by the indicated procedure. Antibody response was measured with the IDEXX PRRS ELISA. Immunization protocols are described in the Procedures section.

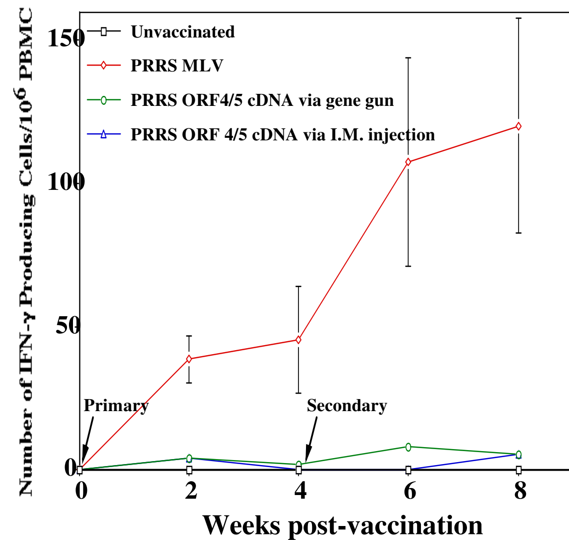

Figure 2. Cell-mediated immune response resulting from immunization with a conventional PRRS MLV vaccine or a DNA vaccine delivered by the indicated procedure. Vaccine-induced interferon- secreting memory T cells were enumerated with an ELISPOT assay. Treatment groups are the same as those in Fig. 1.

Because the poor immune response to the PRRS DNA vaccine precluded any conclusion, we changed our approach in order to achieve our goal of comparing the two methods of naked DNA delivery into pigs. The new methodology was based on the observation that when exogenous IL-12 is given in conjunction with a vaccine that stimulates a poor cellular immune response, such as an inactivated vaccine against PrV or a PRRS MLV vaccine, the strength of this response is enhanced (Zuckermann et al., 1998a; Zuckermann et al., 1998b; Zuckermann and Murtaugh, unpublished observations). Thus, we reasoned that that the administration of IL-12 in the form of expressible cDNA should also increase the intensity of the cell-mediated immune response to a PRRS MLV vaccine. Moreover, the enhancing effect should be directly proportional to the efficiency of the DNA delivery method.

Accordingly, four groups comprised of five PRRS seronegative pigs each were immunized with a commercial PRRS MLV vaccine and given simultaneously at an adjacent site either placebo (saline), rpoIL-12 protein, or a plasmid encoding for poIL-12 administered either intramuscularly with a needle and syringe or intradermaly with a gene gun. As a negative control, a group of pigs was not vaccinated. By two weeks post-immunization all of the vaccinated pigs had become seropositive based on the PRRS IDEXX ELISA (S/P ratio >0.7), and after an additional two weeks the S/P ratio increased to >1.5. In contrast the unvaccinated control pigs remained seronegative throughout the study (data not shown). In

contrast to the strong humoral response detected by the ELISA assay, the titer of virus-neutralizing antibodies ranged from low (1:4) to non-detectable (<1:4) in all vaccinated pigs. Thus, the administration of IL-12 in the form of either soluble protein or expressible cDNA had no effect on the extent of the virus-neutralizing response.

To measure the number of virus-specific memory T-cells induced by the PRRS MLV vaccine, and the relative ability of single cells to secrete IFN- , the IFN- ELISPOT assay was used. In this assay a blue spot whose size is proportional to the amount of cytokine secreted identifies the presence of a single IFN- -secreting cell. Thus, with this assay the frequency of virus-specific IFN- -secreting cells can be determined. Based on this method pigs immunized with the PRRS MLV vaccine exhibited a positive IFN- response by two weeks after treatment. However, the frequency of virus-specific memory T cells was surprisingly low (<70 per million mononuclear cells), and the size of the spots generated in response to stimulation with the PRRS virus antigens were relatively small as compared to those resulting from stimulation with the polyclonal activator PHA. For comparison, pigs immunized with a single dose of PrV MLV vaccine usually develop >300 IFN- -secreting cells/million mononuclear cells. Moreover, spots resulting from these cells are of similar quality as those obtained from PHA-stimulated T cells (for an example see www.cvm.uiuc.edu/faculty/zuckermann.html). Thus, the relative intensity of the cellular immune response to the PRRS MLV vaccine is clearly low. Co-administration of recombinant IL-12 with the vaccine had a slight enhancing effect on the response but it was not statistically significant. Similarly, the intramuscular injection of a plasmid with IL-12 cDNA also had a slight enhancing effect on the response but also was not significantly different from the control. In contrast, intradermal administration of plasmid containing the IL-12 cDNA with the gene gun significantly increased (p<0.03) the strength of the response to the vaccine to almost 300 IFN- -secreting cells/million mononuclear cells (Fig. 3). The transient kinetics of this response are consistent with our previous observations on vaccination studies against PrV (Zuckermann et al., 1998a; Zuckermann et al., 1998b).

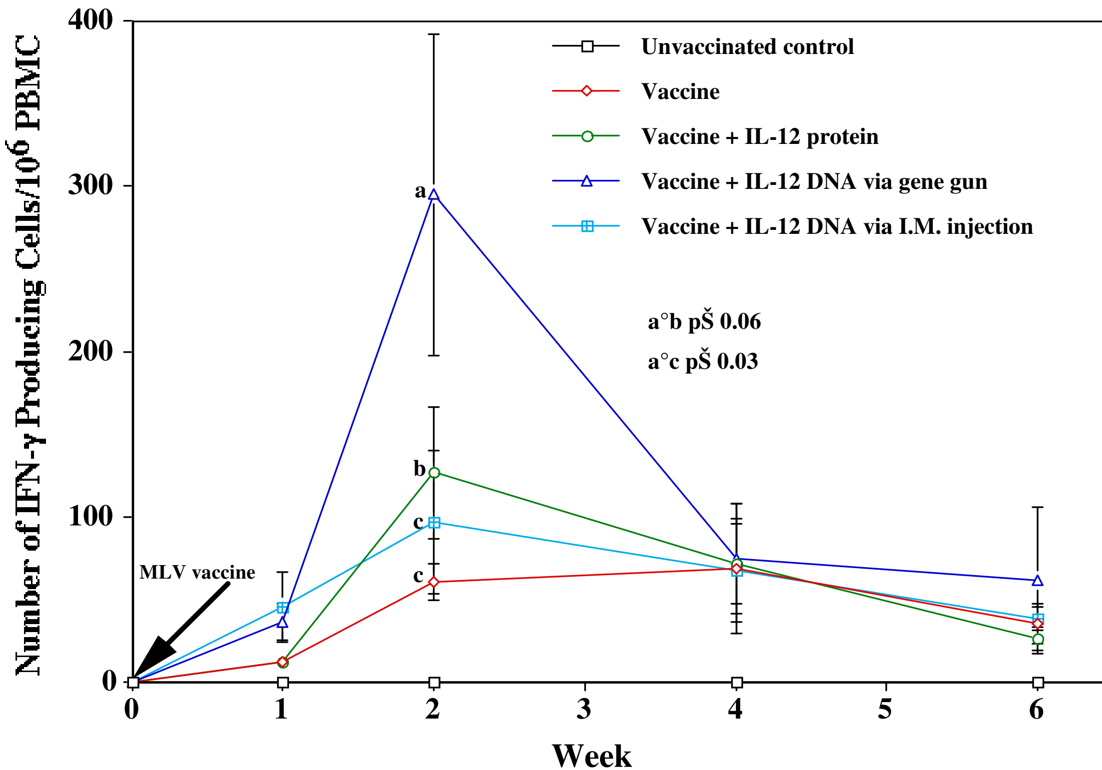

Figure 3. Cell-mediated immune response to a PRRS MLV vaccine administered alone or coadministered with poIL-12 in the form of soluble protein or expressible cDNA as an adjuvant.

These results demonstrate that in swine biolistic delivery is more efficient than intramuscular injection of DNA. The fact that the IFN- response but not the titer of virus neutralizing antibodies was enhanced by the administration of IL-12 cDNA suggests that these two responses are independently regulated. This conclusion is further supported by a similar observation made in response to PrV (Zuckermann et al, 1998a). In conclusion the use of exogenous IL-12 in the form of expressible cDNA has the potential to be used for enhancing the development of cell-mediated immunity against PRRS virus. We predict that such an event will have a positive effect on the extent of protective immunity induced by the vaccine and thus increase the effectiveness of currently available PRRS MLV vaccines.

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