Biologic Properties of Midwestern Isolates of Pseudorabies

Pamela F. Leslie

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BIOLOGIC PROPERTIES

OF

MIDWESTERN ISOLATES OF PSEUDORABIES

BY

PAMELA F. LESLIE

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Department of
Microbiology, South Dakota State
University

1975

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BIOLOGIC PROPERTIES
OF
MIDWESTERN ISOLATES OF PSEUDORABIES

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirement for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor

Date

Head, Microbiology Department

Date
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Also, I would like to thank Esther and Russell Leslie, my parents, for their support and encouragement during my graduate studies.

PFL
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>History</td>
<td>3</td>
</tr>
<tr>
<td>Classification</td>
<td>3</td>
</tr>
<tr>
<td>Characteristics of Modification</td>
<td>4</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>Cell Culture</td>
<td>7</td>
</tr>
<tr>
<td>Medium</td>
<td>7</td>
</tr>
<tr>
<td>Virus</td>
<td>7</td>
</tr>
<tr>
<td>Plaque Assay</td>
<td>9</td>
</tr>
<tr>
<td>Enhancement of Plaque Size Difference</td>
<td>9</td>
</tr>
<tr>
<td>Adsorption to Rabbit Brain Tissue</td>
<td>10</td>
</tr>
<tr>
<td>Rabbit Inoculations</td>
<td>11</td>
</tr>
<tr>
<td>Successive Passages in Cell Cultures</td>
<td>11</td>
</tr>
<tr>
<td>Immuno-electron microscopy</td>
<td>12</td>
</tr>
<tr>
<td>Mutagenesis with NG and Ts Screening</td>
<td>13</td>
</tr>
<tr>
<td>Heat Test</td>
<td>14</td>
</tr>
<tr>
<td>Trypsin Test</td>
<td>14</td>
</tr>
<tr>
<td>RESULTS</td>
<td>15</td>
</tr>
<tr>
<td>Adsorption to Rabbit Brain Tissue</td>
<td>15</td>
</tr>
<tr>
<td>Successive Passages in Cell Cultures</td>
<td>15</td>
</tr>
<tr>
<td>Immuno-electron microscopy</td>
<td>20</td>
</tr>
<tr>
<td>Chemical and Physical Agents</td>
<td>28</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>30</td>
</tr>
<tr>
<td>Characteristics of Modification</td>
<td></td>
</tr>
<tr>
<td>SUMMARY</td>
<td>35</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>36</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. PRV infectivity titers after 1 hr adsorption</td>
<td>16</td>
</tr>
<tr>
<td>II. PRV infectivity titers after 1 hr and 2 hr adsorptions</td>
<td>17</td>
</tr>
<tr>
<td>III. Properties of PRV plaque size variants</td>
<td>23</td>
</tr>
<tr>
<td>IV. Properties of PRV Ts isolates</td>
<td>29</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Rabbit showing pruritis</td>
<td>19</td>
</tr>
<tr>
<td>2.</td>
<td>Monolayer of cells infected with PRV</td>
<td>22</td>
</tr>
<tr>
<td>3.</td>
<td>Plaque plates showing PRV size variants</td>
<td>25</td>
</tr>
<tr>
<td>4.</td>
<td>Electron micrographs of PRV</td>
<td>27</td>
</tr>
</tbody>
</table>
INTRODUCTION

Pseudorabies is an acute infection of domestic and wild animals caused by herpesvirus. In swine, which are the natural hosts for the disease, the virus produces a fatal encephalitis in piglets. Death loss in litters 3-4 weeks old is estimated from 40-60% (?). Pseudorabies causes abortions in sows infected early in pregnancy and causes mummified and macerated fetuses in sows infected later in pregnancy (11).

Distribution of pseudorabies is widespread. The disease occurs throughout central and eastern Europe where it causes significant losses in cattle and swine. Outbreaks have occurred in the British Isles where recently a more virulent virus strain than previously known to that area has been recognized (2). While pseudorabies is a more severe disease of swine in Europe than in the United States, the American strains of the virus appear to have acquired enhanced virulence over the past 10-12 years. This is reflected in the mortality of pigs in all age groups, in contrast to the past when only suckling pigs died of the disease (17).

Previously, pseudorabies was not considered to be a problem in the midwest but prevalence of the disease has increased over the last five years. In 1974 alone, eight isolations of pseudorabies virus were made at the South Dakota Animal Disease Research and Diagnostic Laboratory from outbreaks in the area of northwest Iowa, Nebraska, and South Dakota. As of February 1, 1975, three isolations of pseudorabies virus have been made from outbreaks in the area. Iowa State Diagnostic Laboratory reported a six-fold increase in pseudorabies cases in 1974.
as compared to the preceding four years (5). A survey of swine in
Indiana indicated a high susceptibility rate for the disease (18).

Because there is no vaccine available for pseudorabies in the
U.S. nor is there an effective treatment for the infection, this study
was designed to determine the characteristics of the pseudorabies
isolates and induce and/or select for a less virulent strain of the
virus. Assays were performed to select for a consistent character-
istic to serve as a marker for a less virulent pseudorabies strain.
This may aid in selection of strains suitable for vaccine development.
LITERATURE REVIEW

Pseudorabies or Aujesky's disease was first described by Aujesky in Hungary in 1902. The disease, which affects the nervous system of animals, was initially compared to rabies virus because of the signs of drooling saliva and jaw uncoordination observed in infected pigs and cows. The disease can affect a wide variety of domesticated animals including swine, cattle, dogs, cats, and sheep. Less often, the disease affects wild animals including mink, rats, and mice. The infection has never been confirmed in man (2).

Classification of pseudorabies virus (PRV) is based on the physical and biochemical properties of the virus. The average diameter of the virus capsid is 110 nm; the capsid is composed of 162 capsomeres. The nucleic acid of PRV is double-stranded deoxyribonucleic acid (DNA). Like other enveloped viruses, PRV is susceptible to ether and chloroform. The virus is stable between pH 5 and pH 9 (9). It is reported to have a half-life of 7 hr at 37°C (3). Unlike most DNA viruses, PRV growth is not inhibited by 5-iododeoxyuridine or 5-bromodeoxyuridine (8).

The virus is capable of replication in many types of cell cultures, including mouse fibroblasts, HeLa cells, rabbit, swine, and monkey kidney cells, and chick embryos (2). Not all tissue culture systems are equally sensitive and the cytopathic effect (CPE) may vary with the cell type (2). Two different types of CPE occur. One type is composed mostly of multinucleated giant cells (syncytia) and the other of rounded, highly refractile cells. The two types may occur in the same
culture but one will predominate (10). The virus produces plaques on a cell monolayer when overlayed with an enriched agar. Plaque morphology is noted to be a function of virus strain (2).

Pathogenicity in rabbits and growth characteristics were markers used by European researchers to differentiate less virulent PRV strains which occur naturally or were induced (2). One marker was pruritis, a severe itching, which occurred in rabbits inoculated intramuscularly or subcutaneously with PRV. This pruritis was associated with two plaque sizes. The naturally attenuated K strain was isolated from a field outbreak of Aujesky's disease (1). Differentiation of the attenuated K strain was based on production of small plaques and rounded cells in pig kidney cell cultures. The SUCH-1 strain produced large plaques in chick embryo cultures (CEC) and grew slowly to low titers (21). It did not produce pruritis when inoculated into rabbits. This naturally attenuated strain was isolated from the organs of a pig infected with PRV. It was selected from field isolates for serial passage in CEC and was found to display low virulence for livestock from the first passages.

Artificially attenuated strains with some of the same properties have been reported (20, 21). Skoda modified a strain designated Bucharest (BUK) by serial passage in CEC (21). The strain was obtained after it had undergone 98 passages in chick embryo chorioallantoic membranes (CAM). It was then passed over 600 times in CEC. After the 115th passage in CEC, the virus did not cause pruritis in inoculated rabbits. The appearance or absence of pruritis was unrelated to infective dose. When assayed for plaque size in CEC, the BUK strain
produced larger plaques than virulent strains used for comparison. No characteristic differences were observed in the CPE produced by the different strains.

Earlier Tokamura differentiated two PRV strains by CPE in monkey kidney cells (22). The PRV strain obtained from the American Type Culture Collection (ATCC) had been rabbit brain-adapted and undergone 18 passages in CAM of chick embryos. After three passages of the virus on monkey kidney cell monolayers, two types of CPE were noted. One clone was of rounded ballooned cells which produced small plaques. The other strain produced giant cells and large plaques. When inoculated into rats, the virus producing large plaques produced pruritis in rats while the other type did not produce pruritis. There was no difference in heat stability.

The K strain was analyzed by Bodon and found to resist the effect of heat and trypsin (3). The virulent virus and K strain were held at 50 C for one hour and then plaque assayed. The titer of the avirulent virus was reduced from 10^7 to 10^6. The virulent virus lost all infectivity. In the trypsin assay, the virus was exposed to 0.5 mg/ml of trypsin at 37 C for one hour. The titer of the avirulent virus was reduced from 10^7 to 10^6. The virulent virus lost all infectivity.

Other virus models provided different means of selecting for less virulent strains. In the reovirus model, temperature sensitive mutants of Reovirus III, unable to multiply at the restrictive temperature (39 C), were found to be less neurotropic (14). Similarly, temperature sensitive mutants have been induced in DNA viruses such as herpes simplex (19).
A poliovirus model compared the capacity of highly virulent and highly attenuated strains to combine with gray matter from the brain of various primate species (16). A quantitative difference proved that the highly attenuated strain would not adsorb to the nervous tissues tested and was more likely to be recovered after the reaction.
MATERIALS AND METHODS

Cell Culture

Secondary pig kidney cells were used for isolation of viruses from brain tissues of animals suspected of having PRV. Cell lines used for successive passages were as follows: Vero Green Monkey kidney cells, passages 120-150, obtained from A.T.C.C.,* HeLa, human epitheloid carcinoma cells, passages 92-110-92-150, obtained from A.T.C.C.,** Primary chick embryo cells (CEC) were provided courtesy of Salsbury Laboratories***. The primary cells were subcultured and used until the 10th passage. Stock cultures of all cell lines were preserved by freezing in growth medium with 7.5% dimethyl sulfoxide at -70 C.

Medium

The growth medium for all the cell lines was as follows: Eagle's Minimum Essential Medium (MEM)**** with Earle's balanced salts, non-essential amino acids, 10% fetal calf serum, 0.5% lactalbumin hydrolysate, 0.01% pyruvic acid, 0.03% L-glutamine, penicillin G, 100 units/ml, streptomycin, 100 ug/ml, and kanamycin, 100 ug/ml. The plaque medium consisted of Eagle's MEM, supplemented with 0.01% pyruvic acid. This was made in a double strength stock solution and mixed with

* CCL-81, American Type Culture Collection, Rockville, Maryland.
** CCL-764, American Type Culture Collection, Rockville, Maryland.
*** Salsbury Laboratories, Charles City, Iowa.
an equal amount of 1.4% ion agar* containing 40 ug/ml of DEAE dextran. Before mixing, the melted agar and 2X medium were brought to 45 C.

**Virus**

Four PRV isolates from tissues of swine showing central nervous system (CNS) symptoms were used for the study; these were designated by accession numbers S.D. 74-3457, S.D. 74-3738, S.D. 74-3959, S.D. 74-4261. They were pooled at approximately the fifth passage in tissue culture and designated Aujesky's I virus pool. Another PRV isolate used for comparison was obtained from a cow with interstitial pneumonia. The virus was isolated from a tissue pool of bovine spleen and lung and designated S.D. 74-7194.

Identification of the isolates was based on appearance of typical PRV cytopathic effect in cell culture. Initial confirmation of identity was made with the fluorescent antibody (F.A.) test in which infected cells were stained with an anti-pseudorabies antibody conjugated with fluorescein isothiocyanate**. The stained preparation was observed using a microscope equipped with an ultra-violet light source. The virus was identified as PRV if the infected cells showed cytoplasmic fluorescence. The original virus suspensions were also inoculated subcutaneously into adult white rabbits. The development of CNS symptoms accompanied by pruritis at the site of inoculation and subsequent death of the rabbit confirmed the isolate as PRV. The bovine PRV isolate was

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** Courtesy of Dr. E. Carbrey, NADC, Ames, Iowa.
confirmed by the F.A. test and immuno-electron microscopy using antiserum against procine PRV.

Plaque Assay

The plaque assay method was used for virus purification and as a means of evaluating effects of various temperatures and chemicals. Vero cells were propagated in 60 mm. plastic petri dishes until complete monolayers were formed. The growth medium was removed from each monolayer and an inoculum of 0.5 ml of each virus dilution was placed on the cells. The virus was allowed to adsorb for one hour at 37 C. After the adsorption period, the excess inoculum was removed, and the plates were rinsed with Hank's balanced salt solution (HBSS). Five ml of agar overlay was then added to each plate and allowed to solidify at room temperature. The cultures were then inverted and incubated at the appropriate temperature in a humidified, 5% CO₂ atmosphere. After development of CPE in the cells, any isolates to be transferred were then removed as described under plaque purification. After 2-3 days, the agar overlay was removed and equal volumes of 10% crystal violet and phosphate buffered formaldehyde, pH 7 were added to fix and stain the cells. After 3-5 min, the stain was removed and the number and morphology of plaques noted.

Enhancement of Plaque Size Difference

To enhance the size difference between large and small PRV plaques, a method similar to Skoda et al. was used (22). After the virus was adsorbed and overlayed with agar, as described under plaque assay, part

* Falcon Plastics, Oxnard, California.
of the cultures were incubated at 30 C for 42 hours and the other incubated at 40 C for 42 hours. After this, both cultures were incubated for 30 hr at 37 C in a 5% CO₂ humidity-controlled incubator. The control virus cultures were incubated at 37 C for 72 hr and used for comparison.

**Plaque Purification**

When the plaques were visible, they were examined using a light microscope to determine any differences in plaque types. A sterile capillary pipette was used to select a sample from plaques exhibiting the greatest difference in size. These samples were each inoculated onto Vero cell monolayers in tissue culture plates. When 80% of the cells were cytopathic, they were frozen, thawed, and again plaqued in order to evaluate plaque size and consistency of characteristics. Three of the most consistent isolates from the successive passage in chick embryo cells were again plaque purified and propagated in cell monolayers.

**Adsorption to Brain Tissue**

Rabbit brains were obtained from adult white rabbits. The brains weighed between 5 and 10 grams. In the first three adsorptions, the whole brains were then ground with a mortar and pestle and mixed with 5x10⁶-7x10⁷ plaque forming units (PFU) of virus. The initial inoculum was the pooled S.D. isolates designated Aujesky’s I. The virus-brain mixture was incubated at 31 C for one hour, centrifuged at 1,100 x g for 15-20 min, and the supernatant fluid reinoculated as described after adsorptions 4-13.
In the fourth through the thirteenth adsorptions, the brains were weighed, then divided into two portions and ground with a mortar and pestle. Virus aliquots containing $2 \times 10^6 - 1.6 \times 10^8$ PFU of inoculum were added to one portion of tissue and incubated for 1 hr at 37°C in a 5% CO₂ humidity-controlled incubator. The suspension was then centrifuged at 4°C in a Sorvall RC-2 refrigerated centrifuge for 15-20 min. at 1,100 x g. The supernatant fluid was then decanted and the adsorption process repeated with the other portion of brain for 2 hr at 37°C. The final supernatant fluid was then decanted and inoculated onto a monolayer of Vero cells. When approximately 80% of the cells were cytopathic, the plate was frozen, thawed, and 1 ml of tissue culture fluid inoculated into a 75 sq. cm. flask of Vero cells. When the monolayer was approximately 80% cytopathic, the flask was frozen, thawed, and 1 ml of tissue culture fluid was inoculated into a second flask of Vero cells. This virus pool provided the inoculum for the next adsorption with brain tissue.

**Rabbit Inoculations**

Random virus suspensions after adsorption to rabbit brain, and various virus isolates were selected for inoculation into rabbits for virulence testing. One-half ml of the virus suspensions was inoculated into rabbits subcutaneously. The rabbits were then observed for CNS symptoms and pruritis.

**Successive Passages in Cell Cultures**

Two cell lines, HeLa and chick embryo cells, were used to determine the effect of continuous passage of PRV in unrelated cell lines. Approximately $1 \times 10^6$ PFU of Aujesky's I was initially inoculated onto a
75 sq. cm. flask of HeLa cells. Similarly, the CEC monolayers were inoculated with approximately $5 \times 10^4$ PFU of Aujesky's I which had been adsorbed to brain tissue eight times and propagated in Vero cells a total of 15 passages. Isolate S.D. 74-7194 was propagated in monolayers of CEC in tissue culture plates. When the CPE reached approximately 80% within 48-72 hr, the cultures were frozen, thawed, and one ml of the cell culture fluid was inoculated onto a fresh monolayer in a flask, or 0.5 ml of the fluid inoculated onto a fresh monolayer in a tissue culture plate. Five ml samples of the cell culture fluid were frozen at -70 C and stored for later evaluation. Viral infectivity titer was checked periodically as described under "Plaque Assay" and plates were screened for CPE and plaque size consistency. Passages with inconsistent plaque size were then assayed and plaque purified as previously described. Selected isolates were inoculated into rabbits as previously described.

**Immunoelectron Microscopy**

In order to determine the relationship of bovine PRV isolate, S.D. 74-7194, to porcine PRV and check the plaque size variants of PRV for extraneous virus, immunoelectron microscopy (e.m.) was employed. Swine antiserum* to PRV was reacted with the above viruses and observed under the electron microscope. The methods employed were essentially those of Richie et al. (15).

Preparation of the virus-infected cells for e.m. was as follows: a 75 sq. cm. flask of 80% virus-infected cells and medium was harvested

* Courtesy of Dr. E. Carbrey, N.A.D.C., Ames, Iowa.
by a freeze-thaw cycle followed by centrifugation at 1,100 x g for 15 min at 5 C in a Sorvall RC-2 centrifuge. The virus and cell pellet were resuspended in 0.3 ml of sterile water.

Negative stain: a 4.0% solution of phosphotungstic acid, pH 6.5, 0.1% bovine serum albumin, water, and virus suspension was mixed 1 drop/1 drop/20 drops/2 drops, respectively. The suspension was sprayed with a nebulizer* onto a 300 mesh carbon and colloidon-coated grid. The grid was immediately examined in a Hitachi HU-12 electron microscope.

Immuno-e.m.: two drops of the water-virus suspension obtained from the virus and cell pellet were mixed with an excess of antibody, 3 drops. The mixture was then incubated at 4 C for 24 hr. In order to retrieve the virus-antibody complex, the mixture was centrifuged at 12,300 x g in a Sorvall SS-3 centrifuge for ½ hr. The antibody-virus pellet was resuspended in 0.2 ml sterile water and mixed with phosphotungstic acid in the same proportions as above for negative staining.

Mutagenesis with NG and Ts Screening

N-methyl-N’ Nitro-N-Nitrosoguanidine** (NG) was used in varying concentrations to induce production of a temperature sensitive (Ts) mutant of PRV. An assay to select for the mutants was designed similar to that of Schaffer et al. (19). Aujesky’s I virus pool and a sample from the eighth brain adsorption were inoculated onto a monolayer of Vero cells and allowed to adsorb for 1 hr at 37 C. The virus samples were then removed and the monolayers rinsed with HESS. Five ml

* Pelco Corp., Austin, Texas.

** Schwarz-Mann, Orangeburg, New York.
aliquots of the appropriate dilutions of NG, 10, 25, 50, 100, ug NG/ml, were inoculated onto the plates which were then incubated for 24-48 hr at 33 C. When 80% of the monolayer was cytopathic, the plates were frozen, thawed, and the tissue culture fluid stored at -70 C as mutagenized stock. This was used to infect fresh monolayers at approximately 10-20 PFU per plate in the manner described for "Plaque Assay". The incubation temperature for the plaque screening was lowered to 35 C. After 72 hr, a random number of well-isolated plaques were outlined. The temperature of incubation was then raised to 39 C and the plates were incubated for 36 hr.

Plaques which did not increase in size when incubated at the restrictive temperature, 39 C, were transferred by capillary pipette to duplicate monolayers of Vero cells. Such isolates were suspected temperature sensitive mutants of PRV. Three of the suspected mutants were later screened as described under "Plaque Size Enhancement" for markers of modification.

**Heat Test**

Two elevated temperatures were used to evaluate heat resistance of PRV. Virus suspensions were incubated for 1 hr at 56 C or 50 C. The suspensions and controls incubated at 25 C were diluted and plaqued as previously described.

**Trypsin Test**

Virus suspensions were incubated at 37 C for 1 hr with 0.5 mg of trypsin/ml of suspension. The suspensions were then diluted and 0.5 ml of the suspension inoculated onto a Vero cell monolayer and incubated for 1 hr at 37 C. The plates were then plaqued as previously described.
RESULTS

Adsorption to Rabbit Brain Tissue

Viral infectivity titers resulting from the attempts to select a non-neurotropic strain of PRV by adsorbing the virus to brain tissue are shown in Tables I and II. When a suspension of the virus pool, Aujesky's I, was incubated with the brain tissue for one hour, the virus infectivity titer of the recovered supernatant fluid fluctuated (Table I). The infectivity titer of a suspension of the virus pool incubated without brain tissue did not decrease after the same period at 37 °C. Rabbits inoculated with the supernatant fluids after adsorption died after developing CNS symptoms and pruritis.

For the fourth adsorption and those following, a modified procedure was used. The virus suspension was incubated with one portion of brain for one hour. The fluids recovered were then incubated with the second portion of brain for two hours. A greater decrease in the viral infectivity titer resulted from this procedure. Throughout nine similar attempts to select a non-neurotropic PRV strain, the viral infectivity continued to fluctuate (Table II). Rabbits inoculated with the supernatant fluids died with clinical CNS symptoms and pruritis. See Figure 1.

Successive Passages

The pooled virus grown in HeLa cells produced rounded and refractile cells within 48 hr after inoculation. The infectivity titer after 52 successive passages through cell cultures did not exceed $6 \times 10^6$ PFU/ml. During this time, no variations in CPE or plaque morphology
<table>
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<th>Adsorption #</th>
<th>Inoculum PFU/ml</th>
<th>Recovery PFU/ml</th>
<th>Rabbit Inoculation</th>
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<tr>
<td>1</td>
<td>3.3x10^6</td>
<td>4.2x10^5</td>
<td>died with pruritis</td>
</tr>
<tr>
<td>2</td>
<td>7.8x10^6</td>
<td>1.3x10^4</td>
<td>N.T.*</td>
</tr>
<tr>
<td>3</td>
<td>1.4x10^8</td>
<td>1.0x10^6</td>
<td>died with pruritis</td>
</tr>
<tr>
<td>25 C control</td>
<td>1.4x10^8</td>
<td>1.7x10^8</td>
<td>N.T.*</td>
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* Not Tested
Table II. PRV Infectivity Titers After Adsorption to Rabbit Brain For 1 Hr Followed by 2 Hr Adsorption

<table>
<thead>
<tr>
<th>Adsorption #</th>
<th>Inoculum PFU/ml</th>
<th>Recovery PFU/ml</th>
<th>Rabbit Inoculation</th>
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</thead>
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<tr>
<td>4</td>
<td>4.3x10^7</td>
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<td>N.T.*</td>
</tr>
<tr>
<td>5</td>
<td>9.8x10^6</td>
<td>1.3x10^4</td>
<td>N.T.*</td>
</tr>
<tr>
<td>6</td>
<td>1.8x10^7</td>
<td>3.0x10^4</td>
<td>N.T.*</td>
</tr>
<tr>
<td>7</td>
<td>1.4x10^7</td>
<td>3.0x10^4</td>
<td>died with pruritis</td>
</tr>
<tr>
<td>8</td>
<td>1.2x10^7</td>
<td>3.0x10^4</td>
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<td>12</td>
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</tr>
<tr>
<td>13</td>
<td>1.1x10^7</td>
<td>1.5x10^3</td>
<td>died with pruritis</td>
</tr>
</tbody>
</table>

* Not Tested
Figure 1. Rabbit showing severe pruritis (note arrow) at site of inoculation with PRV. (48 hr post inoculation.)
were noted. The virus pool consistently produced virus plaques at 37 C. When incubated at 40 C, the virus pool produced significantly larger plaques. Immunoelectron microscopy of the suspension revealed that the virus had been passaged 29 times in vivo. The test result virus reaction was positive. The rabbit inoculated with virus strain B approximately 2x10^6 PFU of Salm-sky's 1 for seven days showed signs of inflammation.

The CNS were initially inoculated and subsequently was infected with 5x10^6 PFU of PRV. After inoculation of tissue culture, granulation (Figure 2), morphology was observed. Inoculation from the follicles of the rabbit.

The PRV, the virus producing infection was inoculated into a rabbit. The rabbit died within 48 hours. CNS symptoms were present.

Immunoelectron Microscopy

Virus inoculated into tissue culture and cell cultures was examined by immun-e.c.m. For which the strain and the susceptibility was determined as PRV. All three tissue cultures from CIV reacted positively. The bovine isolate, B.2.1987, reacted positively with 5x10^6 PFU as pictured in Figure 3.
were noted. The virus pool consistently produced large plaques at 37 C. When incubated at 40 C, the virus pool continued to produce large plaques. Immuno-electron microscopy was performed on Aujesky's I which had been passaged 29 times in HeLa cells. The antiserum-virus reaction was positive. A rabbit inoculated subcutaneously with approximately 3x10^6 PFU of Aujesky's I HeLa passage 52 died exhibiting pruritis.

The CEC were initially inoculated with approximately 3x10^4 PFU of PRV. After 38 passages, the virus infectivity titer was 9x10^5 PFU/ml of tissue culture fluids. The virus produced rounded cells with much granulation after 48-72 hr post-inoculation onto a monolayer of cells (Figure 2). At the 37th and 38th passage in CEC, a variation in plaque morphology was noted. Three variants were isolated by plaque purification from PRV in CEC at passage 37. Data describing the characteristics of the isolates is summarized in Table III and Figure 3.

The PRV isolate S.D. 74-7194, was propagated in CEC for 15 passages. The virus produced rounded cells and complete CPE on the cell monolayer after 24 hr. When the virus which had been passed 12 times through CEC, was inoculated into a rabbit, the animal died after developing clinical CNS symptoms and pruritis.

**Immuno-electron Microscopy**

Viruses isolated from large and small plaques on CEC were checked by immuno-e.m. for extraneous virus and for positive antigenic identity as PRV. All three virus isolates from CEC reacted positively. The bovine isolate, S.D. 74-7194, reacted positively with PRV antiserum as pictured in Figure 4.
Figure 2. Monolayer of Vero cells infected with PRV showing (a) rounding and (b) granulation of cells.
Table III. Properties of PRV Plaque Size Variants Isolated CEC Passage-37

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Plaque Size</th>
<th>Titer PFU/ml</th>
<th>Rabbit Inoculation</th>
<th>Immuno-e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>54/1a</td>
<td>small</td>
<td>4.0x10^5</td>
<td>died without pruritis</td>
<td>+PRV</td>
</tr>
<tr>
<td>61/3-5</td>
<td>large</td>
<td>4.6x10^6</td>
<td>died without pruritis</td>
<td>+PRV</td>
</tr>
<tr>
<td>52/5c</td>
<td>large</td>
<td>1.3x10^6</td>
<td>died with pruritis</td>
<td>+PRV</td>
</tr>
</tbody>
</table>
Figure 3. Vero cell monolayers stained with crystal violet showing (a) large (.9-1.2 mm) and (b) small (.4-.7 mm) plaques produced by PRV variants from CEC passage 37 (magnification 4X).
Figure 4. Electron micrographs showing (a) uncoated PRV virion (280,000X) and (b) bovine PRV coated with antibody to PRV (120,000X).
**NG Treatment**

Three virus isolates which exhibited reduced ability to grow at 40 C were obtained from the NG treatment and screening. Each of the isolates had been subjected to 10 μg/ml of NG previously. The isolates were then assayed for plaque size enhancement. The results are summarized in Table IV.

**Heat Test**

Neither the large nor the small plaque isolate from CEC-37 were found to be heat resistant. No plaques were formed by either isolate after 1 hr at 50 C or 56 C indicating inactivation. The bovine isolate S.D. 74-7194, was also inactivated after 1 hr at 50 C.

**Trypsin**

The large and small plaque isolates, and S.D. 74-7194 were tested and found to be resistant to 0.5 mg/ml of trypsin at 37 C for 1 hr. The infectivity of the viruses did not decrease.
Table IV. Properties of PRV Ts Isolates from NG Treatment

<table>
<thead>
<tr>
<th>Isolate #</th>
<th>Plaque Size at 37 C</th>
<th>Titer PFU/ml</th>
<th>Plaque Size 40 C/37 C</th>
<th>Titer PFU/ml</th>
<th>Rabbit Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>large</td>
<td>2.2x10⁵</td>
<td>small</td>
<td>2.0x10⁵</td>
<td>N.T.*</td>
</tr>
<tr>
<td>8</td>
<td>large</td>
<td>1.0x10⁵</td>
<td>small</td>
<td>1.2x10⁴</td>
<td>died with pruritis</td>
</tr>
<tr>
<td>10</td>
<td>large</td>
<td>4.0x10⁵</td>
<td>large</td>
<td>3.0x10⁵</td>
<td>N.T.*</td>
</tr>
</tbody>
</table>

* Not Tested
DISCUSSION

Various biologic markers have been associated with PRV strains considered attenuated. These markers are lack of pruritis in inoculated rabbits, plaque size, CPE and infectivity titer, temperature and trypsin resistance of PRV. Pseudorabies virus isolates known to be virulent were examined for these markers. They were compared to markers exhibited by PRV isolates which had undergone successive passages through cell cultures, or undergone chemical treatments, or had been adsorbed to brain tissue. Through these methods, it was hoped to determine a consistent series of markers indicating reduced virulence of PRV.

The "p" marker, or ability to produce pruritis was generally not exhibited in pigs. Natural transmission of infection to cattle, sheep, and dogs, resulted in pruritis in those animals (2). Pruritis was also present in inoculated rabbits. The initial S.D. PRV isolates did not produce pruritis (p-) in pigs, but produced pruritis (p+) in inoculated rabbits. Brain-adsorbed PRV cultured in Vero cells retained the positive "p" marker in rabbits. Similarly, PRV passaged 52 times through HeLa cells retained the positive "p" marker. In agreement with Skoda's work (21), successive passage in chick embryo cells modified PRV. One large plaque isolate and one small plaque isolate from PRV passaged through CEC did not produce pruritis when inoculated into rabbits.

Plaque morphology was one characteristic associated with attenuation but two plaque sizes were observed. Bartha et al. reported that the K strain produced small plaques in pig kidney cell culture (1). In
contrast, Skoda's SUCH-1 and BUK-200 strains produced large plaques in cultures of chick embryo cells (21).

In this study, the PRV propagated in HeLa cells was never observed to produce different plaque morphology on Vero cells. After 37 successive passages through CEC, two virus plaque sizes were distinct on Vero cells. Two large and one small sized plaques were selected and reproduced after passage. The small plaque isolate and one large plaque isolate were found to produce the "p-" marker in rabbits. Because the "p-" accompanied one large plaque isolate but not both, plaque morphology alone was not considered a reliable marker for modification.

Lomniczi's work supported this when he noted plaque morphology to be a function of cell line (12).

Differences in CPE in cell lines as well as the virus titer of the different PRV strains, have been compared. In general, granulation with rounding of cell, and syncytia formation were the two types of CPE observed. Kaplan noted that both types were observed in the same monolayer but one will predominate (10). When Tokamura differentiated two distinct strains of PRV by type of CPE induced, he suggested that the original stock contained two strains which could be differentiated after adaptation in monkey kidney cells, or that a mutation occurred which led to the production of large plaques (22). Because of later experiments with other viruses, host controlled variation was recognized which may explain the modification of virus properties after passage in cell lines of an unrelated host species (6).

Skoda et al., compared the type and onset of CPE and found that the attenuated SUCH-1 and BUK, and virulent strains produced rapid onset
and development of CPE marked by foci of degenerated, rounded and refractive cells (21). The virulent CVHD produced the same CPE but grew more slowly in CEC. An explanation for the lack of syncytia noted by Skoda et al. (21) was proposed by Zuffa et al. in 1968 who concluded that PRV did not induce syncytia on CEC (23). They noted that a virulent strain on primary monkey kidney cells and calf kidney cells produced cell membrane lysis and formation of polykaryocytes while BUK strains produced rounding and clumping of cells. In contrast to Zuffa et al. (21), Lomniczi noted that cultures of virulent PRV developed CPE more rapidly and formed syncytia on pig kidney cells. He concluded that the virus yield was greater in avirulent strains even though the onset of CPE was slower. Because CPE and infectivity titer of the viruses were functions of cell type, no differentiation between virulent and avirulent PRV strains could be made by comparing CPE and titer.

Temperature responses were also one means to evaluate PRV strains. The technique of enhancement of plaque size as described by Skoda et al. increased the difference between the large and small plaques in European strains at 40 C/30 C (21). Similarly, the size difference between the isolates from CEC passage 37 at 40 C/37 C. No other successive passages in the other cell lines exhibited such plaque size differences.

Temperature sensitive mutants have been induced in other viruses (13, 14) including herpes virus (19). In other viruses, these mutants have been found to be less neurovirulent (14). In the NG-treated samples, some degree of plaque size difference was noted after plaque size difference enhancement at 40 C/37 C. In one sample, a drop in infectivity titer of the virus was also noted indicating a possible
sensitivity to increased temperature. However, the same sample proved to be neurovirulent when inoculated into a rabbit. It has been suggested that it is the degree of defectiveness and not the loss of a specific function which determines the attenuation of a virus in vivo (13). More work needs to be done in this area of Ts PRV.

Susceptibility of PRV to heat outside living systems was determined by Bodon et al. (4). They concluded that virulent PRV lost infectivity after exposure to 50 C for 1 hr. They suggested heat resistance was a property of attenuated strains. Neither PRV plaque variant of CEF-37 nor S.D. 74-7194 were resistant to 50 C.

Bodon et al. reported the attenuated strains were resistant to 0.5 mg/ml of trypsin for 1 hr at 37 C (4). He also found that a fresh field isolate of PRV was partially resistant to trypsin and suggested that was a sign of decreased virulence of the virus. The small and large plaque virus isolates and S.D. 74-7194 were found to be resistant to the same exposure to trypsin.

To determine the degree of modification of PRV variants, antigenic identity of the viruses was made through immuno-e.m. The PRV plaque variants, both small and large, gave positive reaction to porcine PRV antisera indicating that they did not have modified antigenic properties. To better characterize the bovine PRV isolate, it was reacted with porcine PRV antiserum. The antiserum-bovine PRV reaction was positive.

Observations by Sabin in his early work with polio virus confirmed a quantitative difference between the combining capacities of highly virulent and highly attenuated strains of polio virus with gray matter
of the brain of primates (16). The highly virulent virus was adsorbed to the tissue while the highly attenuated virus was recovered after the reaction.

If neurotropic PRV was adsorbed onto the rabbit brain, a decrease in virus infectivity titer was expected. In the present study, the adsorption produced a virus titer decrease, but the recovered virus did not have any markers associated with decreased virulence of PRV. Further work in this area may be significant if the plaque variants from CEC prove to be less neurovirulent in swine and can then be correlated to recovery from nerve tissue.
SUMMARY

It has been shown that field isolates of PRV were modified by successive passage in chick embryo cell culture. Such modification was recognized by a change in plaque morphology, lack of pruritis in inoculated rabbits, and trypsin resistance. These markers of modification may or may not prove to be directly related to virulence in swine.


