Characterization of virulent and attenuated strains of pseudorabies virus for thymidine kinase activity, virulence and restriction patterns

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CHARACTERIZATION OF VIRULENT AND ATTENUATED STRAINS OF PSEUDORABIES VIRUS FOR THYMIDINE KINASE ACTIVITY, VIRULENCE AND RESTRICTION PATTERNS

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Characterization of virulent and attenuated strains of pseudorabies virus for thymidine kinase activity, virulence and restriction patterns

by

Michael Dean McFarland

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

Pseudorabies virus is a neurotropic herpesvirus natural to swine which causes an acute, often fatal infection of young swine, lower mortality but high morbidity in older swine, and abortion and stillbirths in pregnant sows (12, 62, 173). Cattle, sheep, goats, dogs, and cats are also susceptible and in these species the disease is uniformly fatal (12, 42, 62, 82).

Since the early 1970s, the incidence of pseudorabies has significantly increased in the U.S. (39, 56, 62). The swine industry is very interested in measures that will reduce losses to the disease. Vaccination can be an effective method of controlling the disease in areas with a high incidence of pseudorabies (40, 109, 171). Attenuated vaccines that are greatly reduced in virulence for swine are commercially available, but these vaccines may retain considerable virulence for other species (98, 174).

Recently the role of the pseudorabies virus-coded enzyme, thymidine kinase, in determining neurovirulence has become recognized (169). The objectives of this study were to:

1) isolate a thymidine kinase-deficient mutant from a virulent PrV parental strain; assess the mutant for reversion to TK⁺; and assess the mutant for virulence in mice and young swine;

2) determine the TK phenotype and virulence for mice of the 3 federally licensed modified live vaccine strains and 2 field isolates;
3) compare the kinetic behavior of TK from the vaccine strains and field isolates;

4) evaluate the TK\textsuperscript{−} mutant for effectiveness as a pseudorabies vaccine in mice and swine; and

5) compare the migration patterns of restriction endonuclease-digested DNA of the strains included in the study.
LITERATURE REVIEW

Introduction

In 1902, the Hungarian veterinarian Aladar Aujeszky was the first to describe in the scientific literature a rapidly fatal disease (pseudorabies), occurring naturally in the bull, cat and dog, and which could be experimentally transmitted to rabbits (6). Von Rätz produced the disease in swine, in 1914 (177). The disease was referred to as infectious bulbar paralysis in the early literature, but in Europe the accepted name for the infection became Aujeszky's disease.

In the United States, a similar fatal disease of cattle, known as "mad itch" had been described in agricultural journals numerous times during the 19th century (68). In 1931, Shope concluded from experimental data that Aujeszky's disease, or "mad itch," and the disease of swine, pseudorabies, were caused by the same filterable virus (155). Shope further concluded that the virus is contagious among swine, that cattle contract the disease from swine through abrasions of the skin, and that the virus is not normally transmitted from cow to cow (156, 157).

Pseudorabies virus

Pseudorabies virus (PrV) (suid herpes virus 1) is classified as an alpha-herpes virus, in the same subfamily as herpes simplex virus (HSV) types 1 and 2, equine herpesvirus type 1, and bovine herpesvirus type 2 (145). Ben-Porat and Kaplan recently presented an extensive review of the biochemical and genetic properties of PrV (16). The mature virion
is approximately 180 nm in diameter and consists of a viral capsid of 110 nm diameter surrounded by a double- or triple-layer, lipid-containing membrane (34, 144).

The viral genome is a double-stranded DNA molecule of approximately $90 \times 10^6$ daltons molecular weight (MW) (147). Structural analysis of the genome reveals a long unique ($U_L$) sequence with a MW of $65 \times 10^6$, an internal inverted repeat sequence (IR) with a MW of $10 \times 10^6$, a terminal repeat (TR) sequence (MW $10 \times 10^6$), and a short unique ($U_S$) sequence (MW $6 \times 10^6$) located between the IR and TR regions (17, 161). Restriction enzyme cleavage maps (Figure 1) were provided by Ben-Porat and Kaplan (16).

Considerable biological, biochemical and genetic similarity has been demonstrated between PrV and HSV (15, 18, 84, 142), suggesting evolution from a common ancestor, and the utility of applying knowledge of the one virus toward better understanding of the other.

**Clinical signs**

Clinical signs associated with pseudorabies are variable and are influenced by the host species (42, 47, 51, 56, 82, 108), the degree of exposure (10), the route of exposure (141, 150), the age of the host (12, 27, 56, 71), and the strain of virus (12, 138, 139).

In cattle, sheep, dogs, cats, rats, and mice the clinical signs are primarily pruritis, convulsions, paralysis and rapid death, reflecting an acute infection of the central nervous system (CNS). Self-mutilation by biting, scratching, and rubbing of the skin against objects is common.
Figure 1. Restriction endonuclease cleavage maps of PrV DNA. Line A: Pst I digest; line B: Bam HI digest; line C: Kpn I digest; line D: physical model of the PrV genome showing the location of the unique long (U_L), unique short (U_S), inverted repeat (IR) and terminal repeat (TR) sequences. From Ben-Porat and Kaplan (16)
In swine, clinical signs are greatly influenced by age. In piglets less than 2 weeks of age, mortality can exceed 80%. Clinical signs reflect acute CNS infection: tremors, convulsions and coma followed by death are typical. In older swine, the CNS is often affected, but to a lesser degree. Incoordination, depression, and occasionally posterior paralysis occur. Common features in older swine are pyrexia, anorexia, sneezing and dyspnea. Vomiting, excessive salivation, blindness and pruritis are occasionally observed in swine infected with PrV. In mature swine, morbidity is often high but mortality is usually low; clinical disease can be mild and adult swine may experience subclinical infection (12, 56, 62, 130).

**Pathogenesis**

In swine, the natural route of exposure is by inhalation or ingestion of the virus (12, 150). Evidence has also been presented suggesting that some strains of PrV may be transmitted sexually (26, 52, 78).

After inhalation or ingestion of PrV, the virus replicates in the nasopharyngeal mucosa and tonsils. The virus next enters the lymphatics and can be isolated from draining lymph nodes. Limited evidence indicates that PrV may invade leucocytes, although detection of viremia is rare (12, 32, 182).

From the nasal and pharyngeal mucosa PrV enters the CNS, in which the principal histopathologic changes are seen (32, 41, 130). Neuro-pathological lesions associated with pseudorabies are a non-suppurative encephalitis, mild myelitis, and ganglioneuritis. Marked perivascular
cuffing is also observed. PrV can be isolated from the medulla and pons 24 hrs after infection. PrV may spread from the CNS to the spinal ganglia and femoral nerves (141).

PrV may also invade the lung, liver, and spleen from which the virus can occasionally be isolated and in which necrotic foci have been observed in histopathologic studies (32, 56, 150). Tropism for the lung is variable among strains; pneumonia can result from PrV infection (9) but significant pathologic changes in the lung are not caused by all strains (141, 150).

Infection of boars and pregnant sows can lead to serious losses in breeding herds. In utero infection with PrV can result in fetal mummification, abortion, stillbirths, and the birth of weak piglets (33, 57, 96, 173, 183). Infection of boars can result in testicular degeneration and infertility (67, 77, 106).

Similar to HSV (91), PrV has been shown to cause persistent and latent infections in its natural host. Sabó and Rajcání recovered PrV from explanted tonsils, cervical lymph nodes, nasal mucosa and gasserian ganglia removed from swine between 160 and 181 days post-inoculation (149). Other workers have reported isolation of PrV from latently infected swine (19, 22, 36, 65, 175). Gutekunst detected latent PrV DNA in trigeminal ganglia from 5 out of 6 swine, 7 months after exposure (63).

**Epidemiology**

Shope recognized that pseudorabies was a natural infection of swine, from which the virus is occasionally transmitted to other
species (156, 157). Virus is present in nasal secretions of infected pigs during the acute phase of infection (111, 119, 156) and may also be shed by recovered swine (36, 128). Although susceptible to pseudorabies, cattle, dogs, and cats have not been shown to transmit the disease once infected (42, 128). Rodents have been suspected to play a role in spreading PrV (12, 118, 127, 158) and raccoons may be able to transmit the virus (83); however, these species are not considered to be a significant factor in the spread of PrV (12, 118, 184). Although susceptible to infection, these species do not shed virus, once infected.

The principal reservoir of PrV is the latently infected, recovered pig. The carrier pig is believed to be the source of infection for numerous outbreaks of pseudorabies (12, 74, 76, 97, 128, 170).

**Immunity**

Infection of swine with PrV results in both humoral and cell-mediated immune responses (4, 11, 66, 121, 136, 181). Infection of nonimmune swine results in the production of neutralizing antibody (NA) in the serum, generally detectable at low levels at 7 days post inoculation (DPI) (66, 112, 121, 136). Neutralizing antibody titers increase dramatically through 14 DPI and peak at 35 DPI (113).

Although NA undoubtedly plays an important role in immunity to PrV, cell-mediated immunity (CMI) may also play a major role in recovery from infection. Cell-mediated immunity to PrV has been demonstrated in the pig after infection (4, 11, 181), but detailed
information on the role of CMI in recovery from PrV infection is not available.

Extensive studies on the immune response of mice to HSV has revealed a predominant role for CMI in recovery from infection (35, 86, 125). Cytotoxic T lymphocytes (CTL) from infected animals specifically lyse syngeneic cell cultures infected with HSV (81, 135, 146), and may play a significant role in recovery from herpes virus infection. Other experiments with mice indicate that lymphocytes bearing antigens common to T-helper cells and delayed hypersensitivity T cells may play an even greater role than CTL in the CMI response to HSV infection (126).

Vaccination

Numerous vaccination studies with PrV have appeared in the literature. Both inactivated and modified live virus (MLV) vaccines are commercially available in many countries and are effective in preventing mortality and decreasing morbidity (40, 75, 109, 114, 115, 132, 134, 148, 160, 176).

The inactivated vaccines normally require 2 injections to induce protective immunity and the MLV vaccines are generally effective after one inoculation. None of the vaccines evaluated to date has prevented infection by virulent challenge virus, and challenge virus may be shed by previously vaccinated swine (114, 115, 148). Virulent challenge virus may also establish latent infections in previously vaccinated animals (123, 176).
Modified live virus vaccines

Bartha was the first to report, in 1961, the isolation of a PrV strain of attenuated virulence (7). Bartha selected a stable small-plaque mutant from a virulent parental strain exhibiting a mixture of large and small plaques in cell culture.

The Bartha strain has been used successfully as a MLV vaccine to prevent serious illness in challenged swine (8, 38, 114, 176). The protection conferred by the Bartha strain is not complete; vaccinated and challenged animals exhibit clinical signs of a mild infection, characterized by fever, some sneezing, reduced appetite, and dullness.

A second attenuated strain was reported by Skoda et al. in 1964 (159). This strain, derived from the Bucharest strain and designated BUK, had been passaged up to 621 times in chick embryo chorioallantoic (CEC) membranes. The BUK strain retained a large plaque morphology in cell culture and was greatly reduced in virulence for swine at passages above 336 in CEC membranes, although the strain retained virulence for rabbits.

The BUK strain has subsequently been widely used as a MLV vaccine (75, 80, 148, 160). As with the Bartha strain, BUK does not induce an absolute immunity. Vaccinated swine may still be infected with a challenge strain, and may experience a mild infection.

Studies indicate that neither the Bartha strain (114, 176) nor the BUK strain (119) are excreted by vaccinated swine. Jamrichová and Skoda (80) recovered virus from the site of inoculation, regional lymph
nodes, and the CNS of pigs inoculated with the BUK strain, indicating limited replication in the pig.

Although the Bartha and BUK strains do not produce clinical disease in swine, Platt et al. (138, 139) reported fatal infections in rabbits and mice with both strains. Other reports of fatal infections caused by PrV MLV in non-swine species have appeared in the literature. Muhm and Beard (124) reported deaths in cats, dogs, rabbits and chickens inoculated with a commercial MLV vaccine strain. König (198) and Van Alstine et al. (174) reported fatal infections in sheep caused by commercial MLV vaccines, accidentally introduced by the use of contaminated syringes and needles.

**Thymidine kinase**

In 1983 Tenser et al. (169) reported that the expression of a PrV-coded enzyme, thymidine kinase (TK), was necessary to establish an acute trigeminal ganglion infection in mice. Mice were inoculated corneally with either TK$^+$ or TK$^-$ PrV, and the ability of the virus to replicate at the site of inoculation and in the trigeminal ganglia was measured. Both TK$^+$ and TK$^-$ strains replicated to high titers in ocular tissues, but only TK$^+$ replicated to high titers in the trigeminal ganglia. Only trace amounts of TK$^-$ PrV were detected in trigeminal ganglia. Mortality rates for mice receiving $1 \times 10^5$ plaque-forming units (PFU) or greater of TK$^+$ or TK$^-$ PrV were 100% and 0%, respectively.
Lomniczi et al. (107) confirmed the role of PrV TK in determining neurovirulence. TK− mutants of the Norden1 (derived from BUK), Bartha, and Kaplan (Ka) PrV strains did not produce fatality in mice or in one-day-old chicks.

Thymidine kinase is an enzyme that functions to salvage thymine as a substrate for DNA biosynthesis. Pre-formed deoxythymidine is phosphorylated by TK to form deoxythymidine monophosphate (dTMP), a necessary precursor of DNA. TK generates dTMP from free thymidine, providing an independent pathway distinct from the cellular thymidylate synthetase pathway (151).

Increased levels of TK are observed in various tissues during DNA synthesis (24, 95, 164, 165, 185), but the TK level is very low in differentiated nerve tissue (3, 102, 185). Mutant cell cultures lacking TK activity can be isolated by prolonged propagation in medium containing 5-bromo-2′-deoxyuridine (BUDR) (87, 88). Cell cultures encoding normal levels of TK, which are grown in the presence of BUDR, have a greatly reduced growth rate and are eventually overgrown by BUDR-resistant cells lacking TK activity, which have a normal growth rate (79, 88).

Numerous herpesviruses in addition to PrV have been shown to induce a virus-coded TK in infected cells, including HSV (43), varicella-zoster virus (129), marmoset herpesvirus (89), herpesvirus

1Norden Laboratories, Inc., Lincoln, NE.
salmiri (73), bovine herpesvirus type 1 (180) and equine herpesvirus type 1 (1).

The relationship between HSV TK phenotype and neurovirulence was first described by Field and Wildy (48), who compared the virulence of two TK− mutants with their TK+ parental strains. Three-week-old BALB/c mice were inoculated either in the ear pinna or intracerebrally (IC) with TK− or TK+ HSV. The TK− mutants were greatly reduced in virulence by the IC route and both TK− mutants failed to produce clinical disease following peripheral inoculation. The TK− mutants did replicate locally in the ear pinna but to a reduced extent relative to the TK+ parental strains, and were cleared from the tissue more quickly.

Tenser et al. (167) and Tenser and Dunstan (166) similarly reported the inability of TK− HSV to replicate in the trigeminal ganglia of mice after corneal inoculation, in contrast to TK+ HSV which did replicate to high titer in the ganglia. Similarly, a TK+ revertant of a TK− mutant reached titers similar to wild-type TK+ HSV. The TK− mutants did, however, reach similar titers in ocular tissue, which supported the hypothesis that HSV TK plays an important role in infections of the sensory ganglia.

Numerous studies have further confirmed the role of HSV TK in determining neurovirulence (13, 14, 46, 50, 58, 59, 92, 93, 110, 140). Price and Khan (140) reported that viral antigen was absent in superior cervical ganglia in mice infected with TK− HSV, even after immunosuppression by cyclophosphamide or prior treatment with the neurotoxin 6-hydroxydopamine, both of which enhanced infection with TK+ HSV. The
TK− strain did, however, replicate at the site of inoculation, providing further evidence that HSV TK facilitates acute, productive infections of neurons.

Ben-Hur et al. (14) demonstrated that TK− HSV produced greater than 50% mortality in 4-day-old mice inoculated corneally with 10^7 PFU/milliliter (ml). At 10 days of age, mice inoculated similarly were not affected by the same exposure, indicating that age is a factor in susceptibility to infection by TK− HSV. In contrast, the mortality rates in 4-day-old and 10-day-old mice inoculated with TK+ HSV were similar, and decreased only slightly for 28-day-old mice, indicating that maturation of the immune system does not explain the significant age-related resistance to TK− HSV. It has been demonstrated that Tk activity in the cerebellum of rats reaches a peak on day 5 or 6 after birth, then declines to negligible levels by week 3 (164, 179, 185). It was concluded that the large increase in mortality in 4-day-old mice is probably associated with high levels of cellular TK that occur in developing brain tissue.

In an experiment with cyclophosphamide-treated mice, Gordon et al. (60) produced a fatal encephalitis by inoculation of a TK− HSV intracranially. Histological examination revealed that non-neural glial cells were permissive and supported replication of HSV TK− virus, indicating that a functional immune system plays a role in resistance to infection by TK− HSV.
Vaccination with TK⁻ HSV

Marcialis (120) isolated a 5-iodo-2'-deoxyuridine (IUdR) resistant HSV mutant which had greatly reduced virulence for mice. The mutant was used to vaccinate mice and rabbits and was effective in protecting mice from paralysis and death and rabbits from conjunctivitis, keratitis and encephalitis after challenge with virulent HSV.

Field and Wildy (48) challenged mice previously inoculated with TK⁻ HSV and demonstrated resistance to challenge with 100 LD₅₀ of the HSV Cl strain 2 to 3 months after immunization.

The results of other studies have confirmed the efficacy of TK⁻ HSV strains to protect mice from severe clinical infection and death when challenged with virulent HSV (92, 93, 94, 110).

Assays for thymidine kinase activity

TK activity may be determined in cytoplasmic lysates by measuring the phosphorylation of ¹⁴C-thymidine (186), ³H-thymidine (151) or [¹²⁵I]iododeoxyuridine (61). The negatively charged product (dTMP) is separated from the uncharged substrate by binding to DEAE-cellulose paper (151). The kinetic behaviors and substrate specificities have been reported for TK from cellular cytoplasm (23, 103), mitochondria (23, 103, 104), marmoset herpesvirus (104) and herpes simplex virus (29, 99, 100).

A thymidine plaque-autoradiography assay was recently developed (168) in which HSV was grown in TK⁻ cells under methylcellulose overlay until visible plaques had formed. The overlay was removed and medium containing ¹⁴C-thymidine was added, followed by incubation at 37 C for
6 hours. TK virus incorporated the $^{14}$C-thymidine, which caused film darkening when the culture dishes were exposed to x-ray film. TK virus does not incorporate $^{14}$C-thymidine, and TK plaques do not cause film darkening.

**Selection of TK herpesviruses**

Numerous nucleoside analogs have been identified which react preferentially with herpesvirus TK and provide a basis for anti-herpesvirus chemotherapy (5, 30, 31, 54). The herpesvirus TK phosphorylates a nucleoside analog to the monophosphate form which is further converted to the triphosphate form. Nucleoside analog triphosphates inhibit viral replication by direct interaction with the viral DNA polymerase enzyme or by incorporation into viral DNA, which functions abnormally (30, 31, 45, 101, 153). Strains of HSV deficient in TK have been isolated by growing virus in the presence of increasing concentrations of BUdR (43, 163), acyclovir (49), and arabinofuranosylthymine (Ara-T) (167). Strains of PrV deficient in viral TK have been isolated by selection in the presence of Ara-T (107, 169).

Revertant TK virus can be selected in TK cell cultures in the presence of hypoxanthine, amethopterin, thymidine and glycine (163) or methotrexate, thymidine, adenosine, guanosine and glycine (28). Methotrexate (amethopterin) or aminopterin each block de novo dTMP biosynthesis. In the presence of methotrexate or aminopterin, the only
source of dTMP for DNA synthesis is by the phosphorylation of free thymidine by TK. TK\(^-\) cells cannot convert thymidine to dTMP; therefore, only TK\(^+\) virus can replicate under such selective conditions.

**Restriction enzyme analysis**

Migration patterns in electrophoretic gels of restriction endonuclease digested PrV DNA have been compared by several investigators (53, 55, 70, 131, 137). Considerable variation existed among field isolates, but it was also noted that certain regions of the genome gave rise to higher frequencies of variability. Greater variability was associated with fragments from the inverted repeat sequences and regions close to the 5'end of the genome. Similar molecular weight changes in restriction fragments have been observed in other herpes viruses (2, 69, 162).

The Bartha and Norden (BUK) strains were shown to contain a major deletion in their genomes, approximately 2.7 x 10\(^6\) daltons, mapping in the unique short region. The deleted sequences contained a genetic determinant of virulence distinct from TK (107), but the nature of the gene product and its role in virulence has not been identified.

**Humoral antibody determination**

Various methods have been developed to detect antibodies to PrV in sera from vaccinated or infected swine. The virus neutralization test (72) is sensitive and is a good method for quantitative antibody determinations. In recent years, the enzyme-linked immunosorbent assay
ELISA has become widely used as an alternative to the virus neutralization test for the detection of antibodies to PrV (121, 172).

Other methods which have been reported for detecting antibodies to PrV include the microimmunodiffusion test (64), indirect solid-phase microradioimmunoassay test (85) and complement fixation test (44).
MATERIALS AND METHODS

Media

Growth medium

Growth medium (GM) for cell cultures consisted of Eagle's minimal essential medium (MEM)\(^1\) with Earle's salts and 2 mM L-glutamine supplemented with 10% fetal calf serum (FCS). Fifty mg of gentamicin sulfate\(^1\) were added to each liter prior to use.

Maintenance medium

Maintenance medium (MM) consisted of MEM with Earle's salts and 2 mM L-glutamine supplemented with 2% FCS, 50 mg gentamicin sulfate, 100,000 units penicillin G\(^2\), 0.1 g streptomycin sulfate\(^3\) and 2.5 mg amphotericin B\(^4\) per liter.

Swab elution medium

Swab elution medium was the same as MM with the exclusion of FCS.

---

\(^1\)GIBCO; Life Technologies, Inc., Chagrin Falls, OH.

\(^2\)Eli Lilly & Co., Indianapolis, IN.

\(^3\)Pfizer Laboratories, New York, NY.

\(^4\)Fungazone; E. R. Squibb & Sons, Princeton, NJ.
Agarose overlay medium

Agarose overlay medium (AOM) was prepared with equal parts of 2X GM and 1.6% liquefied agarose. The agarose was liquefied by autoclaving at 15 psi for 15 minutes and was cooled to 45°C.

Carboxymethyl cellulase overlay medium

Carboxymethyl cellulose (CMC), medium viscosity, was added to distilled H₂O (1 g/100 mL), autoclaved at 15 psi for 15 minutes, cooled to room temperature, then placed at 4°C overnight. The liquefied CMC was added to 2X GM in equal parts and was warmed to 37°C prior to use.

Cell Cultures

MDBK cells

The Madin-Darby bovine kidney (MDBK) cell line was used for virus propagation, titration of virus, and plaque cloning of virus. MDBK cells were propagated at 37°C in 75-cm² flasks. Confluent monolayers were treated with trypsin-versene solution (TVS) (Table 1), diluted 1 to 4 in GM and dispensed into flasks, 6-well plates or 850-cm² roller bottles.

1. SeaKem ME; Marine Colloids Division, FMC Corp., Rockland, ME.
2. Sigma Chemical Co., St. Louis, MO.
3. Falcon; Becton Dickenson & Co., Oxnard, CA.
4. Linbro; Flow Laboratories, Hamden, CT.
Table 1. Trypsin-verse solution

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<tr>
<td>Na$_2$EDTA$^a$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.58 g</td>
</tr>
<tr>
<td>Trypsin$^b$</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Distilled H$_2$O</td>
<td>q.s. to 1000 ml</td>
</tr>
</tbody>
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Adjust pH to 7.3; filter, sterilize, and store at -20°C

$^a$Fisher Scientific; Fairlawn, NJ.

$^b$Difco Laboratories, Detroit, MI.
**LMTK^- cells**

LMTK^- cells\(^1\) (88) were propagated in GM in 75-cm\(^2\) flasks. Monolayers were trypsinized, diluted 1 to 3 in GM and dispensed into plastic flasks or 15 x 60 mm tissue culture dishes\(^2\). The culture dishes containing GM were incubated overnight prior to seeding.

All six-well plates and 15 x 60 mm dishes were maintained at 37 C in a humidified chamber with 5% CO\(_2\) atmosphere.

**Viruses**

**Field strains**

The Indiana-Funkhauser (IN-Fh) strain of PrV (152) was isolated in 1975 and was received at the 16th cell culture passage,\(^3\) and was passaged once in MDBK cells.

The NVSL 85-12 strain was isolated in 1984 from a pig and was received at the 3rd cell culture passage,\(^4\) and was passaged twice in MDBK cells.

---

\(^1\)Obtained from Dr. S. Kit, Baylor University, Houston, TX.

\(^2\)LUX; Lab-Tek, Naperville, IL.

\(^3\)Obtained from Dr. E. C. Pirtle, National Animal Disease Center, Ames, IA.

\(^4\)Obtained from Dr. H. T. Hill, Virus Diagnostic Laboratory, Iowa State University, Ames, IA.
Vaccine strains

The modified live PrV vaccine strains\textsuperscript{1,2,3}, in the lyophilized state, were obtained commercially, were reconstituted as directed and then were passaged once in MDBK cells.

Virus propagation

Confluent monolayers of MDBK cells were inoculated with virus at a multiplicity of 0.01 to 0.1 plaque forming units (PFU) per cell. Virus was allowed to adsorb to the cells for 60 minutes at 37°C. Twenty ml of GM were added to each 75-cm\textsuperscript{2} flask after inoculation.

Virus infected cells were incubated at 37°C until 100% cytopathic effects (CPE) was observed. Cell debris was removed by centrifugation at 500 x g for 10 minutes and the virus suspension was divided into aliquotes in plastic tubes and was frozen at -70°C.

Virus plaque titration

Ten-fold dilutions of virus stocks were prepared in MEM. One-tenth ml of diluted virus was adsorbed to each of 3 wells of confluent MDBK cells grown in 6-well plates. Virus was adsorbed for

\textsuperscript{1}PR-Vac; Norden Laboratories, Inc., Lincoln, NE.

\textsuperscript{2}PseudoVax; Pitman-Moore, Inc., Washington Crossing, NJ.

\textsuperscript{3}Pseudorabies Vaccine; BioCeutic Laboratories, Inc., St. Joseph, MO.
60 minutes at 37 C, at which time 2 ml of AOM were added per well. The plates were incubated at 37 C in 5% CO₂ for 72 hours and then the cells were fixed and stained with 0.1% crystal violet in 10% formalin.

**Plaque-passaging of virus**

Well isolated viral plaques under AOM were picked with a sterile micropipette tip and were eluted in 2 ml of GM. The eluted virus was "plaque titrated" as above.

**Isolation of TK5Al**

IN-Fh PrV was grown one passage in MDBK cells and GM containing 5 µg/ml of 5-bromo-2'-deoxyuridine (B UdR). A second passage was made in the presence of 10 µg/ml BuDr. The virus suspension was plaque titrated under AOM containing 10 µg/ml BuDr. Several large plaques were picked and passaged once with BuDr at 100 µg/ml. Each clone of BuDr-resistant virus was passaged twice more in the presence of GM with 100 µg/ml BuDr and 200 µg/ml of arabinofuranosylthymine (Ara-T).

Three clones of BuDr and Ara-T resistant virus were plaque passaged twice at 38.5 C to insure against temperature-sensitive mutants. Several plaques were picked, passaged once in MDBK cells and GM, and then were assayed for TK activity by thymidine plaque autoradiography.

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1 Sigma Chemical Co., St. Louis, MO.
The clone identified as TK5Al was judged to be deficient for TK activity and a stock culture was prepared by one additional passage in MDBK cells.

Assay of TK5Al for reversion to TK+

A 75-cm² confluent monolayer of LMTK⁻ cells (4 x 10⁷ cells) was inoculated with 1.2 x 10⁷ PFU of TK5Al PrV. Virus was adsorbed at 37 C for 60 minutes, the inoculum was removed, and the monolayer was washed once with MEM. The inoculum was replaced with 20 ml of GM containing 5 x 10⁻⁵ M hypoxanthine, 5 x 10⁻⁷ M methotrexate, 1 x 10⁻⁶ M thymidine and 1 x 10⁻⁶ M glycine (HMTG). The flask was incubated at 37 C for 3 days and was observed daily for CPE. On day 3, the flask was frozen at -70 C and then was thawed to rupture cells. Cell debris was removed by centrifugation at 1000 x g for 10 minutes, and 1 ml of supernatant was used to inoculate a second LMTK⁻ monolayer. The second, and a third, blind passage in the presence of HMTG was conducted as above. One ml of supernatant from the third passage with HMTG was used to inoculate a 75-cm² culture of MDBK cells. The inoculum was adsorbed at 37 C for 60 minutes, the inoculum was removed, and the monolayer was washed with MEM. The inoculum was replaced with 20 ml of GM. The flask was observed for CPE for 6 days.

As a control, 4 x 10⁵ PFU of NVSL 85-12 PrV was passaged 3 times in LMTK⁻ cells in the presence of HMTG, followed by one passage in MDBK

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¹Lederle Parenterals, Inc., Carolina, P.R.
cells with GM as above, with the exception that flasks were frozen when 100% CPE was observed.

To determine whether TK5Al reverts to TK\(^+\) under nonselective conditions, TK5Al was passaged 10 times in MDBC cells in the presence of GM. The TK phenotype of virus in the 10th passage supernatant was determined by thymidine plaque autoradiography.

**Thymidine Kinase Assays**

**Thymidine plaque autoradiography**

The procedure for thymidine plaque autoradiography has been described previously (168). LMTK\(^-\) cells grown in 15 x 60 mm dishes were inoculated with 0.1 ml of virus suspension diluted to contain 50 to 150 PFU. Virus was adsorbed for 60 minutes at 37\(^\circ\) C and then plates were overlaid with 5 ml CMC overlay medium. Dishes were placed at 37\(^\circ\) C in 5% CO\(_2\) for 72 hrs. The CMC overlay was decanted and the cell monolayer was washed once with MEM, followed by incubation for 6 hours with 2 ml MEM containing \(^{14}\)C-thymidine (0.5 \(\mu\)Ci/ml; 55 mCi/mmole\(^1\)). The medium was removed and cells were fixed and stained with 0.1% crystal violet in 10% formalin. Excess crystal violet was removed by 3 washes with H\(_2\)O. The plates were air dried overnight.

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\(^1\)Amersham Corp., Arlington Heights, IL.
The rims of the plastic dishes were removed with scissors, and dish bottoms were placed in an X-Omatic film cassette\(^1\) and the cell monolayer was exposed to XAR-5 x-ray film\(^1\) for 3 to 7 days at room temperature. After exposure, the film was developed, then was photographed while illuminated by a light box.

**Thymidine kinase kinetic assay**

The procedure of Závada et al. (186) was adapted for determining PrV TK activity in cytosol extracts. Confluent monolayers of LMTK\(^-\) cells grown in 75 cm\(^2\) flasks were infected with PrV at a multiplicity of infection (MOI) of 5-10 PFU/cell and incubated for 18 hours at 37 C. Supernatant was removed, and monolayers were washed twice with Tris\(^2\)-buffered saline (TBS) (0.14 M NaCl, 0.01 M Tris-HCl, pH 7.5). The cells were scraped free with a rubber policeman into 5 ml of TBS containing 5 \(\mu\)M thymidine and 2 mM dithiothreitol.\(^3\) The cells were pelleted at 500 x g for 10 minutes and were washed once in cold lysis buffer (LB) (0.01M Tris-HCl, 2 mM dithiothreitol, 5 \(\mu\)M thymidine). The cells were pelleted, resuspended in 1 ml LB and allowed to swell on ice for 10 minutes, followed by disruption in a Dounce homogenizer cooled to 4 C. One-tenth volume 1.4 M NaCl was added, and the cell

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\(^1\)Eastman Kodak, Rochester, NY.

\(^2\)Trizma base; Sigma Chemical Co., St. Louis, MO.

\(^3\)Sigma Chemical Co., St. Louis, MO.
debris was pelleted at 10,000 x g for 10 minutes. The supernatant was further centrifuged at 105,000 x g for 1 hour at 4 C. The supernatant containing PrV TK was frozen at -70 C, in 100-μl aliquots.

For each enzyme reaction mixture, a 25-μl volume of cell extract was added to 62.5 μl of reaction buffer (100 mM Tris-HCl, 10 mM MgCl₂, 10 mM ATP; pH 8.0), plus 17.5 μl of sterile distilled H₂O. Substrate (¹⁴C-thymidine¹) was prepared in distilled H₂O (1:5, 1:10, 1:25, 1:50, 1:75) and 20 μl were added to each reaction mixture. The reactions were conducted in 1.5-ml micro-centrifuge tubes² that were incubated in a 37 C water bath for 5, 10, 15 and 20 minute time intervals. Reactions were stopped by immersing the tubes in boiling water for 3 minutes followed by centrifugation at 6500 x g for 1 minute to remove denatured protein. For zero-time reactions, the tubes were immersed in boiling water immediately after addition of the substrate. Fifty-μl volumes of reaction supernatant were spotted onto 2 x 2 cm squares of DE-81 papers.³ Papers were air-dried, washed 3 times for 5 minutes in 4 mM ammonium formate, 10 minutes in distilled H₂O, and 10 minutes in absolute methanol. The papers were air-dried and placed in 20-ml

¹ 50 μCi/ml; 55 mCi/mmol; Amersham Corp., Arlington Heights, IL.
² Treff Lab, Tekmar Co., Cincinnati, OH.
³ Whatman; VWR Scientific, San Francisco, CA.
scintillation vials containing 10 ml of scintillant. Each sample was counted for 1 minute in a liquid scintillation counter. Michaelis constants (Km) and maximal velocities (Vmax) for the enzyme reactions were obtained from Lineweaver-Burk plots (105).

Restriction Endonuclease Analysis

Virus production and purification

For each PrV strain, one 850 cm$^2$ roller bottle containing a monolayer of MDBK cells was infected with an MOI of 0.001. The inoculum was replaced with 60 ml of GM after virus had adsorbed for 2 hrs at 37 C. Infected cultures were incubated at 37 C until 100% CPE was observed. The cells were scraped into the medium, pelleted by low speed centrifugation, were saved for isolation of nucleocapsids. The virus-containing supernatant was layered over 40% sucrose in 0.2 M phosphate buffered saline, pH 7.5, and was centrifuged for 1 hour at 25,000 rpm in an SW28 rotor. The supernatant was discarded and the virus pellet was resuspended in 2 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6).

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1Kimble; Owens-Illinois, Toledo, OH.

2Ready-Solv HP; Beckman Instruments, Inc., Fullerton, CA.

3Viral DNA was extracted according to the method of D. R. Jutting, Diagnostic Virology Laboratory, NVSL, Ames, IA, personal communication.

4Beckman Instruments, Inc., Fullerton, CA.
Nucleocapsid purification

Cells from each PrV-infected 850 cm$^2$ roller bottle were suspended in 2 ml of LCM buffer (5% Na deoxycholate; 30 mM Tris-HCl, pH 7.5; 125 mM KCl; 0.5 mM Na$_2$EDTA; 3.6 mM CaCl$_2$; 5 mM MgCl$_2$; 5% NP-40; 46 μl mercaptoethanol/100 ml) and were extracted with 1 ml of Freon (1,1,2-trichloro-1,2,2-trifluoroethane). Phases were separated by centrifugation at 800 x g for 5 minutes. The upper aqueous phase containing nucleocapsids was divided into two portions and each was layered over glycerol gradients (15 ml 45% glycerol, 15 ml 5% glycerol, in LCM buffer). Nucleocapsids were pelleted by centrifugation for 1 hour at 25,000 rpm in an SW28 rotor. The pellet was suspended in 1 ml of TE buffer and was added to the virion suspension from above.

Extraction of viral DNA

Extractions were performed according to Van Alstine et al. (174), with slight modifications. To 3 ml of virion and nucleocapsid suspension were added 20 μl 20% sodium dodecylsulfate and 200 μl Proteinase K$^1$ (1 mg/ml). Tubes were "vortexed" and then were incubated at 37 C for 1 hour. One hundred μl additional Proteinase K were added and tubes were incubated until cloudiness disappeared (1 to 3 hours). After digestion, DNA was extracted once with TE-saturated redistilled phenol,$^2$ once with chloroform/TE-saturated phenol (1:1) and once with

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$^1$Sigma Chemical Co., St. Louis, MO.

$^2$Ultra-Pure; Bethesda Research Laboratories, Gaithersburg, MD.
2% isoamyl alcohol in chloroform. One-tenth volume of 3M sodium acetate was added and the DNA was precipitated by the addition of 2.5 volumes of cold (-20 C) absolute ethanol. Tubes were placed at -20 C overnight and the DNA was pelleted by centrifugation at 12,000 x g for 30 minutes at 0 C. Pellets were air-dried and were resuspended in 2 ml of TE buffer.

Restriction enzyme digestion of DNA

The procedures for restriction endonuclease digestion of PrV DNA and for agarose gel electrophoresis have been reported previously (131, 137). Approximately 1 µg of viral DNA (30 µl) was digested with 2 µl of Bam HI endonuclease, Kpn I endonuclease, or Pst I endonuclease. The digestion buffer was 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (prepared at 10 X concentration) for Kpn I, or with 50 mM NaCl for Bam HI. Buffer for Pst I was purchased commercially.¹

Reaction mixtures were adjusted to 50 µl with distilled H₂O and were incubated at 37 C for 4 hours. Reactions were stopped by the addition of Loening's buffer (30 mM Na₂HPO₄, 1 mM Na₂EDTA, 36 mM Tris, pH 7.7) containing 60% sucrose and 0.02% bromphenol blue.

Bacteriophage lambda DNA¹ was similarly digested with endonuclease Hind III¹ as a molecular weight marker (131, 137).

¹Bethesda Research Laboratories, Gaithersburg, MD.
Agarose gel electrophoresis

DNA digests were electrophoresed in 0.8% agarose\(^1\) in Loening's buffer for 24 hours at 40 volts. The gel was soaked for 1 hour in ethidium bromide (1 \(\mu\)g/ml in distilled \(H_2O\)), rinsed once in distilled \(H_2O\), and photographed through a Kodak 23A red filter\(^2\) while illuminated by an ultraviolet light source.\(^3\) The photographic film was Royal X, ASA 1250.\(^2\)

Animals

Mice

Five- to six-week-old female CF-1 strain mice\(^4\) were inoculated within 2 days of arrival.

Swine

Seven-week-old crossbred swine of both sex (25 to 37 lbs) were purchased from a breeder at Manilla, IA. All pigs were serologically negative for antibodies against PrV.

\(^1\)Seakem ME; Marine Colloids Division, FMC Corp., Rockland, ME.
\(^2\)Eastman Kodak, Rochester, NY.
\(^3\)Ultraviolet Transilluminator; LaJolla Scientific Co., Inc., LaJolla, CA.
\(^4\)Sprague-Dawley, Indianapolis, IN.
Mouse Virulence Test

Ten-fold dilutions of each PrV strain were prepared in MEM and were inoculated into the peritoneal cavity of CF-1 mice, 0.2 ml/mouse. Groups of 5 or 10 mice were inoculated per dilution. Mice were observed for death, 3 times daily, for 14 days post inoculation (DPI), with the exception of mice receiving the BioCeutic strain, which were observed for 21 DPI. Mice found dead were incised through the peritoneal wall and cranium and were preserved in 10% formalin for histopathological examination.

Vaccination of Mice with TK5A1

Groups of 10 mice were inoculated intraperitoneally (IP) with 0.2 ml volumes of 10-fold dilutions of TK5A1 prepared in MEM. At 15 DPI, 4 mice from two groups were exsanguinated to obtain serum with which to determine the humoral response to vaccination. The remaining vaccinated mice and 4 non-vaccinated controls were challenged with 10 LD$_{50}$ of NVSL 85-12 PRV (0.2 ml, IP). Challenged mice were observed for death, daily, for 15 days. At 15 days post challenge (DPC), 4 mice from each of 2 groups were bled and all survivors were euthanatized by cervical dislocation.

Vaccination of Swine with TK5A1

Nine pigs were randomly assigned to 2 groups and were placed in separate rooms. Five pigs were inoculated in the neck muscle with
2.4 \times 10^7 \text{ PFU of TK5Al PrV. The pigs were observed daily for clinical signs of disease, rectal temperatures were recorded, and nasal swabs were taken for 14 DPI. Ten ml of blood were collected from the vena cava at 0, 7 and 14 DPI.}

Four pigs assigned to the non-vaccinated control group were similarly bled and rectal temperatures were recorded. Nasal swabs were collected at 0, 7 and 14 DPI.

At 21 DPI, all pigs were swabbed, bled, had rectal temperatures recorded, and then were challenged intranasally with 3 \times 10^5 \text{ PFU of NVSL 85-12 PrV. Challenge virus was diluted in MEM, and each pig received 0.5 ml in each nostril. Rectal temperatures were recorded for all pigs, and nasal swabs were taken daily through 14 DPC, or until death. Ten-ml blood samples were drawn from surviving pigs at 7, 14 and 21 DPC.}

\textbf{Collection of nasal swabs and virus isolation}

Nasal swabs were taken and swabs were placed in 2 ml swab elution medium for 45 minutes at 4 \text{ C. Tubes were vortexed briefly, swabs were removed, and fluids were centrifuged for 10 minutes at 1000 x g. One-half ml of supernatant was inoculated onto a confluent monolayer of MDBK cells grown in 6-well plates. Plates were incubated at 37 \text{ C for 60 minutes, fluids were removed and 2 ml volumes of MM were replaced. Plates were further incubated at 37 \text{ C in 5\% CO}_2 for 4 days and were examined daily for CPE. Plates containing wells negative for CPE by day 4 were frozen at -70 \text{ C, were thawed and} 0.5 \text{ ml of supernate was}}
inoculated onto a fresh monolayer. Second passage plates were observed daily for CPE for 4 days.

**Serology**

**Virus neutralization test**

Serum samples from mice and swine were tested for neutralizing antibody to PrV by a method described previously (72).

**Enzyme-linked immunosorbent assay**

Serum samples from swine were tested for antibody specific for PrV by the enzyme-linked immunosorbent assay (ELISA) method of Snyder and Erickson.

**Histopathology**

Tissue samples from mice preserved in 10% formalin were dehydrated in graded alcohol solutions, cleared with xylene, infiltrated with paraffin, and were embedded in paraffin blocks for sectioning. Five μm sections were cut with a microtome, affixed to slides and stained with hematoxylin and eosin.

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1 Conducted by Dr. H. T. Hill, Virus Diagnostic Laboratory, Iowa State University, Ames, IA.

2 M. L. Snyder, and G. A. Erickson, Diagnostic Virology Laboratory, NVSL, Ames, IA, personal communication.

3 Conducted by Dr. L. D. Miller, Department of Veterinary Pathology, Iowa State University, Ames, IA.
RESULTS

Isolation and Characterization of a Pseudorabies Virus

Mutant Lacking Thymidine Kinase Activity

Several clones of the IN-Fh strain of PrV were selected for resistance to BUdR and Ara-T. A clone identified as TK5A1 was determined to lack TK activity by thymidine plaque autoradiography (Figure 2). Plaques formed by the Tk⁰ parental strain cause film darkening on X-ray film, (plate 3), because ^14C-thymidine is incorporated into DNA during virus replication. Plaques formed by the mutant TK5A1 do not darken the film, reflecting the loss of TK activity. The mutant replicated as well in MDBK cells as did the parental strain (Table 2). The TK5A1 strain was titrated at 37 C and at 38.5 C, and titered equally at both temperatures, demonstrating that the mutant is not temperature sensitive at 38.5 C.

Mutant TK5A1 was passaged 10 times in MDBK cells and then was examined by thymidine plaque autoradiography for reversion to Tk⁰ (Figure 3). No plaques possessed a Tk⁰ phenotype, indicating that the mutant did not revert to Tk⁰ at a high frequency.

To select for a Tk⁰ revertant in the stock culture of TK5A1 PrV, 1.2 x 10⁷ PFU of the mutant were inoculated onto LMTK⁻ cells and passaged 3 times in medium containing HMTG. No virus was recovered from 1 ml of the 3rd blind passage supernate. In a preliminary experiment, 2 x 10⁵ PFU of TK5A1 PrV were similarly tested, and was also negative for PrV in the 3rd passage supernate. Tk⁰ PrV (NVSL 85-12
Figure 2. Thymidine plaque autoradiography assay: A) crystal violet-stained cell monolayers; B) x-ray film exposed to cell monolayers. Plate 1: uninfected LMTK- cells; Plate 2: TK5A1 strain PrV. Plate 3: IN-Fh strain PrV.
Table 2. Mortality in mice after inoculation with virulent and attenuated strains of PrV

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dilution of Virus (Log&lt;sub&gt;10&lt;/sub&gt;)</th>
<th>PFU/</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVSL 85-12</td>
<td>1.4 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>29/30&lt;sup&gt;d&lt;/sup&gt; 22/30 6/30 0/10</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>IN-Fh</td>
<td>3.4 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>19/20 24/30 3/30 1/30</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>Norden</td>
<td>7.6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>9/10 4/10 0/10</td>
<td>265</td>
<td></td>
</tr>
<tr>
<td>Pitman-Moore</td>
<td>2.7 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>9/10 4/10 0/10</td>
<td>941</td>
<td></td>
</tr>
<tr>
<td>BioCeutic</td>
<td>1.1 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>9/10 5/10 0/10</td>
<td>274</td>
<td></td>
</tr>
<tr>
<td>TK5Al</td>
<td>3.3 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0/15 0/15</td>
<td>&gt;10&lt;sup&gt;7&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>PFU/ml.

<sup>b</sup>Two-tenths ml inoculum/mouse.

<sup>c</sup>LD<sub>50</sub> calculated by the method of Reed and Muench (143).

<sup>d</sup>Number dead/number inoculated.
Figure 3. Thymidine plaque autoradiography assay: A) crystal violet-stained cell monolayers; B) x-ray film exposed to cell monolayers. Plates 1, 2, 3, 5, 6: 10th passage TK5Al PrV. Plate 4: IN-Fh strain PrV.
strain) replicated well in all passages, and was present in high concentration in the 3rd passage supernate.

**Virus Propagation**

Virus stocks were prepared for 2 virulent and 3 commercial attenuated vaccine strains of PrV, and for the TK5Al mutant strain (Table 2). Virus titers ranged from $7.6 \times 10^6$ to $1.4 \times 10^8$ PFU/ml.

**TK Phenotype of Selected Virulent and Attenuated Strains of PrV**

Two virulent and 3 commercial attenuated vaccine strains were determined to have a TK$^+$ phenotype by thymidine plaque-autoradiography (Figure 4).

**Kinetic Behavior of Thymidine Kinase from Selected Strains of PrV**

The kinetic behavior of PrV TK from infected cell lysates was determined for 2 virulent and 3 commercial vaccine strains. Lineweaver-Burk plots (Figure 5) of initial velocity studies were used to determine $K_m$ and $V_{max}$ values for the enzyme from each strain (Table 3). Mean $K_m$ values ranged from 2.9 to 3.9 μM. $V_{max}$ values ranged from 1.7 to 6.7.
Figure 4. Thymidine plaque autoradiography assay; A) crystal violet stained cell monolayers; B) x-ray film exposed to cell monolayers. Plate 1: NVSL 85-12 strain PrV; plate 2: uninfected LMTK− cells; plate 3: IN-Fh strain PrV; plate 4: Norden strain PrV; plate 5: Pitman-Moore strain PrV; plate 6: BioCeutic strain PrV
Figure 5. Lineweaver-Burk plot of initial velocity data of NVSL 85-12 strain PrV thymidine kinase.
Table 3. Kinetic properties of pseudorabies virus thymidine kinase

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Km (μm)</th>
<th>Km Mean</th>
<th>Vmax</th>
<th>Vmax Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVSL 85-12</td>
<td>3.5</td>
<td>3.7</td>
<td>3.5 ± 0.20</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>3.3</td>
<td></td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td></td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>IN-Fh</td>
<td>4.2</td>
<td>3.3</td>
<td>3.8 ± 0.47</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td></td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Norden</td>
<td>3.0</td>
<td>2.7</td>
<td>2.9 ± 0.15</td>
<td>4.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td></td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Pitman-Moore</td>
<td>3.7</td>
<td>4.0</td>
<td>3.9 ± 0.17</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
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<td>4.0</td>
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<td>5.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>BioCeutic</td>
<td>3.2</td>
<td>2.8</td>
<td>2.9 ± 0.26</td>
<td>5.0 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td></td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Reciprocal of counts per minute, x 10<sup>-3</sup>. 
Mouse Virulence Test

Female CF-1 mice were inoculated IP with $\log_{10}$ dilutions of the 6 FrV strains included in the study, and were observed for death daily. The $LD_{50}$ value for each strain was calculated and used to determine a $PFU/ID_{50}$ value, providing a relative measurement of virulence for the mouse (Table 2). The $PFU/ID_{50}$ values were lower for the 2 virulent strains than for the 3 commercial vaccine strains. The value for the Pitman-Moore strain was considerably higher than for the other 2 vaccine strains, but the difference may not be significant because of the small number of mice used to determine the $LD_{50}$. The TK5Al strain was avirulent for the mouse, by this route of inoculation.

Histopathology was performed on selected mice that died after inoculation with FrV strains. In addition, 14 mice inoculated with the TK5Al strain were euthanized at 15 DPI and were similarly examined. Lesions observed are summarized in Table 4. Adrenal necrosis with intranuclear inclusion bodies was the most frequent lesion observed, followed by perivascular cuffing in the brain.

Response of Mice to Vaccination with TK5Al FrV and Challenge with Virulent Pseudorabies Virus

Response of mice to vaccination

No morbidity or mortality was observed in mice vaccinated with TK5Al FrV. Neutralizing antibody titers were either undetectable or were very low in mice bled at 15 days post vaccination (DPV) (Table 5).
Table 4. Histopathology results from mice dying after inoculation with strains of pseudorabies virus

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Virus Dilution</th>
<th>Adrenal Gland</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN-Fh</td>
<td>$10^{-3}$</td>
<td>N 4/15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C 2/19</td>
</tr>
<tr>
<td>IN-Fh</td>
<td>$10^{-4}$</td>
<td>N 2/16</td>
<td>G 1/17</td>
</tr>
<tr>
<td>NVSL 85-12</td>
<td>$10^{-4}$</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>BioCeutic</td>
<td>$10^{-1}$</td>
<td>X 1/4</td>
<td>0/4</td>
</tr>
<tr>
<td>BioCeutic</td>
<td>$10^{-2}$</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Norden</td>
<td>$10^{-1}$</td>
<td>N 3/4</td>
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<tr>
<td>Norden</td>
<td>$10^{-2}$</td>
<td>N 1/2</td>
<td>0/2</td>
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<tr>
<td>Pitman Moore</td>
<td>$10^{-1}$</td>
<td>0/3</td>
<td>0/3</td>
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<tr>
<td>Pitman Moore</td>
<td>$10^{-2}$</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>TK5Al&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$10^{0}$</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>TK5Al&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$10^{-1}$</td>
<td>0/10</td>
<td>C 1/10</td>
</tr>
</tbody>
</table>

C = Perivascular cuffing  
N = Necrosis and intranuclear inclusion bodies  
X = Necrosis and neutrophilic infiltration but without intranuclear inclusion bodies  
G = Ganglion necrosis  

<sup>a</sup>Number with lesions/number observed.  
<sup>b</sup>Mice euthanized 15 dPl.
Table 5. Neutralizing antibody response in mice vaccinated with TK5Al PrV and challenged with NVSL 85-12 PrV

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Time of Bleeding</th>
<th>Dilution Log_{10} Vaccine</th>
<th>VN Titre&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
<td>8</td>
<td></td>
<td>-1</td>
<td>&lt;2</td>
</tr>
<tr>
<td>9</td>
<td>15 DPC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-1</td>
<td>&lt;2</td>
</tr>
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</tr>
<tr>
<td>16</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup>Reciprocal of serum dilution.

<sup>b</sup>Days post vaccination.

<sup>c</sup>Days post challenge.
Response of mice to challenge with NVSL 85-12 PrV

Mortality rates of mice challenged with virulent PrV are shown in Table 6. All control mice died, as well as all mice that were vaccinated with TK5Al PrV diluted $10^{-4}$ and $10^{-5}$ and challenged. Mice which were vaccinated with undiluted TK5Al or the $10^{-1}$ dilution were completely protected upon challenge. Mice vaccinated with TK5Al PrV diluted $10^{-2}$ and $10^{-3}$ showed decreasing levels of resistance to challenge.

Selected mice which had been vaccinated and had survived challenge were bled at 15 DPC, and were tested for production of neutralizing antibody. Virus neutralization titers in these mice were either undetectable or were very low (Table 4).

Response of Swine Vaccinated with TK5Al PrV and Challenged with Virulent Pseudorabies Virus

Response of swine to vaccination

Five 7-week-old pigs were inoculated in the neck muscle with $2.4 \times 10^7$ PFU of TK5Al PrV. No clinical signs of illness were observed in the pigs for 14 DPV. Rectal temperatures recorded from vaccinated and control animals are shown in Table 7. Mean temperatures of the vaccinated and control groups are shown in Figure 6. Temperatures in the vaccinated group were slightly elevated on days 7-9 post vaccination, but did not rise above 40.0 C at any time.

Humoral antibody responses in vaccinated pigs are summarized in Tables 8 and 9. Low levels of neutralizing antibody were first
Table 6. Mortality of vaccinated mice after challenge with 10 LD$_{50}$ of NVSL-85-12 PrV

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>\text{Log}_{10} \text{ Dilution}</th>
<th>\text{Mortality} #\text{dead}/#\text{inoculated}</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>4/4</td>
</tr>
<tr>
<td>TK5Al</td>
<td>undiluted</td>
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<td>&quot;</td>
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<td>1/10</td>
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<td>&quot;</td>
<td>-5</td>
<td>10/10</td>
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Table 7. Rectal temperatures of swine after vaccination with TK5Al PrV

<table>
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<th>Pig Number</th>
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<th>43</th>
<th>44</th>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>38.4</td>
<td>38.8</td>
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<td>39.0</td>
<td>39.1</td>
<td>39.3</td>
<td>39.0</td>
<td>39.6</td>
<td>39.1</td>
<td>39.1</td>
</tr>
</tbody>
</table>

\(^a\)Not done.
Figure 6. Mean daily temperatures of pigs vaccinated with TK5Al PrV, and nonvaccinated controls.
Table 8. Neutralizing antibody titers in pigs vaccinated with TK5Al PrV and challenged with virulent pseudorabies virus

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>Days Post Vaccination</th>
<th>Days Post Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>41</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>42</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>43</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>44</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>48</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

4 control pigs Neg Neg Neg Neg ...\textsuperscript{c} ... ...  

\textsuperscript{a} Reciprocal of serum dilution.  
\textsuperscript{b} Died before 14 days post challenge.  
\textsuperscript{c} All control pigs died before 7 days post challenge.
Table 9. ELISA indices for serum samples from pigs vaccinated with TK5Al PrV and challenged with virulent pseudorabies virus

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>Days Post Vaccination</th>
<th>Days Post Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>41</td>
<td>0.98</td>
<td>4.01</td>
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<td>42</td>
<td>1.21</td>
<td>2.78</td>
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<td>43</td>
<td>0.98</td>
<td>1.68</td>
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<td>44</td>
<td>1.26</td>
<td>1.10</td>
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<td>48</td>
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<td>2.26</td>
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<td>40c</td>
<td>1.36</td>
<td>1.03</td>
</tr>
<tr>
<td>45c</td>
<td>1.06</td>
<td>1.11</td>
</tr>
<tr>
<td>46c</td>
<td>2.09e</td>
<td>1.22</td>
</tr>
<tr>
<td>47c</td>
<td>1.40</td>
<td>1.40</td>
</tr>
</tbody>
</table>

*a* Index values greater than 1.80 were considered positive for antibody against PrV.

*b* Died before 14 days post challenge.

*c* Nonvaccinated control.

*d* All control pigs died before 7 days post challenge.

*e* Nonspecific reaction.
detected at 14 DPV in 2 of 5 pigs, and were detected in the serum from 4 of 5 pigs at 21 DPV. One pig remained seronegative through 21 DPV.

Three of 5 vaccinated pigs were positive for antibody to PrV at 7 DPV by the ELISA method. One vaccinated pig was seronegative by ELISA through 21 DPV.

The ELISA index for the first bleeding from pig 46 was 2.09 (Table 9). Infrequently, nonimmune sera will yield index values as great as 2.10, due to a nonspecific reaction in the ELISA test. This was confirmed as a nonspecific titer by negative indices for subsequent bleedings.

Nasal swabs taken from all pigs were negative for PrV isolation through 14 DPV.

Response of swine to challenge with virulent PrV

Vaccinated pigs were challenged with virulent PrV at 21 DPV. Nonvaccinated control animals were similarly challenged. Rectal temperatures recorded post challenge are shown in Table 10, and mean daily temperatures are shown in Figure 7. Clinical signs observed in PrV challenged swine are presented in Table 11. Fever was defined to be temperatures above 40.0 °C, and was observed in all animals by 4 DPC. Peak temperatures were approximately the same in the vaccinated and control groups. Vaccinated animals appeared depressed at 4 DPV and exhibited mild dyspnea at 5 DPV, but, with the exception of pig 44, experienced only mild clinical signs and recovered quickly. Clinical signs in control pigs post challenge were typical for severe
Table 10. Rectal temperatures of swine after challenge with NVSL 85-12 PrV

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>Vaccinated</th>
<th>Nonvaccinated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days Post-Challenge</td>
<td>41</td>
</tr>
<tr>
<td>0</td>
<td>39.0</td>
<td>39.8</td>
</tr>
<tr>
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<td>39.0</td>
</tr>
<tr>
<td>14</td>
<td>38.8</td>
<td>39.4</td>
</tr>
</tbody>
</table>

^aDead.
Figure 7. Mean daily temperatures of pigs after challenge with virulent PrV
Days Post Challenge

- Nonvaccinated
- Vaccinated
Table 11. Clinical signs in swine after challenge with NVSL 85-12 PrV

<table>
<thead>
<tr>
<th>Pig Number</th>
<th>Vaccinated</th>
<th>Nonvaccinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days Post-Challenge</td>
<td>41 42 43 44 48</td>
<td>40 45 46 47</td>
</tr>
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<tr>
<td>2</td>
<td>F  F  F  F  F</td>
<td>FAD FAD AD FAD</td>
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<td>FAD FAD FAD FAD</td>
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<td>T B</td>
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<tr>
<td>14</td>
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</tbody>
</table>

F = Fever  D = Depressed
M = Death   B = Dyspnea
A = Anorexia T = Tremors
N = Nasal Discharge
pseudorabies, characterized by CNS involvement, and by severe respiratory distress.

Humoral antibody responses in PrV challenged swine are shown in Tables 8 and 9.

Virus isolation results from nasal swabs taken from challenged swine are summarized in Table 12. Vaccinated animals shed virus from 1 DPC through 5-7 DPC, with the exception of pig 48, from which virus was isolated intermittently through 12 DPC. Nonvaccinated animals shed virus from 1 DPC through day of death.

Restriction Enzyme Analysis

Figure 8 shows the Bam HI digests of the six PrV strains included in the study. The IN-Fh strain and the TK5Al mutant have patterns that differ only slightly. The Bam HI L and N fragments of TK5Al were slightly larger in MW, than those of the parental IN-Fh strain. The NVSL 85-12 strain differs from IN-Fh in the N and K or L fragments. The Norden and Pitman-Moore strains of PrV differ slightly from each other in the Bam HI digest. The stepladder pattern below the D fragment has been described previously for the Norden strain, and its parental strain, BUK (131). The BioCeutic strain of PrV differs considerably from the other strains included in the study when digested with Bam HI, indicating that it is not closely related to either the other attenuated strains or the 2 virulent strains isolated from the midwestern United States.
Table 12. Isolation of pseudorabies virus from nasal swabs taken after challenge

<table>
<thead>
<tr>
<th>Days Post-Challenge</th>
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<th>Nonvaccinated</th>
</tr>
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<td></td>
<td>41 42 43 44 48</td>
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<td></td>
</tr>
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<tr>
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</tr>
</tbody>
</table>

*(+) Denotes virus isolated.
(-) Denotes virus not isolated.

Dead.
Figure 8. Bam HI digests of PrV DNA. Lane A: NVSL 85-12 strain; lane B: IN-Fh strain; lane C: TK5Al strain; lane D: Norden strain; lane E: Pitman-Moore strain; lane F: BioCeutic strain
The Pst I digests of the 6 PrV strains are shown in Figure 9. The TK5Al and IN-Fh strains differ only slightly from each other. The NVSL 85-12 strain is somewhat similar, yet quite distinct in some of its pattern. It is significant that the Pst I A fragment found in the NVSL 85-12, IN-Fh and TK5Al strains is not present in the 3 commercial vaccine strains. It has been reported previously that the Pst I A fragment contains a gene that influences virulence, and that is distinct from TK (107). Although the BioCeutic strain is not closely related to the Norden and Pitman-Moore strains, it has also undergone a major change within the Pst I fragment.

When the strains of PrV were digested with Kpn I (Figure 10), it was again evident that strains TK5Al and IN-Fh are closely related, and that the Norden and Pitman-Moore strains are also closely related to each other. The NVSL 85-12 and BioCeutic strains differ from each other and each of the other strains in the study.
Figure 9. Pst I digests of PrV DNA. Lane G: NVSL 85-12 strain; lane H: IN-Fh strain; Lane I: TK5Al strain; lane J: Norden strain; Lane K: Pitman-Moore strain; Lane L: BioCeutic strain
Figure 10. Kpn I digests of PrV DNA. Lane M: NVSL 85-12 strain; lane N: IN-Fh strain; lane O: TK5Al strain; lane P: Norden strain; lane Q: Pitman-Moore strain; Lane R: BioCeutic strain
A TK-negative mutant of PrV (TK5A1) was isolated from the virulent field strain, IN-Fh, by selection in the presence of B<sub>UdR</sub> and Ara-T. Mutant TK5A1 was examined for reversion to TK<sup>+</sup>; the mutation was stable through 10 passages in MDBK cells (Figure 3). No revertent TK<sup>+</sup> virus was selected from 1.2 x 10<sup>7</sup> PFU of TK5A1 stock culture inoculated onto LMTK<sup>−</sup> cells and passaged 3 times in medium containing HMTG, indicating that if TK5A1 PrV has a reversion frequency, it is probably less than 10<sup>−7</sup>.

TK5A1 PrV was avirulent for adult mice when inoculated IP (Table 2). This was similar to the findings of Lomniczi et al. (107) and Tenser et al. (169) who reported that the loss of TK activity correlated with loss of virulence for the mouse. Studies by Field and Hill (47) and Fraser and Ramachandran (51) demonstrated that the nerve ganglia and the CNS are the principal sites of PrV replication in the mouse, and McCracken et al. (108) similarly documented the neuronal spread of PrV in calves. Studies with TK<sup>−</sup> mutants of HSV suggested that the viral enzyme was essential for establishing a neuronal infection (140, 164, 167). The findings presented here support the role for PrV TK in determining virulence.

Mutant TK5A1 was tested for its ability to stimulate protective immunity in mice and swine. As shown in Table 6, the TK5A1 strain provided complete protection to mice against challenge when the mice were vaccinated with an undiluted preparation or a 10<sup>−1</sup> dilution. The effectiveness of TK5A1 as a vaccine was clearly dose-dependent, as
would be expected of a live virus vaccine with limited ability to replicate in its host. The immunity stimulated in the mouse by TK5Al may be primarily a CMI response. Levels of neutralizing antibody in sera from vaccinated mice were either very low or undetectable, even at 15 DPC (Table 5). It is known that the CMI response plays the major role in immunity of mice to HSV infection (125, 126, 135); Sethi et al. (154) were able to protect mice from HSV challenge by the adoptive transfer of immune cytotoxic T-cells. It is likely that a similar mechanism was stimulated by vaccination of mice with TK5Al.

Use of the TK~ PrV mutant as a vaccine for swine was also examined. After vaccination in the neck muscle, 7-week-old pigs did not develop clinical signs of infection, and only a slight increase in body temperature was observed at days 7-9 post vaccination (Figure 6).

Challenged, vaccinated pigs and nonvaccinated control pigs developed fevers at 3 DPC (Table 10, Figure 7), which declined by 6 DPC. Vaccinated pigs, with the exception of pig 44, experienced mild clinical signs upon challenge, and recovered quickly (Table 11). Non-vaccinated control pigs were noticeably ill at 2 DPC, and developed severe clinical signs, culminating in death by days 6 and 7 post challenge, indicating that the strain used for challenge, NVSL 85-12, is a highly virulent strain for swine.

Although vaccination of pigs with the TK5Al strain conferred good protection against mortality and serious morbidity, it did not prevent infection by the challenge virus. The clinical signs observed in vaccinated pigs after challenge (Table 11) were consistent with mild
pseudorabies. Vaccinated pigs also shed virus from the nasal cavity for 5 to 12 DPC (Table 12). These findings are consistent with other reports on the inability of modified live virus vaccines (40, 75, 114, 115) or inactivated vaccines (40, 115, 134, 182) to prevent infection by virulent pseudorabies challenge virus. However, nasal swabs from vaccinated pigs positive for virus isolation appeared to contain less virus than swabs from nonvaccinated pigs. In a study by Maes et al. (119), vaccinated pigs shed virus for a shorter period of time than did nonvaccinated, challenged pigs. Maes recovered virus in nasal swabs as late as 19-21 DPC.

The neutralizing antibody titers in vaccinated pigs (Table 8) were 2- to 8-fold greater than those reported by Skoda et al. (160), Howarth (75) and McFerran et al. (115) in studies with the BUK strain. The differences noted may have been caused by a more limited ability of the BUK strain to replicate in the pig, or may be due to differences in the animals used or in the method of the virus neutralization test. However, considerable evidence is available that TK⁻ HSV (48, 58, 164, 167) and PrV (169) replicate well in nonneural tissue, which may result in higher levels of immunity than those caused by virus strains containing mutations in other loci which influence virulence.

It was also observed that the VN titer of pig 48 did not rise dramatically after challenge, and pig 48 also shed virus longer than the other vaccinated pigs (Table 12), suggesting a relationship between VN titer and viral clearance.
The reason pig 44 did not respond to vaccination is not clear. It is possible that the vaccine was administered to a site less accessible to the immune system, or that the pig was not able to mount an adequate immune response because of a physiological or genetic anomaly. Pig 44 survived 1 to 2 days longer than nonvaccinated pigs, and did not develop serious clinical signs until 6 DPC, indicating some resistance to infection. In addition, pig 44 did not exhibit signs of CNS infection, in contrast to nonvaccinated animals. The results suggest that the pig demonstrated signs of partial resistance to the challenge virus, but was unable to launch a full immune response because of an undetermined immune suppression or genetic immune deficiency.

The 3 commercial MLV vaccine strains expressed TK activities, as did 2 virulent field isolates (Figure 4). Analysis of the kinetic behavior of TK from the vaccine and virulent strains revealed only minor differences in Km values (Table 3). The small differences in Km values probably have little biological significance.

The Km values determined for the PrV strains are approximately 7 to 10 fold greater than values reported for HSV (Table 13). The difference is perhaps due to the more stringent neurotropism of HSV, which is thus more dependent on viral TK activity for optimum survival in the host. The PrV Km values are somewhat similar to the values reported for marmoset herpesvirus TK (104) and the TK from eucaryotic cell cytoplasm (23, 103), and considerably less than the values for 3 of the 4 mitochondrial TKs (23, 104).
Table 13. Km values for thymidine of thymidine kinase from various sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Km (μm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV type 1 clinical isolate</td>
<td>0.54(^a)</td>
<td>117</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>0.57(^b)</td>
<td>117</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>0.29(^c)</td>
<td>117</td>
</tr>
<tr>
<td>&quot; &quot; strain KOS</td>
<td>0.14</td>
<td>117</td>
</tr>
<tr>
<td>&quot; &quot; &quot; &quot;</td>
<td>0.59</td>
<td>29</td>
</tr>
<tr>
<td>HSV type 2 strain 333</td>
<td>0.35</td>
<td>29</td>
</tr>
<tr>
<td>Marmoset herpesvirus</td>
<td>4.7</td>
<td>104</td>
</tr>
<tr>
<td>Eucaryotic cell cytoplasm(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; e</td>
<td>2.6</td>
<td>103</td>
</tr>
<tr>
<td>Mitochondria(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; f</td>
<td>5.2</td>
<td>103</td>
</tr>
<tr>
<td>&quot; e</td>
<td>10.4</td>
<td>104</td>
</tr>
<tr>
<td>&quot; g</td>
<td>12.4</td>
<td>23</td>
</tr>
<tr>
<td>&quot; g</td>
<td>13.1</td>
<td>23</td>
</tr>
</tbody>
</table>

\(^a\) Following acyclovir (ACV) therapy, viral TK isolated from the patient had a Km value of 25.5 μm.

\(^b\) Following ACV therapy, viral TK isolated from the patient had a Km value of 1.96 μm.

\(^c\) Following ACV therapy, viral TK isolated from the patient had a Km value of 1.41 μm.

\(^d\) Blast cells of acute myelocytic leukemia.

\(^e\) LA9 cell culture.

\(^f\) Chick embryo cells.

\(^g\) LMTK- cell culture.
Determination of the Km is independent of enzyme concentration. The Vmax, however, is dependent on enzyme concentration, and varies among replications (Table 3). The 3rd replication of the BioCeutic strain produced much lower counts in the initial velocity assay than were observed during the first 2 replications. The cells from which the enzyme was extracted for the 3rd assay showed much less CPE after overnight incubation, probably due to a lower MOI, and consequently yielded less viral TK in the extract. The Km value for the 3rd replication was very similar to those of the first 2 assays.

Restriction enzyme (RE) analysis revealed that the Norden and Pitman-Moore PrV strains are closely related (Figures 8, 9, 10). The BioCeutic strain is significantly different in RE patterns from the other 5 strains examined, indicating a distant evolutionary relationship. It is significant, however, that the BioCeutic strain, as well as the Norden and Pitman-Moore strains, lack the distinct Pst I A fragment that is found in the other 3 strains (Figure 9). Geck et al. (52, 53) observed that the absence of the Pst I A fragment was a marker for the Bartha K strain, and could be used to distinguish it from other avirulent and virulent strains of PrV. Lomniczi et al. (107) used marker rescue of a wildtype PrV Pst I A fragment to restore virulence to the Bartha strain, proving that a gene that influences virulence lies in that region of the genome. Very recent evidence indicates that a glycoprotein may be the gene product encoded in the Pst I A fragment that plays a role in determining virulence (122) and may function in release of virions from infected cells (21).
RE analysis also revealed minor variations between the IN-Fh strain and the TK5Al TK\textsuperscript{−} mutant derived from it (Figures 8, 9, 10). Ben-Porat et al. (20) also noted that RE pattern differences existed between a TK\textsuperscript{−} mutant and its parental strain. Ben-Porat reported reiterations of the Bam HI E fragment in DNA from the TK\textsuperscript{−} mutant, which was not observed in the parental strain pattern. Ben-Porat concluded that the change in the RE pattern may be related to a biological function associated with the loss of TK activity. In the present study, the Bam HI E fragment did not change after selection for a TK\textsuperscript{−} mutant. The changes in the TK5Al RE pattern are associated with an increase in MW of Bam HI fragments L and N (Figure 8). Both the L and N fragments map within the repeat sequences of the genome (Figure 1). The Bam HI fragment E also maps within the repeat sequences, contiguous to the L and N fragments. It has been observed by Wathen and Pirtle (178) that the repeat region is associated with minor MW changes in restriction fragment mobility; they reported that similar changes occurred in the Bam HI L and N fragments after passage of a strain of PrV in vivo. Similar changes occur in HSV (37) and equine herpes virus type 1 (2), and are probably not associated with the TK gene function, which maps at approximately 0.45 map units (18).

The six PrV strains included in the present study were compared for virulence for the mouse (Table 2). The commercial vaccine strains, which express TK activity similar to the virulent strains but contain a major deletion in the Pst I A region of the genome, are reduced in virulence for the mouse approximately 2 to 10 fold, compared to the
NVSL 85-12 and IN-Fh strains. The TK5A1 strain, which lacks TK activity but retains a Pst I RE pattern similar to the virulent strains, is avirulent for the mouse by IP inoculation. Although the gene contained in the Pst I A fragment of the genome influences virulence, particularly for swine, the TK gene obviously has a much greater influence on virulence. It is the belief of this author that modified live vaccines for PrV would be much safer if they were made deficient in TK activity. As noted in 2 reports (98, 174), fatal infections in sheep occasionally occur from the use of attenuated PrV vaccine virus, and in a recent incident in Illinois, one or more cattle died as the result of vaccination with a PrV live-virus vaccine.¹ Some interest has been shown in vaccinating cattle (25) and dogs (133) against pseudorabies, but the results have not been satisfactory. It is possible that one or more inoculations with a TK⁻ vaccine strain, such as TK5A1 or a similar strain, would stimulate protective immunity in these species.

While this work was in progress, two reports appeared in the literature concerning the vaccination of swine with TK⁻ live-virus vaccines. McGregor et al. (116) vaccinated pigs with a TK⁻ mutant of the Kaplan (Ka) strain of PrV selected in the presence of Ara-T. The vaccine was effective in preventing mortality in 8-week-old pigs challenged with the Sullivan PrV strain. Ganglia from vaccinated

¹Dr. G. A. Erickson, USDA, NVSL, DVL, Ames, IA, personal communication.
animals were co-cultivated with PrV-susceptible cell cultures in an attempt to reisolate latent vaccine virus. No vaccine virus was isolated. Similarly, ganglia from vaccinated and nonvaccinated animals taken post challenge were assayed for latent virus, and the percentage of reisolations was greatly reduced for the vaccinated group, indicating that vaccination with a live virus vaccine greatly reduces the colonization of ganglia by challenge virus.

Kit et al. (90) produced a TK⁻ vaccine virus by using recombinant DNA techniques to delete a portion of the TK gene of the BUK strain of PrV. The use of this approach prevents the reversion of the mutant to TK⁺, which might occur with a TK⁻ strain selected by drug resistance. Vaccination of swine with the TK⁻ BUK strain stimulated the production of low levels of neutralizing antibody and was effective in preventing mortality in animals challenged with the virulent IN-Fh strain.

The question arises as to the relative effectiveness of a TK⁻ mutant of the already attenuated BUK strain which originated in central Europe, and has undergone countless passages in vitro since its isolation, relative to a TK⁻ mutant of a virulent PrV strain isolated recently in the United States. It is possible that selection only for the TK⁻ phenotype may allow more replication in the region of inoculation than would a strain also having a deletion in the Pst I A region of the genome, as does the BUK strain. Greater replication might result in a stronger primary immune response. It is also possible that the BUK strain does not share all the antigenic determinants that play a role in the immune response to PrV strains endemic in the United
States. Therefore, selection of a TK⁻ strain of PrV from a region with endemic pseudorabies might provide a vaccine with a greater number of shared antigenic determinants with strains circulating in that area, resulting in greater effectiveness.

In conclusion, the TK5Al strain may 1) be able to replicate in the host to a greater extent than strains derived from the Bartha or BUK strains, resulting in a stronger primary immune response; 2) share more antigenic determinants with PrV strains circulating in the United States than strains derived from the Bartha or BUK strains; and 3) be safe for use in non-swine species.

Further studies should be conducted on TK⁻ PrV strains derived from field isolates to determine whether the points listed above are verifiable.
SUMMARY

A thymidine kinase (TK)-negative mutant of the Indiana-Funkhauser strain of pseudorabies virus (PrV) was isolated and examined for reversion to TK*. The TK\(^-\) mutant was avirulent for mice inoculated intraperitoneally, and for 7-week-old swine inoculated intramuscularly. The TK\(^-\) mutant stimulated protective immunity in mice and swine challenged with lethal doses of a virulent PrV challenge strain.

Three commercially available modified live-virus (MLV) vaccine strains and 2 field isolates were determined to encode TK activity by thymidine plaque autoradiography. Enzyme kinetic behavior of the TK extracted from LMTK\(^-\) cells infected with the MLV vaccine strains and 2 field isolates revealed only minor differences in Km values.

The TK\(^-\) mutant, 2 field isolates and 3 vaccine strains were compared by restriction endonuclease (RE) analysis and for relative virulence for mice. The 3 vaccine strains were 2 to 10 fold less virulent for mice compared with the field isolates; in contrast, the TK\(^-\) mutant did not cause morbidity or mortality in mice.

RE analysis revealed that all 3 commercial vaccine strains lacked the Pst I A fragment characteristic of the other strains examined. It was also observed that 2 of the vaccine strains, Norden and Pitman-Moore, are closely related, and that the BioCeutic strain differed significantly from the other strains examined.

Small changes in molecular weight (MW) were observed in 2 Bam HI fragments of the TK\(^-\) mutant when compared to Bam HI fragments of its parental strain. The fragments, which were greater in MW, mapped in
the repeat sequences of the genome. The changes observed may be independent of the TK phenotype.
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