Title: A study on the feasibility of using a porcine alveolar macrophage cell line to produce a PRRS modified live virus vaccine – NPB #05-200

Investigator: Federico A. Zuckermann

Institution: University of Illinois

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Industry summary:
The goals of this project was: 1) to determine the feasibility of using an innovative porcine alveolar macrophage cell line, designated ZMAC-1, for the production of a PRRS modified live virus (MLV) vaccine and 2) to compare the efficacy of vaccine virus grown in this alternate host to that propagated in the only other cell line known at the initiation of this study to support the growth of PRRS virus, namely the simian cell line MA-104 and its derivative the MARC-145 line. Initially, the ZMAC-1 cells were found to be readily susceptible to the MLV vaccine Prime Pac PRRS (Schering-Plough Animal Health). Moreover, after the third passage of the virus in ZMAC-1 cells, a yield comparable to that achieved when using MARC-145 cells was obtained. To evaluate the vaccine potential of both ZMAC-1 cell-grown virus, a standard immunization-challenge study was conducted. In this case, six 8 week-old pigs were injected with an equivalent dose of the Prime Pac vaccine grown in either ZMAC-1 or MARC-145 cells while two additional groups of three animals were not immunized. Four weeks later, all vaccinated and one of the PRRS virus-naive groups were challenged with an “atypical PRRS abortion storm” virus isolate (NADC-20). One outcome of the study was that the Prime Pac MLV vaccine grown in either cell line was equally effective at preventing the body weight loss of PRRS virus-naive pigs that had been exposed to the heterologous virus 7 days earlier. However, the vaccine virus grown in ZMAC-1 cells was significantly more effective than the MARC-145 derived one at reducing the extent of viremia and also at eliminating virulent virus from the lungs at 7 and 10 days post-challenge, respectively. The observation that the type of cell line used to grow the PRRS MLV vaccine can improve the level of protective immunity elicited by the same vaccine virus against a genetically divergent virulent PRRS virus has important implications for the prospect of developing a highly effective vaccine against this pathogen. Namely, the results of this study suggest that the effectiveness of a PRRS MLV virus vaccine is not, as it is commonly believed, only determined by its genetic similarity to the challenge virus, but is also influenced by how it is produced. The results of this study provide great hope that an effective MLV vaccine against PRRS virus can be developed.
III. Scientific Abstract:
An innovative porcine alveolar macrophage cell line, designated ZMAC-1, was generated and its utility to manufacture an effective PRRS modified live virus (MLV) vaccine was examined. This cell line was found to be 100% susceptible to infection by PRRS virus, as evidenced by the successful immunofluorescence staining for viral proteins at 20 hr after infection. Moreover, based on multiple step growth curve analyses, the first round of PRRS virus replication was determined to be completed by 9 hr after infection and the yield of virus progeny during the second round of replication at 19 hr after infection. To compare the efficacy of stocks of the MLV vaccine Prime Pac PRRS (Schering-Plough Animal Health) prepared in either ZMAC-1 or the simian cell line MARC-145, a standard immunization-challenge study was conducted. Six 8 week-old pigs were initially vaccinated intramuscularly with an equivalent dose (10^4 TCID_{50}) of the Prime Pac vaccine grown in either ZMAC-1 or MARC-145 cells, while two additional groups of three animals were not immunized. All of these animals, as well as one of the two groups of unvaccinated controls, were challenged 4 weeks later with 10^4 TCID_{50} of an “atypical PRRS abortion storm” virus isolate (NADC-20). While the unvaccinated animals experienced an average body weight (BW) loss of –5±4 lb by 7 days after the virulent virus challenge, the PRRS virus-naïve controls had gained on average 19.7±6 lb during this time interval. In contrast, at 7 days post-challenge, the animals vaccinated with the MLV virus grown in either ZMAC-1 or MARC-145 cells exhibit average BW gains of 8.2±5.2 and 9.3±3.6, respectively. Thus, statistically the Prime Pac MLV vaccine grown in either cell line was equally effective at reducing the negative effect of the exposure of pigs to a highly virulent PRRS virus on their growth. Remarkably, analyses of the virus load in serum and lung lavage samples from PRRS virus-immunized and challenged animals revealed that the vaccine virus grown in ZMAC-1 cells was significantly (P=0.015) more effective at reducing the extent of viremia at 7 days post-challenge and also at eliminating virulent virus from their lungs by 10 days post-challenge. The observation that the type of cell line used to grow the PRRS MLV vaccine can improve the level of protective immunity elicited by this product against a genetically divergent virulent PRRS virus has significant implications for the prospect of developing a highly effective vaccine against this pathogen. Namely, the results of this study suggest that the effectiveness of a PRRS MLV virus vaccine is not only, as it is commonly believed, determined by its genetic similarity to the challenge virus, but is also influenced by how it is produced.

IV. Introduction:
The significance given by the Pork Checkoff to a new generation of PRRS virus vaccines is clearly exemplified by the prominent rank of this topic in the list of research priorities of this NPB PRRS Initiative Research Objectives. In mid-1994, the first PRRS MLV vaccine (Ingelvac PRRS MLV) was released. Since then, the use of attenuated viruses as vaccines has become customary in North America and Europe. It is well accepted that these agents are effective in conferring appropriate levels of homologous protective immunity while affording variable degrees of protection against challenge by heterologous strains (Mengeling et al., 1996; Mengeling et al., 1999). Unfortunately, as result of patent litigation most manufacturers have ceased production of their PRRS vaccines – an action that has virtually brought to a halt the incorporation of innovations in the area of PRRS virus biologics (Hill et al., 2004). However, due to advances in the study of this pathogen, a resurgence of interest by major companies in the development of an effective PRRS vaccine can be perceived. Recently, a porcine alveolar macrophage cell line designated ZMAC-1 was developed at the principal investigator’s laboratory and was found to not only be susceptible to infection by PRRS virus but also to release significant amounts of virus progeny. Consequently, this project was proposed as a means to test the feasibility of using this cell line to produce a MLV vaccine against PRRS virus.
V. Objectives:

1. Determine the growth characteristics of attenuated PRRS virus strains in the porcine macrophage cell line ZMAC-1.

2. Determine the immunogenicity and efficacy of a PRRS modified live virus vaccine produced in the porcine macrophage cell line ZMAC-1.

VI. Materials and Methods:

Cells. Porcine alveolar macrophage cells were selected from the lung lavage population of SPF pigs on the basis of their inability to adhere to plastic surfaces. The cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum, sodium pyruvate and non-essential amino acids, and maintained at 37°C in a 5% CO₂ atmosphere. A robust third generation cell line, designated ZMAC-1, was established in May of 2006 and has since been continuously growing. Stocks representative of different temporal subdivisions of this cell line have been cryo-preserved.

Virus. The MLV vaccine Prime Pac PRRS (Schering-Plough Animal Health) was sequentially passaged three times in ZMAC-1 cells. Although the titer was only $10^4$ TCID₅₀/ml after the first two passages, the virus appeared to have adapted during the third passage as the titer had increased to $10^6.25$ TCID₅₀/ml. Accordingly, this progeny was used for vaccination. Stocks of the Prime Pac vaccine were also prepared in MARC-145 cells as previously described (Osorio et al., 2006) and attained a titer of $10^6$ TCID₅₀/ml. The “atypical PRRS abortion storm” virus isolate NADC-20 (Harms et al., 2001) was grown in ZMAC-1 cells and exhibited a maximum titer of $10^{7.6}$ TCID₅₀/ml.

Vaccination and challenge study: Eighteen 8-week-old SPF pigs (free of all major swine pathogens including PRRS virus, mycoplasma and circovirus) were randomly allocated into 6 isolation cubicles (3 pigs per cubicle) at a suite in the Bioccontainment Facility at the University of Illinois. Animals from four cubicles were injected once in the rump area with a 2 ml solution containing $10^4$ TCID₅₀ of Prime Pac virus grown in either ZMAC-1 or MARC-145 cells, for a total of 2 cubicles per vaccine formulation (6 pigs total). The six remaining pigs in the two other cubicles were not immunized and served as unvaccinated controls. Four weeks after vaccination, all of the immunized animals as well as three of the control pigs housed in one cubicle were challenged with $10^4$ TCID₅₀ of PRRS virus strain NADC-20. While the unvaccinated and challenged animals served to establish the severity of infection by the NADC-20 PRRS virus, the PRRS virus-naïve pigs in the remaining cubicle were used to provide normal clinical parameters of growth and health. The degree of protective immunity elicited by the vaccine was determined based on a comparison of body weight (BW) changes and the appearance of depression and respiratory signs. These parameters were monitored daily for ten days after the challenge. The level of viremia was determined at 0, 4, 7 and 10 days after challenge by measuring infectious units in MARC-145 cells. Ten days after the challenge, the animals were euthanized and the viral load in the bronchoalveolar lavage (BAL) fluid was determined by using real time PCR and virological methods as previously described (Zuckermann et al., 2007).

Statistical analysis. Analysis of Variance was used to determine significant differences among groups of pigs in regard to weight gain. Group means were compared by Fisher’s least significant difference procedure utilizing Stat View software (SAS). To minimize the effect of BW weight differences between animals at the time of challenge, the data was calculated as the difference between the BW of the animals at the time of challenge and 7 days later.
VII. Results:

Objective 1. Determine the growth characteristics of attenuated PRRS virus strains in the porcine macrophage cell line ZMAC-1.

The goal of this aim was to ascertain the robustness of the ZMAC-1 cell line and its susceptibility to infection by PRRS virus. In addition, we sought to determine optimal conditions for the growth of attenuated PRRS virus in the ZMAC-1 cells and to demonstrate that stocks of MLV vaccine prepared in this cell line could potentially be used for commercial purposes.

Characterization of the porcine alveolar macrophage cell line ZMAC-1. The ZMAC-1 macrophage line has exhibited a very robust growth pattern with a doubling time of approximately 72 hours (Fig. 1). We have been able to adapt the cells to be grown in 75 cm² flasks and to keep this type of cell culture in continuous production for the last 15 months. To date, we have generated more than 1 billion cells from an initial starting population of a few thousand. In order to ensure the perpetuity of this valuable cell line more than 100 frozen cells stocks have been prepared. Every lot has at least 10 vials, and each vial contains at least 2-3 million cells. Upon thawing a representative vial of every lot, we have determined that these vials have >90% viable cells which exhibit vigorous growth within 4 days after re-initiation of their culturing. This cell line has been confirmed to be of swine origin by the reactivity of 100% of the cells in the population with the monoclonal antibody K252.1E4, which is specific for porcine CD45 (Schnitzlein and Zuckermann, 1998; Zuckermann et al., 2001). In addition, the ZMAC-1 cells express the following cell surface markers: CD14, CD163, CD172, MHC class, whose presence is characteristic of macrophages (data not shown).

Figure 1. Growth kinetics of the ZMAC-1 cell cultures of ZMAC-1 cells in 75 cm² flasks 10⁵ cells/ml medium were combined with an fresh medium to achieve a cell density of 1-1.6 x 10⁵ cells/ml. Cells were counted at day 0, 3 and 6 days after delivered into the culture flask.

Growth of PRRS virus in ZMAC-1 cells. The ZMAC-1 cell line is 100% susceptible to infection by PRRS virus, as evidenced by the successful immunofluorescence staining for viral proteins at 20 hr after infection (Fig. 2). Moreover, we have determined that the first round of replication of PRRS virus is completed by 9 hr after infection (data not shown) and that the peak yield of virus progeny is achieved by the second round of replication at 19 hr after infection (Fig. 3).

Figure 2. Expression of PRRS virus nucleocapsid protein in ZMAC-1 cells. At 20 hr after infection with PRRS virus strain NADC-20 at MOI of 1, the cells were fixed and stained with FITC-labeled, anti-PRRS virus nucleocapsid mAb SDOW17.
Figure 3. Multiple step growth curves of PRRS virus in ZMAC-1 cells. A suspension at 1x10^6/ml was infected at an MOI of 0.02 with the attenuated PRRS virus isolate Prime Pac. After one hr incubation at 37°C the inoculum was removed by centrifugation and the cells suspended in medium at a concentration of 1x10^6/ml. One-tenth ml aliquots were removed at the indicated times after determining the presence of (TCID_{50} in MARC-145 cells).

Objective 2. Determine the immunogenicity and efficacy of a PRRS modified live virus vaccine produced in the porcine macrophage cell line ZMAC-1.

The goal of this aim was to compare the levels of protective immunity elicited by the same PRRS MLV vaccine, in this case the Prime Pac PRRS virus, produced in either the ZMAC-1 cell line or in MARC-145 cell line.

To test the efficacy of the Prime Pac PRRS virus vaccine, that had been prepared in either ZMAC-1 or MARC-145 cells, groups of pigs were either immunized with vaccine virus derived from one of the two cell lines or mock-treated. At the time of challenge, 4 weeks after immunization, the average BW of all 18 pigs in the study was 117±8.6 lb. Since no significant differences between the average BW of vaccinated and non-vaccinated animals was observed, vaccination with the MLV propagated in either Z-MAC1 or MARC-145 cells had no obvious impact on animal growth. In contrast, at 7 days after challenge with the virulent NADC-20 isolate, the unvaccinated pigs had an average BW loss of –5±4 lb while the unchallenged animals gained on average 19.7±6 lb (Fig. 4). An average BW gain of 8.2±5.2 and 9.3±3.6 lb, that were not statistically different from each other, were also noted for the groups that had previously received the MLV virus vaccine prepared in either ZMAC-1 or MARC-145 cells, respectively. Thus, regardless of the type of cell used to propagate the virus, the Prime Pac MLV vaccine was equally effective at diminishing the negative effect of the exposure to virulent PRRS virus on growth, as evidenced by the significant BW loss of PRRS virus-naïve pigs that had been exposed to a heterologous virulent virus 7 days earlier. Remarkably, analyses of the virus load in serum and lung lavage samples from PRRS virus-immunized and challenged animals revealed that the vaccine virus grown in ZMAC-1 cells was significantly (P=0.015) more effective at reducing the extent of viremia at 7 days post-challenge (Figure 5) and also at eliminating virulent virus from their lungs by 10 days post-challenge (Figure 6).

Figure 4. Weight change in pigs after exposure to wild-type PRRS virus. The PRRS virus-naïve (n=3) and each type of cell-generated were measured immediately days after challenge with the virus isolate NADC-20. also made at these time points.
unchallenged, PRRS virus-naïve animals (n=3). Changes in weight during the 7-day interval were averaged for members of each group and these values ± the standard error are shown. * Indicates that the group mean is statistically different (P<0.01) from the challenged, PRRS virus-naïve control animals. ** Indicates that the group mean is statistically different (P<0.01) from the unchallenged, PRRS virus-naïve control animals.

**Figure 5.** Extent and frequency of viremia in pigs after exposure to wild-type PRRS virus. Serum samples were collected from PRRS virus-immunized animals prior to and at the indicated time points for the unchallenged, PRRS virus-naïve control animals. The level of virus load in the serum was determined by performing titration in MARC-145 cells and then averaging. The data represents the average level of viremia for each group. The ratio next to the symbols indicates the number of viremic pigs (numerator) and the total number of pigs per group (denominator).

**Figure 6.** Virus load in the bronchialveolar lavage of pigs after exposure to wild-type PRRS virus. Bronchoalveolar lavage was collected from PRRS virus-naïve and previously immunized pigs at 10 days after challenge with the wild-type PRRS virus isolate NADC-20. Samples were also obtained at this time from unchallenged, PRRS virus-naïve animals. The level of virus load in the BAL of each animal was determined by performing titration in MARC-145 cells.

**VIII. Discussion.**
The observation that the cell line used to grow the same PRRS MLV vaccine strain can improve the level of protective immunity elicited by this product against a genetically divergent virulent PRRS virus has important implications for the prospect of developing a highly effective vaccine against this pathogen. Namely, the results of this study suggest that the effectiveness of a PRRS MLV virus vaccine is not, as it is commonly believed, only determined by its genetic similarity to the challenge virus, but is also influenced by how it is produced. A reasonable interpretation for the observations described above is that the biological properties of the vaccine virus were modified in a desirable way by simply being grown in ZMAC-1 cells. As a result of this change, a more effective protective immune response developed in the vaccinated animals. Thus, the results of this study provide great hope that a more effective MLV vaccine against PRRS virus can be created.
IX. References.
PRRSV: comparison of commercial vaccines in their ability to induce protection against current PRRSV strains of high virulence. Allen D. Leman Swine Conference 25:176-182.