Evaluation of pseudorabies virus excreted and nucleocapsid proteins as subunit diagnostic antigens

by

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EXPLANATION OF THESIS FORMAT

The following thesis consists of a general introduction, a review of the literature, two separate manuscripts (Sections I and II), a general summary, literature cited, and acknowledgements. The master's candidate, Michael James Mc Ginley, is the senior author and principal investigator for each of the manuscripts.

GENERAL INTRODUCTION

Pseudorabies virus (PRV) causes an economically important disease of swine that is generally controlled by vaccination. While vaccination with currently available modified live or killed commercial vaccines provides adequate protection from disease the differentiation of vaccinated pigs and virus infected pigs is not possible. Consequently, vaccinated pigs must be labelled as latent carriers and potential disseminators of PRV. The use of PRV subunit vaccines with a defined antigenic composition makes differentiation possible by utilizing non-vaccine component PRV proteins as diagnostic antigens. As a result, subunit vaccines and associated diagnostic antigens can greatly aid in the control and possible eradication of PRV from the swine population.

Subunit vaccinated pigs will be exposed to minimum infective doses of PRV under field conditions. Consequently, the host immune response may suppress virus infection before sufficient amounts of PRV diagnostic antigen are produced. This may result in an undetectable antibody response to the diagnostic antigen in latently infected subunit vaccinated pigs. In the following study the ability of low doses of pseudorabies virus (PRV) to induce detectable antibody levels to a 98K diagnostic antigen (DA) and to establish latency in subunit vaccinated pigs was investigated. In addition, the efficacy of PRV nucleocapsid proteins as diagnostic antigens was evaluated under similar PRV exposure conditions.

LITERATURE REVIEW

Introduction

Pseudorabies (Aujeszky's disease) is an economically important disease of swine caused by the pseudorabies virus (PRV), a member of the family Herpesviridae.[28] Since its initial description in 1902 by the Hungarian veterinarian Aladar Aujeszky[3] the worldwide incidence and economic impact of pseudorabies have increased.[101] Slaughter survey sera from the U.S. indicated 0.56% of pigs were positive for PRV in 1974.[22] This figure rose to 3.73% in 1978 and 8.39% in 1981.[91] Economic losses to the Iowa swine industry alone were estimated at $33.9 million in 1981.[13] A 1985 study, taking into account losses due to PRV infection of breeding stock, estimated the economic loss in Iowa to be $107 to $117 million per annum.[30]

The control of pseudorabies can be accomplished by the elimination of infected pigs from the herd or by vaccination to prevent the spread of virus within the herd.[91] In both cases distinguishing PRV infected pigs from PRV vaccinated pigs is of primary importance. A major limitation of currently available modified live or killed commercial vaccines[64,90,94] is that vaccinated pigs cannot be differentiated from virus infected carrier pigs.[44] The use of subunit vaccines with limited and defined antigenic composition in conjunction with a non-vaccine diagnostic antigen makes this distinction possible. This concept has been demonstrated in PRV subunit vaccinated pigs using an early PRV specific excreted protein not present the vaccine preparation.[70] The following

review of the literature will concentrate on the pseudorabies virus and its protein composition, the concept of subunit vaccines, and a general description of what is known about the porcine immune response to pseudorabies virus.

Pseudorabies Virus

Pseudorabies virus (suid herpesvirus I) is a member of the family Herpesviridae.[78] The virus has been further classified into the subfamily alpha herpesvirinae based on its wide host range, short replication cycle, rapid cytopathic effect leading to cell death, and its ability to establish latent infections.[1,25,58,78,79,80] Representative members of the alpha herpesvirinae related to pseudorabies virus include herpes simplex virus types 1 and 2, equine herpesvirus type 1, and bovine herpesvirus type 1 and 2.[26,38,43,78]

Pseudorabies virus has an icosahedron capsid symmetry of 162 capsomeres with an approximate average diameter of 180 nm as measured by phosphotungstic negative staining and electron microscopy.[9,42,82] The capsid surrounds a double-stranded DNA genome with a molecular weight of 90 x 10^6 daltons.[9] The genome consists of four distinct regions; a unique long region (UL) of 65 x 10^6 daltons, an inverted repeat region (IR) and terminal repeat region (TR) of 10 x 10^6 daltons each, and a unique short region (US) of 6 x 10^6 daltons located between the IR and the TR regions.[9,11,89] The nucleocapsid (capsid and genome) is surrounded by an envelope consisting of a double or triple lipid-containing membrane rich in glycoproteins which is derived from the

nuclear or cytoplasmic membrane of virus infected cells.[8,21,75]

Pseudorabies Virus Proteins

The number of polypeptides identified from purified PRV preparations has changed over time with advances in separation techniques and improved resolution.[9] Original estimates indicated approximately 10 polypeptides were associated with purified virions.[7] Subsequent studies have identified a minimum of 20 and as many as 27 polypeptides in purified PRV preparations.[9,66,68,88] Several structural proteins have been identified; four of these are phosphoproteins[9] and four major and three minor structural proteins are glycosylated.[9,32,53] In addition, several non-structural viral proteins have been identified.[23,40,69,70,73,93] The following review of pseudorabies virus proteins will focus on the structural proteins associated with the virus envelope and nucleocapsid as well as the non-structural virus-directed proteins.

Pseudorabies virus envelope proteins

The PRV envelope fraction contains approximately 50% of the virus protein and all of the glycoproteins.[32,39] The virus envelope has been shown to contain four major and three minor structural glycoproteins as well as one non-glycosylated protein.[32] A proposed nomenclature for the pseudorabies virus envelope proteins[32] is summarized in Table 1.

Table 1. The envelope proteins of pseudorabies virus

Major glycoproteins		Minor glycoproteins	
125K[a]	(gIIa)	130K[b]	(gI)
98K	(gIII)	98K[b]	(gIV)
74K[a]	(gIIb)	62K[b]	(gV)
58K[a]	(gIIc)		

Nonglycosylated protein
115K

[a]These three major glycoproteins are covalently linked by disulfide bridges.

[b]The three minor glycoproteins are noncovalently linked and form a complex with the 115K nonglycosylated protein.

The four major envelope glycoproteins are highly sulfated and have molecular weights of 125K (gIIa), 98K (gIII), 74K (gIIb), and 58K (gIIc).[23,32,47] Analysis of detergent extracted virus envelopes by radioimmunoprecipitation with monoclonal antibodies reactive to envelope fractions has shown that the 125K, 74K, and 58K glycoproteins reside as a complex covalently linked by disulfide bridges.[32] Partial peptide

mapping of these three glycoproteins shows extensive sequence homology indicating that the individual peptides may have originated from a single precursor protein.[32,47,53] The fourth major glycoprotein (98K, gIII) is not complexed to any other protein.[32] The three minor envelope glycoproteins with molecular weights of 130K (gI), 98K (gIV), and 62K (gV) form a noncovalently linked complex with a 115K non-glycosylated protein.[32,47,53]

Because the envelope glycoproteins are on the surface of the virion, they probably play a primary role in eliciting a host immune response.[32,84,98] Monoclonal antibody studies have demonstrated that antibody to the major 98K (gIII) glycoprotein will inhibit virus absorption and neutralize virus infectivity in the absence of complement.[32] Wathen et al. demonstrated that the 58K (gIIc) protein may also induce serum-virus neutralizing antibody.[97] Similar studies indicate antibody directed to the other envelope glycoproteins have little serum-virus neutralizing activity.[9,32] The possible role of these envelope proteins in inducing cell-mediated immune responses has not been investigated to date.

Pseudorabies virus nucleocapsid proteins

Viral nucleocapsids consist of nucleic acid and associated proteins surrounded by a protein capsid.[25,50] Early studies on the protein composition of PrV nucleocapsids described three major proteins with molecular weights of 142K, 35K, and 32K and a minor protein with a molecular weight of 62K.[8,39] Subsequent studies by Stevely identified a

total of eight nucleocapsid proteins.[88] Four of these were in close agreement with earlier studies. In addition, four minor proteins with molecular weights of 120K, 82K, 41K, and 22.5K were resolved.[88]

Pseudorabies virus non-structural proteins

A recent review by Ben-Porat and Kaplan[9] summarizes the information available to date on the character and function of PRV specific non-structural proteins and organizes them into four general categories: immediate early (IE) protein, DNA-binding proteins, virus enzymes, and excreted glycoproteins.

The IE protein of PRV is a multifunctional, 180K protein that appears to regulate several processes in PRV infected cells.[9,34] The IE protein has been shown to be a requirement for orderly PRV RNA transcription[34] and in fact acts as a repressor of IE mRNA transcription.[24] In addition, the IE protein contributes to the disruption of cellular mRNA-ribosome complexes which in turn inhibits cellular protein synthesis.[10,11,34]

To date, at least 16 DNA-binding proteins specified by PRV have been described.[9] These DNA-binding proteins include both structural and non-structural proteins. Two major non-structural DNA-binding proteins with molecular weights of 136K and 15K have been described in detail.[9] The major 136K DNA-binding protein synthesized by PRV is responsible for initiating the first round of virus DNA synthesis and later rounds of virus DNA replication, as well as stabilizing progeny virus DNA.[9,12] The 15K DNA-binding protein is found in association with the large

concatameric PRV DNA (polymerized copies of PRV the genome) found in infected cells late in the infection.[9] The 15K protein appears to function in the stabilization of the DNA.[9] The removal of the 15K protein and a closely associated 10K structural protein from concatameric DNA by isopycnic centrifugation in cesium chloride results in double-stranded breaks in the viral DNA.[9]

A limited number of PRV specific enzymes have been described to date.[9] Those that have been described are thymidine kinase (TK), PRV-induced DNA polymerase, and a PRV-induced DNase.[9] The PRV TK enzyme generates the DNA precursor deoxythymidylate (dTMP) from free thymidine.[41,99] The PRV TK provides a thymidine utilization pathway independent of the cellular thymidilate synthetase pathway.[85] The PRV-induced DNA polymerase is antigenically distinct from cellular DNA polymerase and provides PRV with a cell independent DNA polymerase.[31] The PRV-induced DNase is an exonuclease that catalyzes the release of deoxyribonucleoside-5'-monophosphates from the 3' end of DNA and is also antigenically distinct from cellular DNase.[9,42]

The primary non-structural protein of PRV is a highly sulfated excreted glycoprotein that is produced and accumulates in large quantities in the medium of PRV infected cells.[23,40,69,70,73,93] Kaplan and Ben-Porat described this protein as a highly sulfated protein with a molecular weight of 90K.[40] Later studies by Platt described a similar protein with a molecular weight of 110K that could be used to detect virus infection in PRV subunit vaccinated pigs.[69,70] In a subsequent study, Rea et al. mapped and sequenced a gene coding for a 95K

glycoprotein.[73] Rea et al. named this protein gX and it appears to be identical to the highly sulfated excreted glycoprotein described previously.[40] Recent work by Thomsen et al. demonstrated that gX did not induce PRV neutralizing antibody raised in animals.[93] The same investigator concluded from studies with gX⁻ mutants that the protein was not directly associated with virulence or virus replication.[93] Although a specific function has not been associated with gX, it has been hypothesized that it may serve as a "scaffolding protein" involved in intracellular virion assembly[23] or it may aid in viral evasion of host immune responses by promoting cell fusion.[20,62]

Subunit Vaccines

Subunit vaccines are defined as vaccines containing only those viral structures necessary for the induction of a protective host immune response.[49,74,83] General methods for the production of these subunit vaccines include the extraction of specific proteins from whole virion preparations,[56] extraction of virus infected cell membranes,[69,77,95] viral peptide synthesis,[16] and various applications of recombinant DNA technology.[14,16,45,46,51,76] The absence of whole virus and viral nucleic acid in subunit vaccines makes them safer than attenuated or inactivated vaccines because they are: non-infective,[19,74,83] less toxic,[81] and incapable of establishing latent infections.[74,83] These qualities make it possible to administer large doses of immunogen from virulent virus without adverse effects intrinsic to traditional vaccines. Additionally, the limited antigenic composition of subunit vaccines makes

it possible to identify virus infected subunit vaccinated animals by serologically testing for antibody to non-vaccine viral components. Subunit vaccine preparations have been evaluated with respect to a number of herpesviruses such as; infectious bovine rhinotracheitis (IBR),[54] equine herpesvirus type 1 (EHV-1),[64] Marek's disease virus,[35,36,37,52,102] herpes simplex virus type 1 (HSV-1),[15,48,72,87,103] herpes simplex virus type 2 (HSV-2),[86,96] and pseudorabies virus (PRV).[68,69,70,77,95]

Initial PRV subunit vaccine preparations consisted of detergent extracts of viral envelope glycoproteins.[56,68,77,95] The use of non-ionic detergents such as Triton X-100 (TX-100) and Nonidet P-40 for protein extraction was initially employed in the isolation of membrane bound cell receptors, transplantation antigens, and histocompatability antigens.[33,55] The technique was subsequently adapted for use in the extraction of protective immunogens[4,18,20,27] from the envelopes of whole virions[56] and/or virus infected cell membranes.[65,68,77,95] In 1980, Rock and Reed first evaluated a TX-100 extract of PRV infected cells and found the extracted viral antigens to be highly immunogenic and protective in mice.[77] This finding was confirmed in 1981 by Turner et al. with a similar NP-40 detergent extract.[95] A similar study by Maes and Schutz showed NP-40 extracts of intact virion induced immunity in swine.[56] They also reported that this immunizing preparation stimulated the production of higher levels of neutralizing antibody than either attenuated or inactivated vaccines.[56] In 1982, Platt demonstrated that the administration of TX-100 extracts of PRV infected cells induced PRV

specific protective immunity in swine.[69] Subsequently, Platt showed that the glycoproteins isolated from crude detergent extracts by lectin affinity chromatography also induce protective immunity.[69] He also demonstrated that pigs which were immunized with lectin purified preparations did not have antibody to a PRV-specific early excreted non-structural protein.[69] These findings led to the successful use of the early excreted protein as a diagnostic antigen to identify virus infected vaccinated pigs.[70]

The Porcine Immune Response to PRV.

The humoral immune response to PRV is generally characterized by serum virus-neutralizing antibody (SN) assays. Low levels of IgM class SN antibody are detectable 7 to 10 days post-infection (p.i.).[29,57,59,67] Serum IgM titers peak at day 10 p.i. and are generally detectable until day 14 p.i. at which time they dramatically decrease.[57] Meanwhile, IgG titers increase dramatically to day 14 p.i. and generally peak by day 35 p.i.[57,60] Serum virus-neutralizing antibody is primarily responsible for neutralizing free virus in blood and tissue fluids.[5]

The role of local secretory immunity is linked to the fact that the primary route of PRV natural infection is via the mucosa of the nasopharynx.[6] Therefore, IgA class antibody in nasal secretions is an important aspect of humoral immunity.[6] The anti-PRV IgA response after initial infection is generally brief.[5] Shortly after infection low levels of antibody remain on the mucosal surfaces.[5] However, re-exposure to PRV results in the induction of high levels of protective anti-PRV

IgA.[5,6] The specific aspects relating to the induction of primary secretory immunity and subsequent memory have not been described in the pig.

The initial low levels and late onset of humoral immune responses have led some investigators to believe that cell-mediated immunity is the primary immune response in the early stages of PRV infection. Cell-mediated immunity (CMI) in response to PRV and other herpesvirus infections has been demonstrated by the following methods: lymphocyte transformation,[100,101] macrophage migration inhibition,[5] delayed hypersensitivity skin reaction,[5] antibody-dependent cell-mediated cytotoxicity,[2] and specific MHC-directed cytotoxic T-lymphocyte killing.[81]

Wittmann et al. demonstrated the early onset of CMI by stimulating the transformation of lymphocytes collected from regional lymph nodes and spleen at 4 days p.i. Serum-virus neutralizing antibody was not detected until day 7 p.i.[101] In addition, Ashworth et al. detected anti-PRV CMI by antibody-dependent cell-mediated cytotoxicity (ADCC) in 50% of non-immune pigs exposed to virus on day 7 p.i. when no SN antibody was detectable and in all pigs when SN antibody titers were uniformly low.[2] This study implies that non-detectable levels of antibody may participate in early cell-mediated killing of PRV infected cells.[2] Demonstrations of MHC specific T-cytotoxic killing in response to PRV infection are not available. However, extensive studies of closely related herpes simplex virus (HSV) types 1 and 2 in mice have shown that cytotoxic T-lymphocytes specifically lyse HSV infected syngeneic cell cultures.[81] These studies

indicated cytotoxic T-lymphocytes play a significant role in recovery from infection.[81] Other studies indicate that lymphocytes bearing T-helper and delayed hypersensitivity markers also play a significant role in recovery from HSV infection.[63]

The humoral immune response of pigs following vaccination is in general similar to that observed in natural infection. Typically, low levels of PRV specific SN antibody are detected following inoculation with PRV modified live[61] or subunit vaccines[69]; however, exposure of vaccinates to virus results in a marked anamnestic SN antibody response.[61,69] The extent of local secretory and cell-mediated immunity induced by vaccination has not been investigated to date.

SECTION I. PSEUDORABIES VIRUS SUBUNIT DIAGNOSTIC ANTIGENS MAY FAIL TO DETECT LATENT INFECTIONS INDUCED BY LOW VIRUS EXPOSURE DOSES

This manuscript has been submitted for publication to the American Journal of Veterinary Research.

SUMMARY

The ability of low doses of pseudorabies virus (PRV) to induce detectable antibody levels to a 98K diagnostic antigen (DA) and to establish latent infections in subunit vaccinated pigs was studied. The relationship of virus exposure dose and vaccine dose to the response of pigs to DA was studied in 18 pigs. Two groups of three pigs were immunized with a total of 200ug of a lectin derived PRV subunit vaccine over a five week period. Two additional groups of three pigs were similarly immunized with a total of 100ug. Two groups of three pigs served as uninoculated controls. One group of pigs from each of the preceding categories was intranasally challenged with $10^{6.0}$ and $10^{2.7}$ PFU of virus. Antibody to DA was detected by enzyme-linked immunosorbant assay (ELISA) and radioimmunoprecipitation (RIP) three to seven days earlier in both vaccine groups that were challenged with $10^{6.0}$ PFU. These results demonstrate that virus challenge dose affects the antibody response to DA.

The establishment of latent infections by low PRV exposure doses and the ability to detect these infections was studied in 10 subunit vaccinated pigs. Each pig was intranasally challenged with $10^{2.3}$ PFU of virus. The serum-virus neutralizing (SN) antibody titer of these pigs rose to a peak at 14 to 21 days post challenge (p.c.) and then steadily declined through day 113 p.c. indicating an absence of viral recrudescence. All pigs were treated with dexamethasone for four consecutive days beginning on day 113 p.c. Latent infections were

identified in eight of 10 pigs (80%) based on the recovery of virus and/or two $\log_2$ or greater increases in SN titer. Antibody to DA was detected in the eight latently infected pigs for periods ranging from 21 to 113 days p.c. before dexamethasone treatment. The antibody titer to DA increased in six of the eight latently infected pigs following dexamethasone treatment. However, no antibody to DA was detected in the remaining two latently infected pigs (25%) despite significant increases in SN titers and the recovery of reactivated virus in one of the pigs. The failure to consistently detect antibody to 98K DA in latently infected pigs demonstrates that the reliability of diagnostic tests that use non-vaccine diagnostic antigen may only be suitable for detecting infections in vaccinated herds and not in individual pigs.

INTRODUCTION

Subunit vaccines may prove to be valuable tools in pseudorabies virus (PRV) control programs. These vaccines will not only provide protection from clinical disease and slow the spread of virus within a herd but because of their limited antigenic composition they will offer the unique advantage of permitting the detection of virus infection in vaccinated pigs. The ability to detect virus infection in subunit vaccinated pigs has been previously documented in earlier work in which an early excreted non-vaccine viral protein was used as a diagnostic antigen.[9] The virus challenge doses used in these experiments were $10^{7.4}$ PFU and greater. Under field conditions the virus exposure doses in vaccinated herds will most likely be lower and possibly approach the minimum infective dose since smaller amounts of virus are shed by vaccinated pigs than by non-vaccinated pigs.[1,7] Consequently, it is conceivable that a virus infection may be suppressed by specific host immune responses before a sufficient amount of non-vaccine viral protein can be generated that will induce a detectable antibody response. Under these conditions subunit vaccinated pigs may remain seronegative to diagnostic antigen but be latently infected. The following study was undertaken to test this hypothesis.

MATERIALS AND METHODS

Virus, Cells, and Medium

Pseudorabies virus (PRV) strain Be^8 was used for both viral antigen production and challenge virus. Madin Darby bovine kidney (MDBK) cells were obtained from the National Veterinary Services Laboratory, Ames, IA. Pig kidney 1a cells were obtained from the Veterinary Medical Research Institute, Iowa State University, Ames, IA. Growth medium consisted of Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum or 10% horse serum (GIBCO Laboratories, Grand Island, NY).

Subunit Vaccine and Diagnostic Antigen

Vaccine preparation has been previously described.[7] Essentially, viral glycoproteins were extracted from solubilized PRV infected pig kidney cell membranes and isolated by lectin affinity chromatography using Lens culnaris agglutinin covalently linked to agarose beads (E-Y Laboratories, San Mateo, CA). Bound glycoprotein was eluted with 2.5% mannose in 0.025M Tris/Tricine (TT) buffer pH 8.4 and concentrated 10 fold by ultra-filtration through a 30K molecular weight cut-off membrane (Diaflo YM30 ultrafilter, Amicon Corporation, MA). The protein concentration of column eluate was determined by a dye binding method described by Bradford.[2] Column eluate was diluted to specific protein concentration in TT buffer and emulsified in an equal volume of Freund's incomplete adjuvant.

Diagnostic antigen (DA) was serum-free maintenance media of MDBK cells collected 6h after PRV infection. This preparation was shown in an earlier study to contain high levels of relatively pure excreted PRV glycoprotein. The molecular weight of this protein was determined to be 110K by size exclusion high performance liquid chromatography,[9] 106K by sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and 98K by SDS-PAGE analysis of ^{35}S methionine labelled immunoprecipitate. The physical and biochemical properties of DA closely resemble those described for the PRV protein gX.[10,12] Normal host cell antigen (NHC) was serum-free maintenance media collected from identically treated normal MDBK cells.

Serum Neutralization

Serum neutralization (SN) titers were determined as previously described[5,9] and are expressed as the reciprocal of $\log_2$.

Enzyme-Linked Immunosorbent Assay

Both DA and NHC were diluted in antigen coating buffer consisting of 0.02M sodium carbonate/bicarbonate buffer pH 9.6 containing 1 mg/ml of water soluble carbodiimide 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide (Sigma Chemical Company, St. Louis, MO) to enhance antigen binding.[3] One hundred ul of each antigen preparation was incubated at 4°C for 16-24h in individual wells of polystyrene microplates (Immulon I microplates, Dynatech Labs Inc., Alexandria, VA). Unadsorbed antigen was removed by

washing all wells three times with 0.01M phosphate buffered saline, pH 7.2 containing 0.5% Tween 20 (ELISA wash buffer). Unreacted sites were blocked by treating individual wells with 2.0% gelatin in antigen coating buffer for 2h at 37°C. Serums were diluted 1/20 in antibody/conjugate diluent consisting of 0.05M Tris, 150mM sodium chloride, 0.01mM EDTA, pH 7.4 containing 0.05% Tween 20 and 1.0% gelatin and added at a rate of 100ul to individual wells coated with DA and NHC. Serum preparations were incubated at 37°C for 15 min, washed eight times with ELISA wash buffer and air dried for 10 min. Goat anti-porcine IgG (H+L) conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was diluted 1/3500 in antibody/conjugate diluent, added at a rate of 100ul/well and incubated at 37°C for 45 min. Plates were washed eight times with ELISA wash buffer as described above. One hundred ul of enzyme substrate that was prepared by adding 50 ul of a 30% concentrated solution of hydrogen peroxide and 20 mg of o-phenylenediamine chromagen to 100 ml of 0.02M citric acid, pH 5.0, was added to individual wells and reacted in the dark at 25°C for 20 min. The resulting color reactions were stabilized with 4.5M sulfuric acid.

Absorbance values were determined with an automatic microplate reader (Dynatech MR600 microplate reader, Dynatech Labs Inc.) with a test filter wavelength of 490 nm. Absorbance values of individual samples were determined by subtracting the optical density (O.D.) value of NHC from the O.D. value of DA and expressed as the mean of four replications. A baseline O.D. reaction was established using 40 known negative serums that gave a corrected O.D. range of 0.000 to 0.100. Corrected O.D.

values of 0.150 or greater were considered positive. Antibody titers were considered to be directly proportional to corrected O.D. values.

Production of Radiolabelled Diagnostic Antigen

The production of radiolabelled DA for immunoprecipitation was the same as the production of unlabelled DA with the following exceptions. Virus inoculum was diluted in D-MEM containing 2% horse serum. After 90 min the inoculum was removed, the cell monolayer was washed three times with serum-free and methionine free D-MEM, and replaced with the same medium at the rate of 5 ml/150 cm^2 flask. After 1h the media was replaced with 5.0 ml of fresh serum free D-MEM containing 100 uCi [^{35}S]Methionine/ml (Amersham Corporation, Arlington Heights, IL). At 6h p.i. the media was harvested, centrifuged at 100,000 x g for 90 min and stored in aliquots at -70^{o}C until used.

Sodium Deodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium deodecyl sulfate-polyacrylamide gel electrophoresis was performed by standard methods.[4] Briefly, DA was diluted in an equal volume of sample preparation buffer consisting of 2% sodium deodecyl sulfate, 4% B-mercaptoethanol, 0.05% bromophenol blue and 50% glycerol in 0.5M Tris pH 6.8. Stacking and separating gels consisted of 4 and 10% acrylamide monomer respectively, cross-linked with bis-acrylamide at a ratio of 30:0.8. All gels were electrophoresed at 25 mA until the samples reached the stacking gel/separating gel interface. Sample

separation was completed by electrophoresis at a constant current of 35 mA.

Radioimmunoprecipitation

Radioimmunoprecipitation was performed as described by Wathen and Wathen.[13] Briefly, 100 ul of radiolabelled DA was combined with 25 ul of test serum. The mixture was gently vortexed, and incubated at 4°C for 16-18h. The antigen-antibody complexes were harvested by adding 25 ul of a 40% vol/vol solution of freshly washed S.aureus protein A-sepharose beads (Sepharose CL-4B, Pharmacia Fine Chemicals, Piscataway, NJ). This preparation was incubated at 4°C for 1 h with periodic gentle agitation and the protein A beads were harvested by centrifugation for 2 min. The beads were washed six times with TT buffer, boiled for 3 min in sample preparation buffer and analyzed by SDS-PAGE. Electrophoresed gels were impregnated with 2,5-diphenyloxazole scintillant in dimethyl sulfoxide, washed with deionized water, dried under vacuum and exposed to XAR5 film (Eastman Kodak Company, Rochester, NY) at -70°C for three to six days.[6]

Experimental Design

Two studies were conducted. The first study was designed to characterize the effect of the level of immunity and virus exposure dose on the antibody response of pigs to diagnostic antigen (DA). The second study was designed to determine if latent infections could be established in subunit vaccinated pigs by low doses of virus without inducing

persistent antibody titers to DA.

Sixteen pigs were used in the first study. Two groups of six pigs each were inoculated subcutaneously twice at 21 day intervals with 100 and 50 ug of antigen respectively. A third group of four pigs served as non-vaccinated controls. All three groups were equally divided, separately housed and nasally challenged with 2.0 ml of virus suspension containing $10^{6.0}$ or $10^{2.7}$ PFU of virulent PRV 21 days after the second vaccine dose. Serum was collected on both days of inoculation, 10 days before virus challenge, the day of challenge, and on days 4, 7, 10, 14, and 21 days p.c. Serums from each group were pooled by day and analyzed for antibody to DA by ELISA and RIP. Serum virus neutralization titers were determined for individual serums.

The virus latency study was conducted with 16 pigs. Ten of these pigs were inoculated s.c. with one 100ug and two 50ug doses of vaccine antigen. Vaccinates and six non-vaccinated controls were separated 21 days after the third vaccine dose and nasally challenged with $10^{2.3}$ PFU of PRV in two ml of maintenance medium. Nasal swabs were collected prior to challenge and on days one through four p.c. and assayed for the presence of virus. Serums were collected on days 0, 4, 7, 10, 14, and 21 p.c. and assayed individually for virus neutralizing activity and antibody to DA.

Challenged vaccinates were subsequently divided into three housing groups of three to four pigs each and maintained through day 113 p.c. During this period SN titers of individual pigs continuously declined indicating the absence of spontaneous viral recrudescence. All

vaccinates were checked for latent infections on day 113 p.c. by injecting them with four daily doses of dexamethasone given i.p. at the rate of 1.5 mg/kg body weight. Previous studies demonstrated that similar doses of dexamethasone successfully reactivated latent PRV infections.[11,14] Nasal swabs were collected prior to treatment and on days one through six post-treatment (p.t.) and assayed for virus activity. Serums were collected on days 0, 10, 14, 21, and 28 p.t. and individually assayed for neutralizing and DA antibody. Pigs were considered to have been latently infected if virus was recovered and/or a two $\log_2$ or greater rise in SN titer occured following dexamethasone treatment.

RESULTS

The Effect of PRV Exposure Dose on the Antibody Response to Diagnostic Antigen (DA)

The effect of virus exposure dose on the antibody response to DA is summarized in Figures 1 and 2. Enzyme-linked immunosorbant assay and RIP data clearly demonstrate that virus exposure dose influences the time of appearance and the relative level of the antibody response to DA in subunit vaccinated pigs. No antibody to DA was detected in either vaccine group by ELISA or RIP on the day of virus challenge. Subsequently, antibody to DA was first detected by ELISA on days 7 and 10 in the low and high vaccine dose groups respectively and by RIP on day 7 following nasal challenge with $10^{6.0}$ PFU of virus. In contrast, antibody to DA was not detected by either ELISA or RIP until day 14 after exposure to $10^{2.7}$ PFU.

Antibody to DA was detected earlier in the high vaccine dose group than in either the low vaccine dose group or non-vaccinated controls following challenge with $10^{6.0}$ PFU virus. Antibody to DA was also detected earlier in both vaccine dose groups than in controls after challenge with $10^{2.7}$ PFU virus. These results were unexpected and suggest that the subunit vaccine may have sensitized these pigs to DA.

Figure 1. Radioimmunoprecipitation detection of post-challenge antibody responses to [^{35}S]Methionine-labelled diagnostic antigen in pooled serum collected from subunit vaccinated pigs. For all vaccine/challenge combinations n=3; Vaccine Dose I= 2x100ug doses; Vaccine Dose II= 2x50ug doses; High Challenge= $10^{6.0}$ PFU of pseudorabies virus (PRV); Low Challenge= $10^{2.7}$ PFU of PRV; -C= normal negative pre-vaccination pig serum; +C= PRV hyperimmune pig serum.

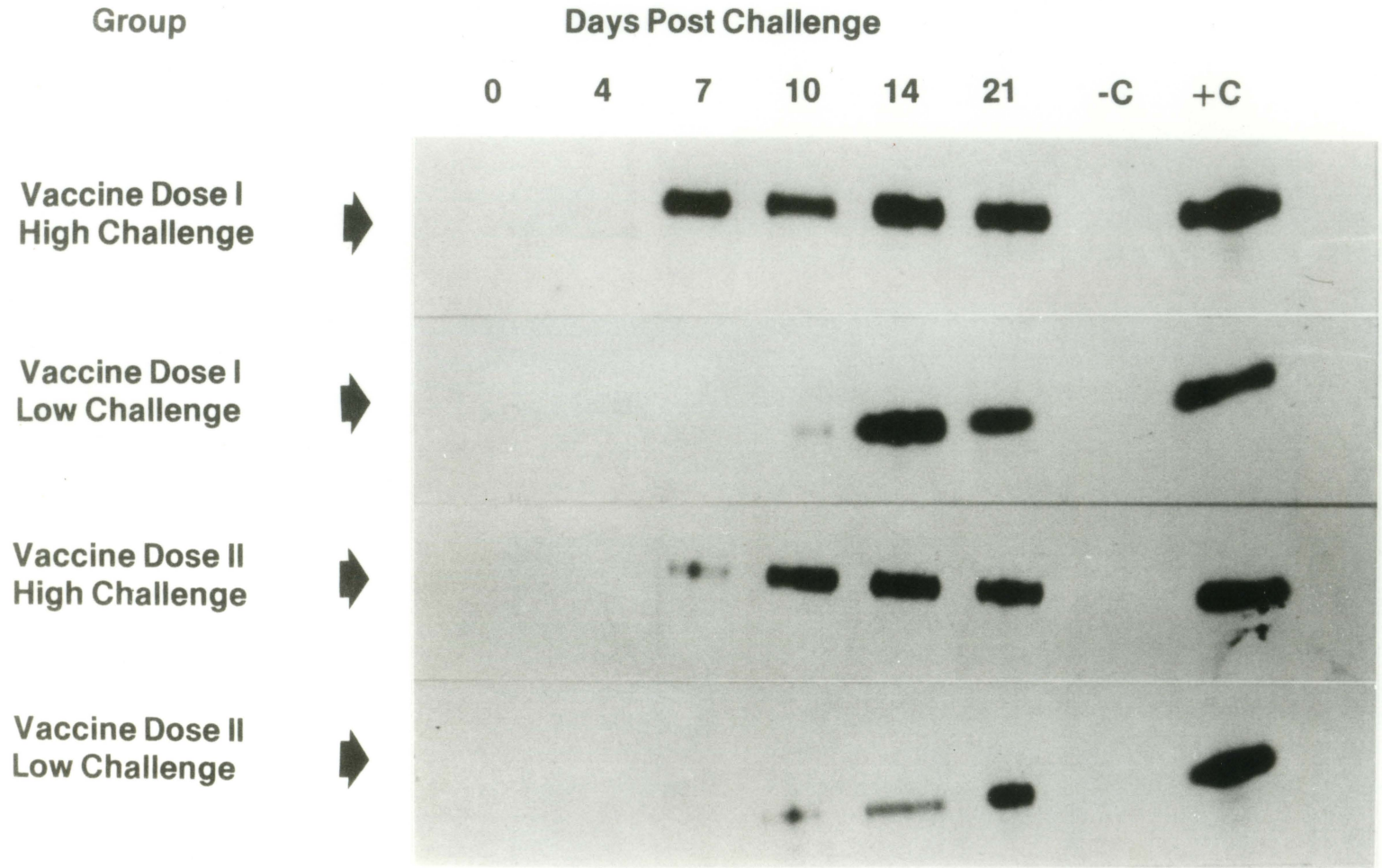
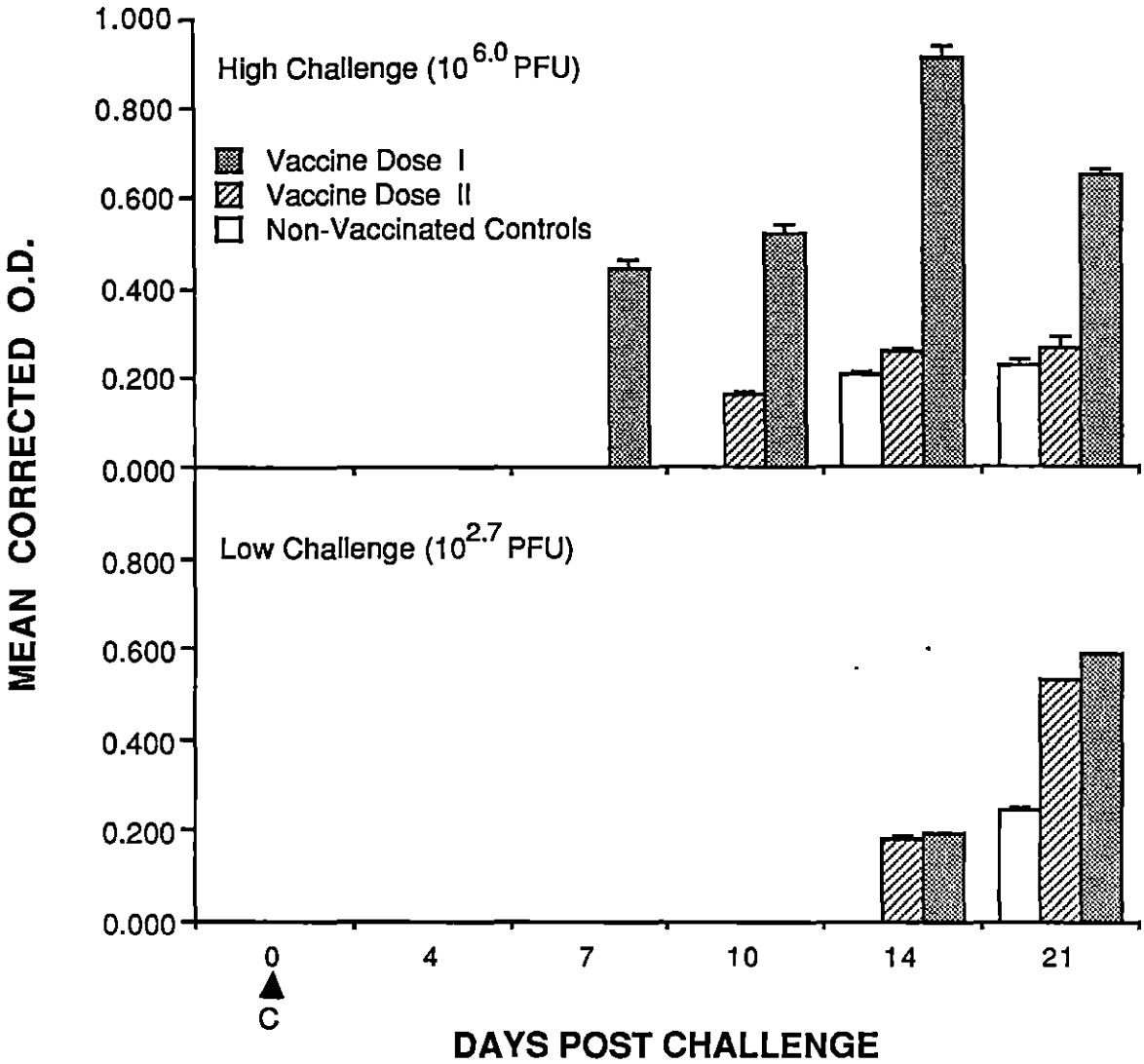

Group
Days Post Challenge
0
4
7
10
14
21
-C
+C
Vaccine Dose I
High Challenge
Vaccine Dose I
Low Challenge
Vaccine Dose II
High Challenge
Vaccine Dose II
Low Challenge

Figure 2. Enzyme-linked immunosorbant assay detection of post-challenge (p.c.) antibody responses to diagnostic antigen in pooled serum collected from subunit vaccinated and non-vaccinated pigs.
Vaccine Dose I= 2x100ug doses; Vaccine Dose II= 2x50ug; C= day of PRV challenge.


1.000
0.800
0.600
0.400
0.200
0.000
High Challenge ($10^{6.0}$ PFU)
Vaccine Dose I
Vaccine Dose II
Non-Vaccinated Controls
0.800
0.600
0.400
0.200
0.000
Low Challenge ($10^{2.7}$ PFU)
MEAN CORRECTED O.D.
0
4
7
10
14
21
C
DAYS POST CHALLENGE

Two $\log_2$ or greater increases in SN titer preceded detection of DA specific antibody by four days following virus challenge with $10^{2.7}$ PFU (Figures 2 and 3). In contrast antibody to DA was detected 4 days before increases in SN titer when vaccinates and controls were challenged with $10^{6.0}$ PFU (Figures 2 and 3).

The Response of Immunized Pigs to Challenge with a Low Dose of Virus

The SN antibody response of immunized pigs following challenge with a low dose of virus is summarized in Figure 4. The group mean SN titer on day 0 was 2.5 $\pm$0.2. This titer rose to a maximum of 6.3 $\pm$0.4 on day 14 p.c. and steadily declined thereafter to a mean value of 3.5 $\pm$0.3 by day 113 p.c. when dexamethasone treatment was initiated. The SN response of individual pigs followed the same general pattern as the mean group response. The absence of periodic increases in SN titer indicated that virus recrudescence did not occur prior to dexamethasone treatment.

Antibody responses to DA are summarized in Figure 5. No antibody to DA was detected by ELISA on the day of challenge with $10^{2.3}$ PFU of virus. Antibody to DA was initially detected by ELISA on day 14 p.c. in eight of 10 pigs. No virus was recovered from nasal swabs during this period. The two remaining pigs seroconverted on day 21 p.c. while one seropositive pig from day 14 became seronegative and remained so through day 113 p.c.

Figure 3. Serum neutralizing antibody responses of individual subunit vaccinated and non-vaccinated pigs following pseudorabies virus (PRV) challenge. For all vaccine/challenge combinations n=3; n=2 for control groups except that n=1 for the control group exposed to $10^{6.0}$ PFU of pseudorabies virus (PRV) after day 10 p.c.; Vaccine Dose I= 2x100ug doses; Vaccine Dose II= 2x50ug doses; day -63= day of initial vaccine inoculation; C= day of PRV challenge.

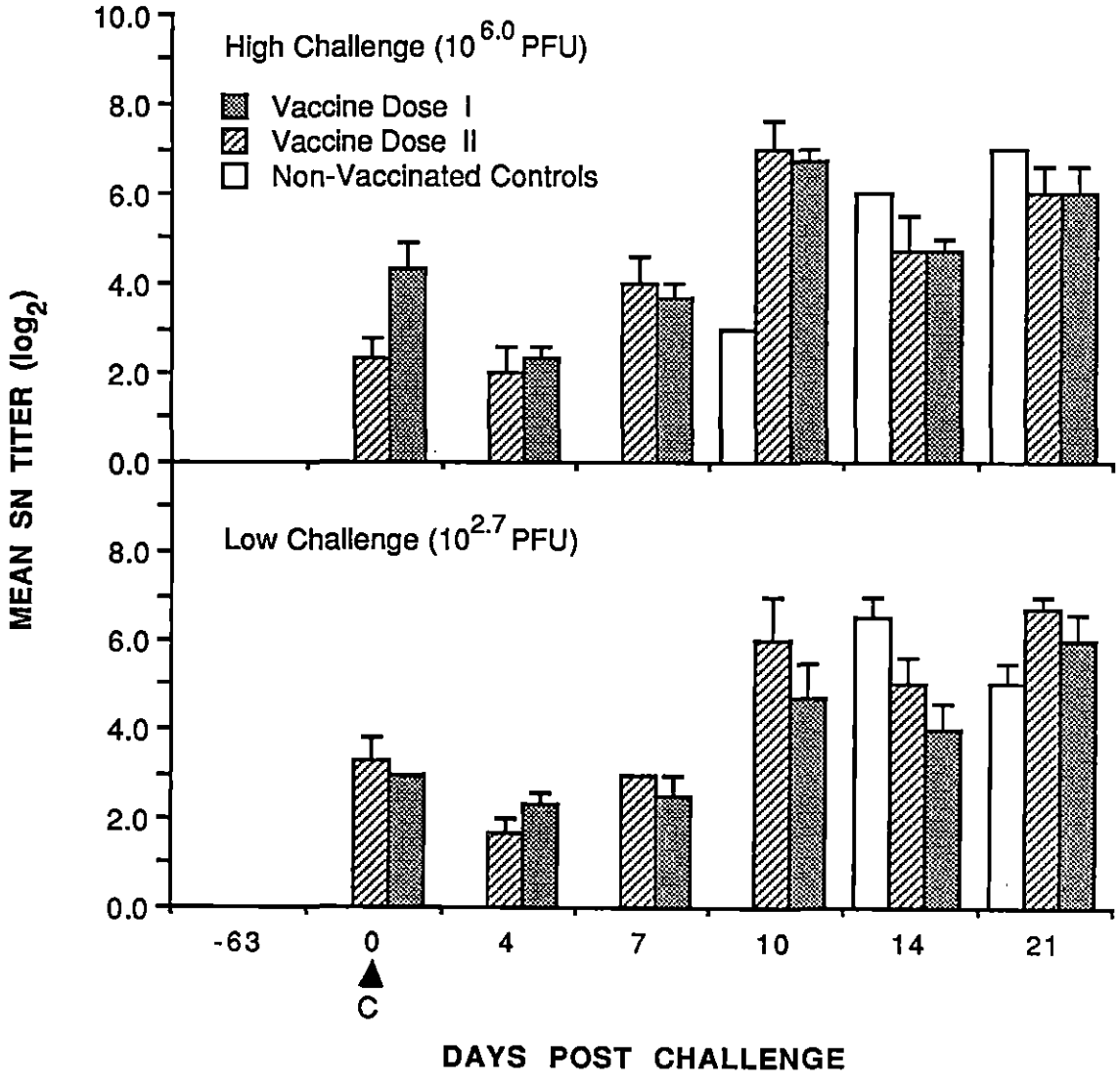

High Challenge ($10^{6.0}$ PFU)
Vaccine Dose I
Vaccine Dose II
Non-Vaccinated Controls
Low Challenge ($10^{2.7}$ PFU)
MEAN SN TITER (log_2)
10.0
8.0
6.0
4.0
2.0
0.0
-63
0
4
7
10
14
21
C
DAYS POST CHALLENGE

Figure 4. Serum neutralizing antibody response of subunit vaccinated pigs (n=10) after exposure to $10^{2.3}$ PFU of pseudorabies virus (PRV) and subsequent dexamethasone treatment. C= day of PRV challenge; D= day of initial dexamethasone treatment.

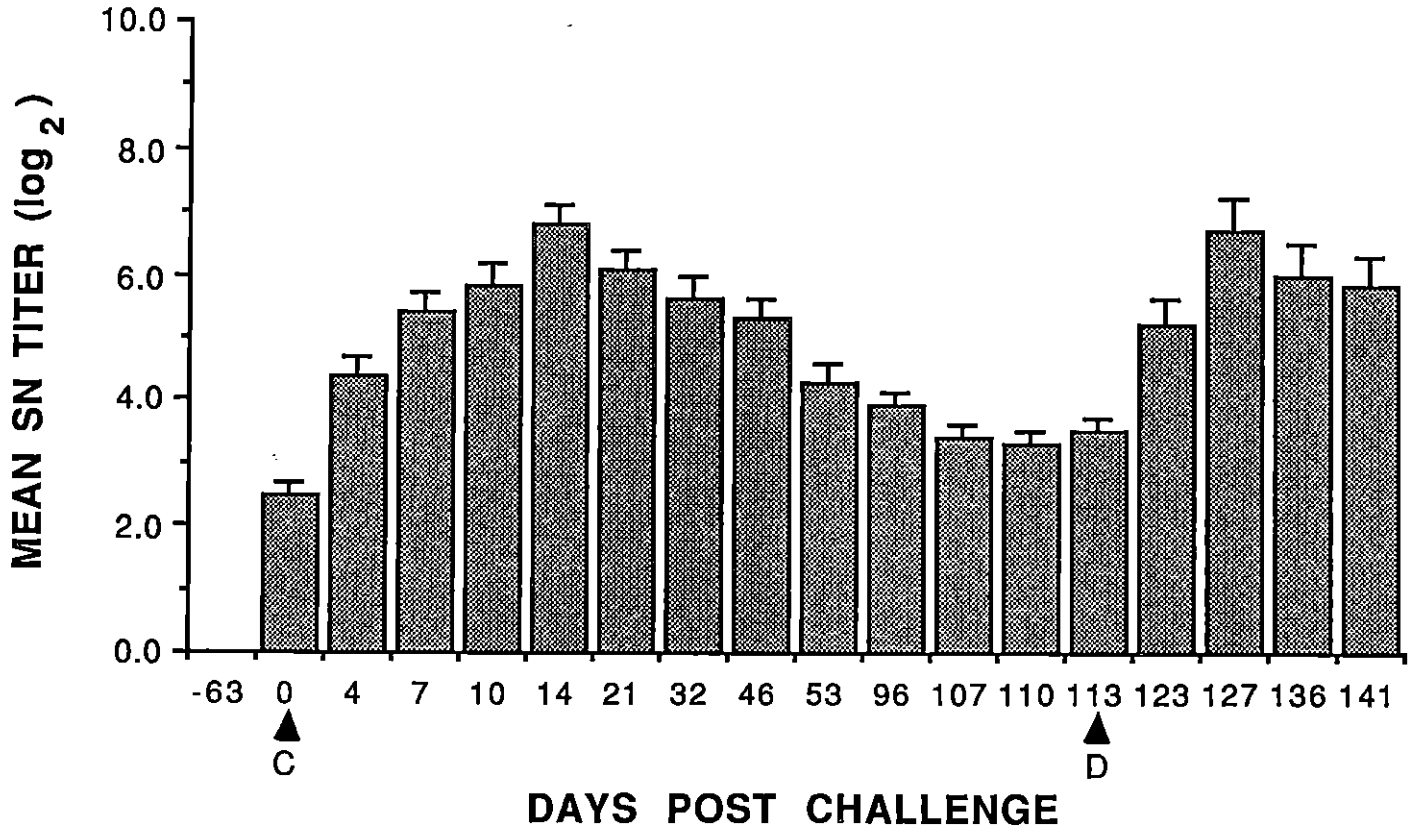
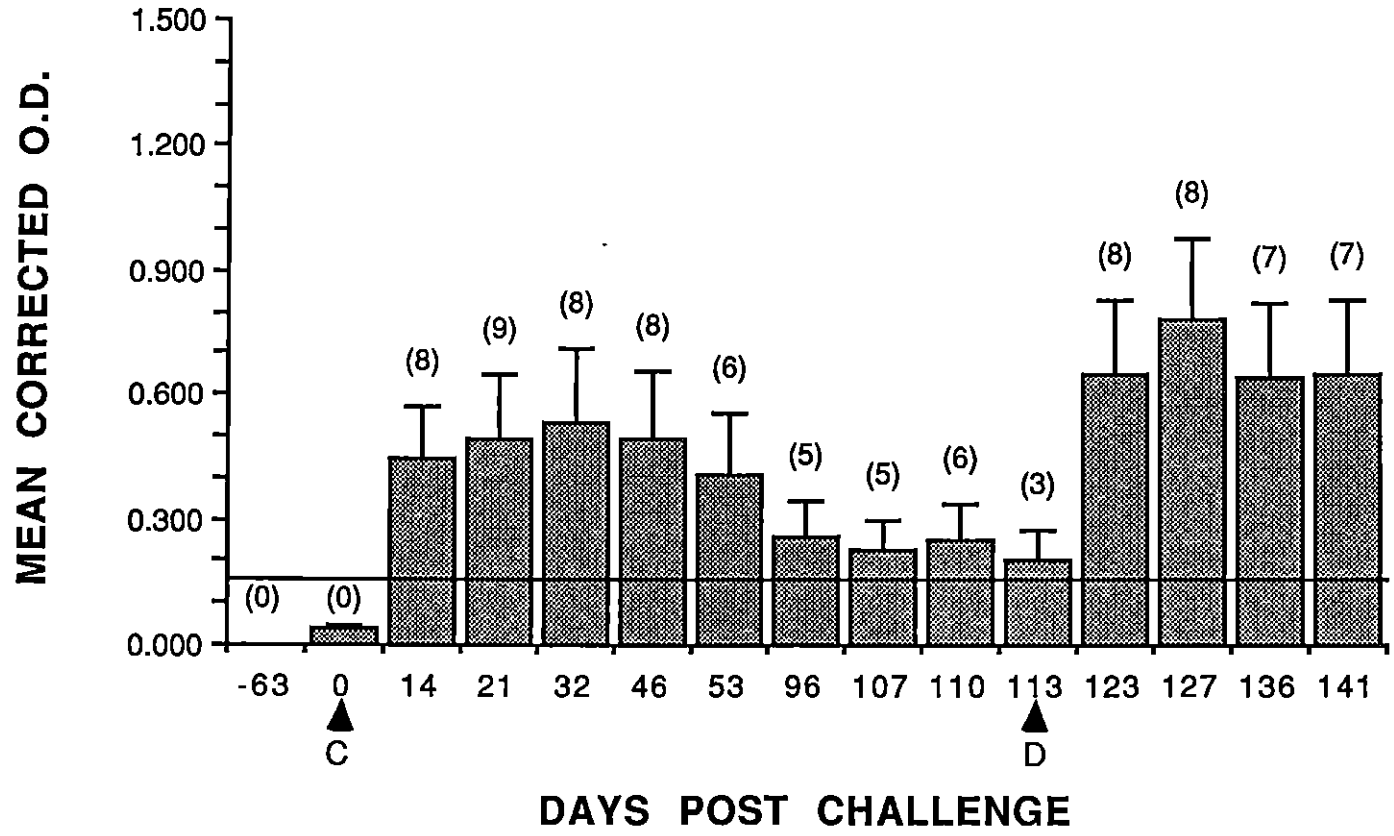

MEAN SN TITER (log_2)
10.0
8.0
6.0
4.0
2.0
0.0
-63 0 4 7 10 14 21 32 46 53 96 107 110 113 123 127 136 141
C
D
DAYS POST CHALLENGE

Figure 5. Antibody response of subunit vaccinated pigs (n=10) to diagnostic antigen after exposure to $10^{2.3}$ PFU of pseudorabies virus (PRV) and subsequent dexamethasone treatment as determined by enzyme-linked immunosorbant assay. Positive threshold equals a corrected optical density of 0.150 and is represented by ———; (#)= number of pigs above the positive threshold; C= day of PRV challenge; D= day of initial dexamethasone treatment.


MEAN CORRECTED O.D.
1.500
1.200
0.900
0.600
0.300
0.000
(0)
(0)
(8)
(9)
(8)
(8)
(6)
(5)
(5)
(6)
(3)
(8)
(8)
(7)
(7)
-63
0
14
21
32
46
53
96
107
110
113
123
127
136
141
C
D
DAYS POST CHALLENGE

Antibody to DA continued to be detected through day 113 p.c. in three of 10 pigs and until days 21, 46, 53, and 110 p.c. in one, two, one, and two pigs respectively.

The same general pattern of antibody response was revealed by RIP. Antibody to DA was initially detected in all 10 pigs by day 14 p.c. and continued to be detected through day 113 p.c. in five pigs and through days 46, 96, and 110 p.c. in one, two and two pigs respectively.

Eight of the 10 pigs were considered to have been latently infected based on increases in SN titer of two $\log_2$ units within the first 10 days after dexamethasone treatment and virus recovery from six of the eight pigs between days one and three p.t. The amount of virus recovered from these six pigs ranged from $10^{1.0}$ to $10^{2.0}$ PFU. Antibody levels to DA as demonstrated by the intensity of the ELISA reactions also rose concurrently with the SN titer in six of eight latently infected pigs (Figure 5). Failure to detect increases in DA antibody by ELISA in the two remaining latently infected pigs was unexpected since virus was recovered and SN titers rose in both pigs after dexamethasone treatment. However, an increase in DA antibody apparently did occur in one of these pigs because RIP detected antibody after but not immediately before treatment.

DISCUSSION

The preceding study has clearly demonstrated that latent infections can be readily established in subunit vaccinated pigs that are exposed to low doses of virus. Furthermore, the ability to identify these infections by assaying for antibody to a non-vaccine early excreted 98K PRV protein using ELISA was variable. Only four of eight latently infected pigs had detectable DA antibody 110 days after challenge. Antibody to DA was detected in the remaining pigs for periods ranging from 14 to 53 days p.c. The variability of this response to DA may have been due to the ability of the immunized pigs to suppress virus replication which in turn limited the amount of DA that was produced in the host. This explanation may also account for the failure of two latently infected pigs to produce ELISA detectable DA antibodies following viral recrudescence. This hypothesis is supported by the fact that a direct relationship exists between virus challenge dose and DA antibody response in both subunit vaccinate and non-vaccinated controls (Figure 1).

The variable nature of the antibody response of immunized pigs to the 98K DA that was observed in the above study is in direct contrast to earlier work in which all similarly immunized pigs responded to high doses of virus.[9] The inability to consistently detect antibody to the 98K DA in pigs exposed to low doses of virus raises the question of whether or not diagnostic tests using this antigen can be used to reliably detect PRV infections in the field. Although the present study

only focused on the antibody response of subunit vaccinated pigs to a single protein it is possible that similar results may have occured if other non-vaccine diagnostic antigens were used. Consequently, it is essential that all potential non-vaccine diagnostic antigens should be thoroughly evaluated for efficacy in immunized pigs that are exposed to low doses of virus before they are approved for use in PRV control programs.

The preceding study also suggests that the ability of diagnostic tests to detect virus infection may be enhanced by sensitizing pigs with amounts of specific diagnostic antigens that do not induce detectable antibody. Sensitizing doses of specific diagnostic antigens could be incorporated in the initial dose of subunit vaccine. Support for this concept is based on the fact that vaccinated pigs responded to DA sooner than non-vaccinated pigs when tested by ELISA (Figure 2). This sensitization is believed to have been due to the presence of shared antigenic epitopes between the subunit vaccine and the DA. Earlier work in this laboratory demonstrated by crossed immunoelectrophoresis that a degree of relatedness did exist between a component of the vaccine and DA.[7] It is also possible that the vaccine was contaminated with small amounts of DA. However, this explanation is not considered likely because multiple vaccine doses did not induce detectable antibody to DA prior to virus exposure.

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SECTION II. WESTERN IMMUNOBLOT ANALYSIS OF THE ANTIBODY RESPONSE OF SUBUNIT VACCINATED PIGS TO THE NUCLEOCAPSID PROTEINS OF PSEUDORABIES VIRUS

SUMMARY

The antibody response to pseudorabies virus (PRV) nucleocapsid proteins (NCP) was analyzed by western-immunoblot in 10 pigs immunized with PRV envelope glycoproteins following nasal challenge with $10^{2.3}$ PFU of virus. Antibody to five NCP with molecular weights of 140K, 63K, 41K, 34K, and 23K was initially detected in all vaccinates and six non-vaccinated controls between days 14 and 21 post-challenge (p.c.). Antibody to 140K NCP was detected in eight of 10 subunit vaccinated pigs from 32 to 113 days p.c. Similarly, antibody to 63K NCP was detected in nine of 10 subunit vaccinated pigs from 32 to 113 days p.c. Nine of 10 subunit vaccinated pigs produced antibody to 41K and 34K NCP from 21 to 113 days and 32 to 113 days p.c. respectively. Antibody to 23K NCP was detected in eight of 10 subunit vaccinated pigs for 113 days p.c. One subunit vaccinated pig did not produce antibody to any NCP for a period longer than 32 days p.c. The antibody responses to two or more individual NCP that included the 23K or 41K NCP were required to detect PRV infection in 9 of 10 subunit vaccinated pigs for 113 days p.c. These results indicate that NCP may be used as a non-vaccine diagnostic antigen in conjunction with PRV envelope glycoprotein based subunit vaccines.

INTRODUCTION

Subunit vaccines may play an important role in PRV control programs because their use will permit the detection of wild type virus infection in vaccinates.[10] The identification of virus infected vaccinates will depend on the ability to detect antibody to non-vaccine virus antigens. Since subunit vaccines will consist of one or more PRV envelope glycoproteins it is very likely that a non-vaccine diagnostic antigen that is compatible with one subunit vaccine may represent an immunogen in another vaccine. As a result control programs will be more difficult to administrate because care will be required to insure that all pigs are vaccinated with the same vaccine to avoid false positive reactions. Diagnostic laboratories will also be required to test for antibody to more than one non-vaccine diagnostic antigen. The use of a universal diagnostic antigen would facilitate the administration of control programs. Pseudorabies virus nucleocapsid proteins (NCP) are logical candidates for a universal diagnostic antigen because they have not been shown to play a role in protective immunity and will most likely not be a component of subunit vaccines. The following study was undertaken to determine if pigs immunized with PRV envelope glycoprotein responded to NCP following virus challenge.

MATERIALS AND METHODS

Virus, Cells, and Medium

Pseudorabies virus (PRV) strain Be[9] was used for viral antigen production and challenge virus. Madin Darby bovine kidney (MDBK) cells obtained from the National Veterinary Services Laboratory, Ames, IA were used for viral antigen production. Pig kidney (PK) 1a cells obtained from the Veterinary Medical Research Institute, Iowa State University, Ames, IA were used to propagate challenge virus. Growth medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY).

PRV Subunit Vaccine

Vaccine preparation has been previously described.[8] Essentially, viral glycoproteins extracted from solubilized PRV infected PK cell membranes were isolated by lectin affinity chromatography using Lens culnaris agglutinin covalently linked to agarose beads (E-Y Laboratories, San Mateo, CA). Bound glycoprotein was eluted with 2.5% mannose in 0.025M Tris/Tricine, pH 8.4 and concentrated 10 fold by ultra-filtration through membrane with a 30,000 molecular weight cut-off limit (Diaflo YM30 membrane, Amicon Corporation, Danvers, MA). The protein concentration of the column eluate was determined by a dye binding method described by Bradford.[2] Vaccine antigen was diluted and emulsified in an equal volume of Freund's incomplete adjuvant.

PRV Nucleocapsid Antigen Preparation

Pseudorabies virus nucleocapsids were isolated using a modification of the method of Gibson and Roizman for isolation of herpes simplex type 1 and 2 nucleocapsids.[3] Cells from 10-850cm^2 roller bottles were harvested 18 to 24 hours after infection and pelleted by low speed centrifugation at 1000 x g for 15 minutes. The cell pellet was washed three times in 500mM NaCl, 20mM Tris, pH 7.5 (TBS), resuspended in 20ml of cell lysis (CL) buffer consisting of 150mM NaCl, 10mM Tris, 2mM $MgCl_2$, pH 7.5, containing 1% Nonidet P-40 and incubated for 30 minutes at 0^{o}C. Infected cell nuclei were isolated by low speed centrifugation, washed twice in CL buffer and lysed in nuclei disruption (ND) buffer consisting of 5% sodium deoxycholate (DOC) in CL buffer. The lysate was incubated at 37^{o}C for 1 hour in the presence of 50ug/ml deoxyribonuclease I (Sigma Chemical Company, St Louis, MO) and clarified by low speed centrifugation. The clarified supernatant was layered onto 5 to 45% glycerol step gradients. Nucleocapsids were pelleted by centrifugation at 24,000 rpm for 90 minutes and resuspended in TBS. Resuspended nucleocapsids were adsorbed for 30 minutes at room temperature with Lens culnaris agglutinin covalently linked to agarose beads (E-Y Laboratories) to remove residual PRV glycoproteins. The nucleocapsid preparation was aliquoted and stored at -70^{o}C.

Sodium Deodecyl Sulfate-Polyacrylamide Electrophoresis (SDS-PAGE)

Pseudorabies virus NCP and molecular weight markers were diluted in an equal volume of sample preparation buffer consisting of 2% SDS, 4% B-mercaptoethanol, 0.05% bromophenol blue and 50% glycerol in 0.5M Tris buffer, pH 6.8, boiled for five minutes and separated under reducing conditions by standard SDS-PAGE methods.[4] Stacking and separating gels consisted of 4 and 10% acrylamide monomer respectively, cross-linked with bis-acrylamide at a ratio of 30:0.8. All gels were electrophoresed at 25 mA until the samples reached the stacking gel/separating gel interface. Sample separation was completed by electrophoresis at a constant current of 35 mA until the dye front had migrated 15 cm. The 15 cm migration limit was imposed on all separations to standardize protein migration patterns for later comparison.

Electrophoretic Transfer of PRV Proteins

Pseudorabies virus NCP and molecular weight markers were electrophoretically transferred to nitrocellulose immediately following SDS-PAGE. Proteins were transferred for 16 hours at 30 V followed by a 2 hour finishing period at 100 V in transfer buffer consisting of 25mM Tris, 192mM Glycine, pH 8.3 and 20% v/v methanol. Following transfer, the nitrocellulose sheets were cut into 0.5 cm strips, air dried, and used immediately. Total NCP profiles were visualized by india ink staining as described by Hancock and Tsang.[5]

Western-Immunoblot Assay

Nitrocellulose strips containing separated NCP were were blocked with 2.5% gelatin in TBS for two hours at 37°C and were washed for three 5 minute cycles in TBS containing 0.05% Tween 20 (TTBS). Serums were diluted 1/100 in TTBS containing 1% gelatin, incubated with the nitrocellulose strips for 1 hour at room temperature, and washed as described above. Specific antigen-antibody reactions were visualized using a biotin-streptavidin detection system. Biotin labelled goat anti-porcine IgG (H+L) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used at a 1/2000 dilution followed by streptavidin-horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories) at a 1/4000 dilution. All incubations were for one hour at room temperature with washing as described above. Enzyme substrate was prepared immediately before use and consisted of 60mg of 4-chloro-1-napthol in 20ml of ice cold methanol and 60ul of cold 30% hydrogen peroxide in 100ml of TBS. Color reactions were developed at room temperature in the dark for 40 minutes and stopped by two brief washes in deionized water.

Serum Neutralization

Serum neutralization (SN) titers were determined as previously described[6,10] and are expressed as the reciprocal of $\log_2$.

Experimental Design

Ten pigs were inoculated s.c. with one 100ug and two 50ug doses of vaccine antigen at three week intervals. Six pigs served as non-vaccinated controls. Vaccinates and controls were separated 21 days after the third vaccine dose and nasally challenged with $10^{2.3}$ PFU of PRV in two ml of maintenance medium. Serums were collected on days 0, 4, 7, 10, 14, and 21 p.c. and assayed for antibody to NCP. Non-vaccinated controls were killed at 21 days p.c. Challenged vaccinates were maintained through day 113 p.c. and the antibody response of these pigs to individual NCP was characterized by western-immunoblot assay. Specific antibody responses to individual proteins were analyzed by the Chi-square test for independence. During this period SN titers of individual pigs continuously declined indicating the absence of spontaneous viral recrudescence which might influence the duration of the antibody response to NCP antigens.

RESULTS

Detection of Antibody in Subunit Vaccinated Pigs to PRV Nucleocapsid Protein (NCP) by Western-Immunoblot

The antibody response of subunit vaccinated pigs as detected by western-immunoblot assay is illustrated in Figure 1. Antibody responses to a total of five NCP with molecular weights of 140K, 63K, 41K, 34K, and 23K were studied. The molecular weight estimations of the NCP utilized in this study are in close agreement with species described in previous studies.[1,11] The antibody response to each of these proteins is summarized in Table 1.

Table 1. The antibody response of 10 subunit vaccinated pigs to nucleocapsid protein (NCP) as detected by western-immunoblot assay

	Days tested post-challenge								
NCP	0	7	10	14	21	32	53	96	113
140K	0[a]	0	0	8	8	8	6	6	4
63K	0	0	0	9	9	9	8	5	5
41K	0	0	0	10	10	9	9	9	7
34K	0	0	0	10	10	10	9	6	6
23K	0	0	0	8	8	8	8	8	8

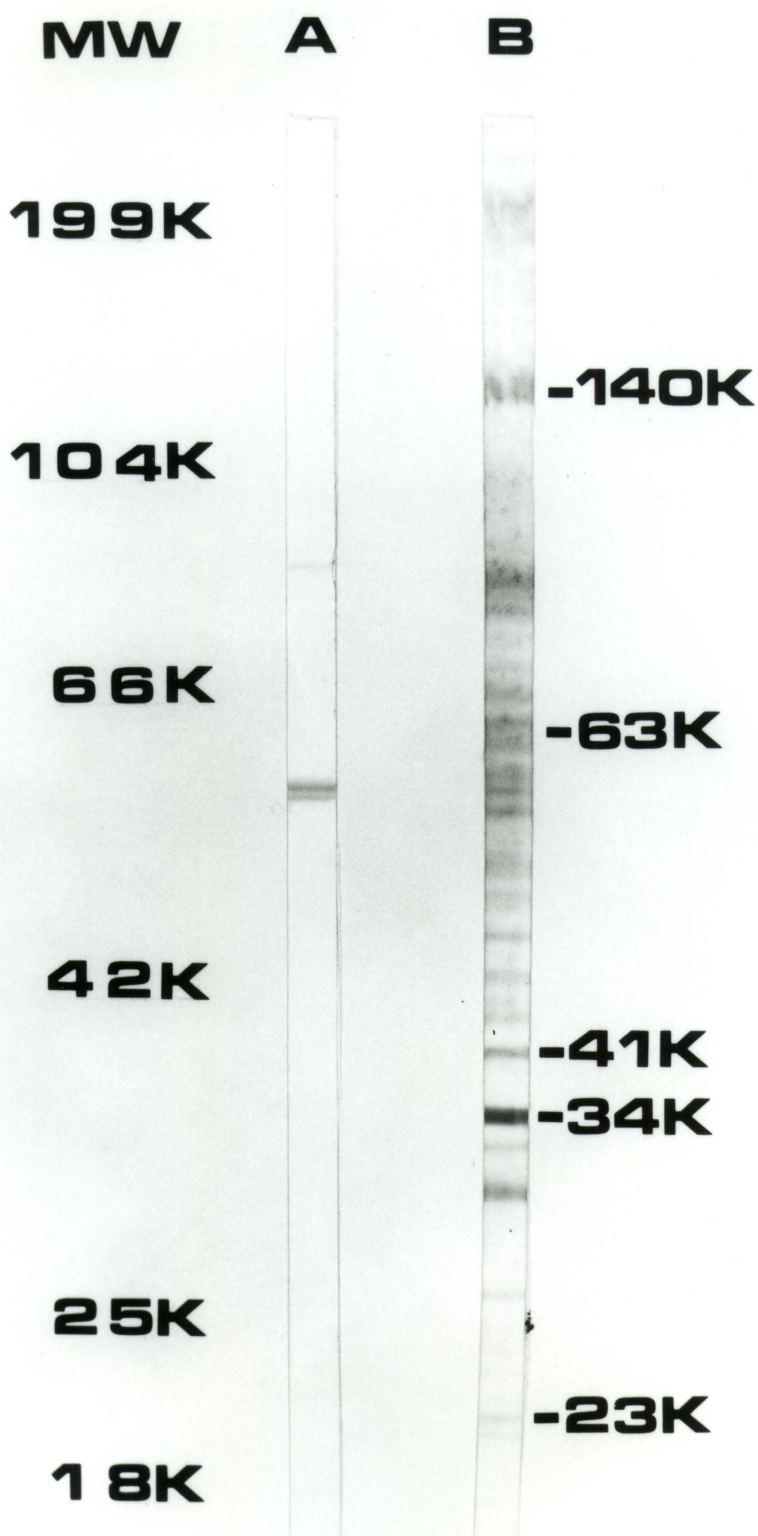
[a]The total number of pigs with detectable antibody to nucleocapsid protein.

Figure 1. Western-immunoblot detection of pre- and post-challenge antibody responses of subunit vaccinated pigs to five pseudorabies virus (PRV) nucleocapsid proteins (140K, 63K, 41K, 34K and 23K).

Lane MW= molecular weight markers

Lane A= PRV subunit vaccinate, pre-challenge

Lane B= PRV subunit vaccinate, 21 days post-challenge


MW
A
B
199K
104K
66K
42K
25K
18K
-140K
-63K
-41K
-34K
-23K

No antibody to any PRV NCP was detected prior to virus challenge. Antibody to NCP was first detected on day 14 or 21 p.c. in both subunit vaccinated and non-vaccinated control pigs. Antibody to the 140K NCP was initially detected in eight of 10 pigs. It ceased to be detected after days 32 and 96 p.c. in two and two pigs respectively, but was detectable in four pigs through day 113 p.c. Similar results were observed with the 63K NCP. Antibody was initially detected in nine of 10 pigs. It ceased to be detected after days 32 and 53 p.c. in one and two pigs respectively, but was detectable in six pigs through day 113 p.c. Antibody to 41K NCP was detected in all 10 subunit vaccinated pigs. Antibody to this protein ceased to be detected after days 21 and 96 p.c. in one and two pigs respectively, but was detected in seven pigs through day 113 p.c. Similarly, all subunit vaccinated pigs produced antibody to the 34K NC protein. Antibody to this protein ceased to be detected after days 32 and 53 p.c. in one and three pigs respectively, but was detectable in six pigs through day 113 p.c. Antibody to the 23K NC protein was initially detected in eight of 10 pigs and remained detectable in all eight through day 113 p.c. One pig did not respond to any NC protein for a period longer than 32 days p.c. Chi-square analysis demonstrated dependency in the antibody response to the 140K and 63K NCP. However, the analysis failed to demonstrate either dependency or independency of the antibody response to other combinations of individual NCP.

DISCUSSION

The following study was conducted to determine if virus infection induced by a low exposure dose could be detected in immunized pigs by testing for antibody to nucleocapsid proteins. Results of this study have shown that pigs will respond variably to five distinct NCP as revealed by the number of pigs with detectable antibody to specific NCP on day 113 p.c. (Table 1). At this time antibody to the 23K, 41K, 34K, 63K and 140K was detected in 8, 7, 6, 5, and 4 pigs respectively suggesting that the 23K, 34K, and 41K NCP were the most immunogenic components of the preparation. However, the antibody response to any of these three antigens alone failed to detect virus infection in all pigs tested on day 113 p.c. This indicates that combinations of individual NCP may be required for the reliable long term detection of PRV infection induced by low exposure doses in subunit vaccinated pigs. Assuming that the antibody responses to the 23K, 34K, and 41K NCP are independent a combination of these three antigens would detect virus infection in 99.8 and 97.6% of pigs at day 96 and 113 p.c. respectively. While these results appear promising additional testing with larger numbers of pigs are required to establish independency in the antibody response to 23K, 34K, and 41K NCP.

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SUMMARY AND CONCLUSIONS

Pseudorabies virus (PRV) subunit vaccinated pigs will be exposed to virus doses approaching the minimum infective dose under field conditions. This may result in PRV infections being suppressed before sufficient amounts of non-vaccine diagnostic antigen are produced. Insufficient amounts of diagnostic antigen would presumably induce low levels of specific antibody. Consequently, latent infections in these subunit vaccinated pigs may go undetected. In the preceding studies two diagnostic antigen preparations were evaluated with these concerns in mind.

Two experiments were conducted in the first study. In the first experiment the effect of subunit vaccine dose and virus exposure dose on the host antibody response to to a non-vaccine 98K early excreted PRV protein diagnostic antigen (DA) was studied in 18 pigs. Two groups of six pigs received high (200 ug total protein) and low (100 ug total protein) doses of subunit vaccine antigen. Two groups of three pigs served as non-vaccinated controls. One group of pigs from each of the preceding categories was intranasally challenged with $10^{2.7}$ or $10^{6.0}$ PFU of virus. Antibody to DA was detected by ELISA and RIP three to seven days earlier in pigs exposed to $10^{6.0}$ PFU. Vaccine dose did not affect DA antibody responses.

In a related experiment, the frequency of latency induced by low PRV exposure doses and the ability of ELISA using DA to detect latent infections was studied in 10 subunit vaccinated pigs. Following

intranasal challenge with $10^{2.3}$ PFU of virus serum-virus neutralizing (SN) and DA antibody titers were monitored for 113 days post-challenge (p.c.). The SN titers of individual pigs peaked between 14 and 21 days p.c. and steadily declined through day 113 p.c. indicating the absence of spontaneous viral recrudescence during the post-challenge period. Antibody to DA was detected in individual pigs for periods ranging from 14 to 113 days p.c. Dexamethasone was used to induce virus recrudescence beginning on day 113 p.c. Eight of 10 pigs (80%) were considered to have been latently infected based on two $\log_2$ or greater increases in SN titer and/or recrudescent virus isolation. Antibody to DA was detected in six of eight latently infected pigs following dexamethasone treatment. No DA antibody was detected in the remaining two pigs (25%) despite increases in SN titer and virus recovery from one pig.

The results of the two experiments in this first study indicate that the antibody response of subunit vaccinated pigs to DA is directly related to virus exposure dose. Furthermore, subunit vaccinated pigs can be latently infected with great frequency following exposure to virus doses approaching the minimum infective dose without developing persistent DA antibody. Both of these findings have important implications in future PRV control and eradication programs were the reliable identification of virus infected vaccinated pigs is critical.

In the second study, a preliminary experiment was conducted to evaluate the performance of selected PRV nucleocapsid proteins (NCP) as non-vaccine diagnostic antigens. The antibody responses of 10 subunit vaccinated and six non-vaccinated control pigs to five NCP were monitored

using western-immunoblot assay. The five NCP were not present in the lectin-derived PRV subunit vaccine preparations. All pigs were challenged with $10^{2.3}$ PFU of virus. No antibody to NCP was detected prior to virus challenge. Antibody to NCP was first detected on day 14 or 21 post-challenge (p.c.) in vaccinates and unvaccinated controls. Vaccinated pigs continued to be monitored for antibody to NCP through day 113 p.c. All pigs responded to one or more NCP. The duration of antibody responses to individual NCP was variable. Antibody responses to two or more NCP were required to detect virus infection in nine of 10 pigs for the entire 113 days p.c. One pig did not respond to any NCP antigen for a period longer than 32 days p.c.

The results of this study indicate that NCP may be used as non-vaccine diagnostic antigens in subunit vaccinated pigs. However, it is clear from this study that a single NCP component will not be adequate.

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