Characteristics of a Swine Papovavirus

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A new member of the papovavirus group has been isolated and appears to infect swine. The new agent, tentatively named swine papovavirus, appears to be very defective and replicates only within a very narrow host cell range. The original source of the isolate is under investigation. Preliminary evidence suggests that the origin of swine papovavirus is either a stable pig kidney cell line or pancreas-derived trypsin.

Papovaviruses have been isolated from many animals including man (2, 6, 9, 12). The papova group of deoxyribonucleic acid (DNA) viruses is known for its potential to transform cells in vitro and cause tumors in vivo (5, 11, 21). Although picorna viruses have been reported from piglet and swine breeding herds (4, 10), there are no reports in the literature of a swine papovavirus (SPV) having been isolated in vitro or seen in tissues by electron microscopy. Parrish (13, 14) reported a transmissible genital papilloma in swine, but the agent was not propagated in vitro. Recently, the agent causing this disease has been classified as a member of the poxviruses by Allison (1) and Andrewes and Pereira (2). During a preliminary study in which we were searching for adventitious agents in swine pancreas-derived trypsin, a virus was isolated whose biological and physical characteristics are the subject of this communication. We are still uncertain as to the exact source of this isolate, but experimental evidence is presented which suggests that the isolate is a swine agent and a new member of the papovavirus group.

MATERIALS AND METHODS

Cell cultures. The virus was propagated in two pig kidney cell lines derived from different clones of a parent line, PK-2a, established by Stice (15). One clone has been referred to as the PK-15 cell line (15) and the second clone as the PS cell line (7). The PK-15 cell line was purchased from Flow Laboratories, Inc., Rockville, Md. This cell line was originally obtained by Flow Laboratories, Inc. from the American Type Culture Collection at the 154th passage level. The PS cell line was provided by D. W. Trent of the University of Texas Medical School at San Antonio who received the cells from E. G. Westaway, Monash Medical School, Victoria, Australia. Other cell lines used in this study were MDCK (dog), HA (human), MA-104 (monkey), and L (mouse). Primary embryonic pig kidney cells and primary embryonic mouse cells were prepared in our laboratory.

Cell monolayers were grown in 32-oz (ca. 0.95 liters) glass bottles and Falcon disposable petri dishes (10 by 35 mm). Growth media consisted of an autoclavable modification of Eagle basal medium (BME; Auto-POW-BME, Flow Laboratories, Inc.) supplemented with virus-screened 10% fetal calf serum (FCS; Industrial Biological Laboratories, Rockville, Md.) and neomycin (20 μg/ml). Confluent monolayers were maintained in BME with 10% FCS.

Viral assay: immunofluorescence. The technique we used in the immunofluorescence focus assay has been described previously (20).

Antibody against SPV was prepared artificially by inoculating 0.2 ml of cesium chloride-purified virus and Freund's complete adjuvant (1:1) into young adult guinea pigs. The inoculation sites (0.05 ml/site) were the rear foot pads and the upper flanks. After 1 month, the guinea pigs received booster shots at the same sites; 10 days later a series of weekly bleedings was initiated. Anti-guinea pig globulin conjugated with fluorescein isothiocyanate was either purchased from a commercial source (Colorado Serum Co.) or prepared in our laboratories. Stained cells were examined in a dark-field fluorescence microscope (Bausch & Lomb, Inc.) with an Osram HBO200 lamp. A Bausch & Lomb no. 5-58 exciter filter was used.

Source of swine sera. Individual lots of swine sera were provided by E. H. Bohl of the Ohio Agricultural Research and Development Center, Wooster, Ohio. Swine serum samples were selected from healthy pigs of different ages and herds.

Source of SV40 and polyoma virus and antiserum. SV40 was a small-plaque strain, provided by P. Gerber, which was propagated in MA-104 cells. Rhesus monkey serum served as a source of naturally occurring antibodies to SV40. Anti-human globulin conjugated with fluorescein isothiocyanate was purchased from Progressive Laboratories, Inc.

Polyoma virus was grown in primary embryonic
mouse cells. Antiserum to polyoma virus was prepared in rabbits. Both antiserum and virus were provided by H. D. Mayor. Anti-rabbit globulin conjugated with fluorescein isothiocyanate was purchased from Progressive Laboratories, Inc.

**Virus purification, concentration, and buoyant density determinations.** Infected PK-15 cells were disrupted by a single freeze-thawing at −50°C, followed by sonic oscillation for 3 min at 38 ma with a Branson sonifier cell disruptor, model no. W140D. After removing large particulate matter by low-speed centrifugation, the virus suspension was placed on a preformed gradient and centrifuged for 3 hr at 100,000 × g in a Beckman model L-265 ultracentrifuge. A modified procedure for purification was also employed. Nonidet P-40, a nonionic detergent, was mixed with frozen-thawed crude virus to a final concentration of 0.1%. This mixture was treated by sonic oscillation for 6 min and was clarified by filtration through Celite no. 512 (Johns-Manville). Then the material was centrifuged on a preformed gradient at 100,000 × g for 14 to 17 hr. Fractions were collected through a puncture in the tube bottoms, and each fraction was examined for virus particles by electron microscopy. Buoyant density measurements were made on virus-containing fractions with a densitometer. The densitometer was calibrated by plotting refractive-index readings against densities of cesium chloride solutions determined by weight-volume measurements.

**Electron microscopy.** The technique for particle counting was a modification of the Sharp method (17) for negative staining as described by Smith and Melnick (19).

**Neutralization tests.** Neutralization tests were carried out by employing an immunofluorescence focus assay method described previously (18). A reduction of 80% or greater in infectious virus titer was considered the neutralization end point.

**Ether sensitivity determination.** We used a standard procedure described by Kapikian (8) for determining ether sensitivity of viruses. This consisted of treating virus suspensions with equal volumes of ether at 4°C for 18 to 24 hr. Herpes simplex virus was used in the test system as a positive control, and assay of this virus was performed as described by Roizman and Roane (16). Treated and untreated SPV was titrated in PS cells, and infectivity was measured by the immunofluorescence focus assay.

**Nucleic acid determination.** PS cells were adsorbed with 0.2 ml of undiluted virus suspension for 1 hr at room temperature and then maintained on BME maintenance media containing 0.1 mg of 5-iodo-2′-deoxyuridine (IUDR) per ml. After 4 days the cell monolayers were scraped and frozen at −50°C. Thawed samples of IUDR-treated and untreated control cells were then titrated in PS cells. Adenovirus type 2 propagated in HA cells was used as a known DNA virus control and reovirus type 3 propagated in PS cells served as a known ribonucleic acid (RNA) virus control. All virus titers were determined by measuring infectivity with the immunofluorescence focus assay.

**RESULTS**

**Source of virus.** SPV was initially isolated by us during a search for adventitious viruses in swine-derived pancreatic trypsin. In this study we exposed PK-15 cells to concentrated trypsin whose activity was inhibited by mixing with FCS. After removing the trypsin, the cells were maintained on BME with 1% FCS. Some treated PK-15 cell cultures slowly developed cytopathic effects (CPE), which we believed to be virus-induced because electron microscopic examination of frozen and thawed material from these cultures revealed numerous cubically symmetrical particles.

**Observation of virus-infected cell cultures.** Infected PK-15 cells maintained in BME medium with 10% FCS (normal cell growth medium) could be propagated and held for prolonged periods of time without obvious CPE. If infected cell monolayers were held in BME maintenance media (1% FCS) with weekly medium changes, cell monolayers slowly degenerated within a period of 3 weeks. Virus titers of supernatant fluids from these cultures averaged about 10⁴ infectious doses per ml when titrated in PS cells. Particle counting of the same material resulted in counts of 10⁵ virus particles/ml. Therefore, the calculated particle-infectivity ratio approached 10⁴ particles per infectious dose.

There was no observable CPE in primary pig embryonic kidney cells. Immunofluorescence staining of pig embryonic kidney cells 4 days post-infection showed scattered foci of fluorescing cells. Fluorescent foci were confined almost entirely to patches of flat epithelial-like cells; spindle-shaped, fibroblast-like cells were rarely involved.

The SPV did not pass successfully in other cell lines, i.e., MDCK, HA, MA-104, and L cells. Growth of virus during passage was determined by immunofluorescence staining of SPV-infected cells of different cell lines. Satisfactory passage was obtained in PK-15 cells, which served as a positive control.

**Immunofluorescence.** The main detection system for SPV was the indirect Coons' method of staining virus-infected cells. Antiserum against SPV, which was produced in guinea pigs, could usually be used successfully for fluorescence work at a dilution of 1:3. Stained, infected cell nuclei displayed maximum fluorescence 96 to 120 hr after virus inoculation.

Antisera against SV40 and polyoma virus did not stain SPV-infected PK-15 cells. Neither embryonic mouse cells infected with polyoma virus nor MA-104 cells infected with SV40 were stained with antiserum against SPV. In all instances infected cells stained positively with homologous antiserum.

**Neutralization tests.** Anti-SPV made in guinea pigs neutralized SPV at a maximum dilution of 1:6. All 10 lots of different swine sera from ani-
mals 6 months to 4 years of age neutralized SPV at a dilution of $\geq 1:2$. Seven of these swine serum samples neutralized SPV at a dilution of $\geq 1:10$ and one serum lot neutralized SPV at a $\geq 1:30$ dilution. We conclude, therefore, that SPV commonly infects swine.

Electron microscopy. The SPV particle, when negatively stained, exhibited cubic symmetry and did not appear to possess an envelope (Fig. 1B). In many instances, however, membrane-like material was found associated with single particles, as well as with clusters of particles (Fig. 1C). The capsomere number and arrangement closely resembled that observed with other

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**Fig. 1.** Morphology of swine papovavirus. (A) a filamentous form showing capsomeres; (B) cubic symmetry particles showing capsomeres; (C) virus particles associated with membranous material. Bars equal 100 nm.
papovaviruses. The average virus size, based on the measurement of 103 negatively stained particles, was 39.6 nm. Approximately 80% of the particles examined had diameters between 36 and 44 nm. (Fig. 2). Filamentous forms were observed which measured 35.8 nm in diameter (Fig. 1A).

Buoyant density. Sonically treated crude virus, clarified by low-speed centrifugation, banded with a peak infectivity at a density of 1.22 g/ml, as can be seen in Fig. 3. Electron microscopic examination of these fractions showed membrane-like material associated with virus particles similar to that seen in Fig. 1C. Ether treatment of banded virus, when rebanded, did not show a significant change in density. When the crude virus was prepared for purification and concentration by a modified procedure employing Celite filtration and addition of Nonidet P-40, the infectivity peak was at a density of 1.35 g/ml. Electron-microscopic examination of these fractions showed virus particles free of membrane-like material.

Ether sensitivity. Treatment of SPV with ether resulted in no significant decrease in infectivity, which suggests that the virus does not contain essential lipids.

Optimum growth temperature. PS cells infected with SPV and held at 33, 36, and 39 C for different time intervals yielded similar titers of infectious virus (ca. 5 × 10⁴/ml) when harvested at the same time intervals.

Nucleic acid type. IUDR at a concentration of 0.1 mg/ml was found to be inhibitory for SPV and adenovirus replication, whereas reovirus type 3 replicated freely under the same conditions (Table 1). RNA viruses which depend on DNA synthesis for the production of new virus progeny might also be inhibited by IUDR. However, no nonenveloped, cubically symmetrical RNA virus is known to be inhibited by IUDR. Therefore, the results of the IUDR experiment would imply that the SPV genome is composed of DNA.

**Fig. 2.** Distribution of particle sizes of swine papovavirus based on the measurement of 103 virus particles.
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FIG. 3. Distribution of swine papova virions after centrifugation over a preformed cesium chloride density gradient. (○) Crude virus treated by sonic oscillation and clarified by low-speed centrifugation; (●) crude virus mixed with Nonidet P-40, treated by sonic oscillation, and clarified by Celite filtration.

Table 1. Effects of 5-iodo-2'-deoxyuridine on the replication of reovirus type 3, adenovirus type 2, and swine papovavirus in tissue culture

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell substrate</th>
<th>IUDR treated</th>
<th>Control</th>
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<tr>
<td>Reovirus 3</td>
<td>PS cells</td>
<td>1.4 × 10⁶</td>
<td>1.0 × 10⁸</td>
</tr>
<tr>
<td>Ad 2</td>
<td>HA cells</td>
<td>&lt;1.8 × 10⁶</td>
<td>2.6 × 10⁷</td>
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<tr>
<td>SPV</td>
<td>PS cells</td>
<td>&lt;6.3 × 10⁶</td>
<td>7.4 × 10⁶</td>
</tr>
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</table>

a Abbreviations: IUDR, 5-iodo-2'-deoxyuridine; Ad 2, adenovirus 2; SPV, swine papovavirus. b Fluorescing foci per ml.

DISCUSSION

A new member of the papovavirus group has been isolated and tentatively named swine papovavirus. The virus has been propagated in two cell lines, PS and PK-15, which were both derived as separate clones from a parent cell line PK-2a. Virus replication has also been demonstrated in primary embryonic pig kidney cell cultures, although virus multiplication seems to be largely limited to epithelial-like cells in these cultures. SPV did not replicate to detectable levels in cell lines from animal species other than swine. In addition to a narrow host range, SPV appears to be a very defective virus because only one particle in approximately 10⁶ is infectious in the assay system we use. The reason for its apparent defectiveness is presently unknown. Two possibilities are apparent: (i) the PK-15 cells are naturally inefficient producers of infectious particles, or (ii) optimal multiplicity of infection has not yet been achieved for maximum infectious virus yields.

The reasons for placing the new isolate in the papovavirus group were based on the characteristics summarized in Table 2. Morphology of the cubic symmetry particle, size, and the presence of filamentous forms are distinctive characteristics of the papovavirus group. The buoyant density of the swine papova virion appears to be within the range of values recorded for other members of the papovavirus group. However, density determinations for others have been done under a variety of conditions that makes strict comparisons difficult. For instance, density measurements have been correlated with physical particles in some cases.
and infectivity measurements in others. Ether sensitivity tests and growth inhibitory studies employing a DNA analogue suggest that SPV is nonenveloped and contains a DNA core; these are properties of the papovavirus group.

Sерological studies suggest that the present isolate is not a previously recognized papovavirus because SPV-infected PK-15 cells are not stained in the indirect Coons' test by antisera to SV40 and polyoma virus. Evidence has been obtained that neutralizing antibodies to SPV are widely distributed in apparently healthy swine. It is likely, therefore, that swine are naturally infected with this virus, possibly without obvious disease (latency is characteristic of papovaviruses in vivo). In most instances the neutralization titers were greater than we were able to obtain artificially in guinea pigs.

We conclude that this agent is SPV on the basis of (i) its physical characteristics, (ii) its nucleic acid type, (iii) the absence of essential lipids, (iv) its replication being restricted to swine cells, and (v) the presence of naturally occurring antibody in swine.

At this time, studies are being conducted to determine the original source of the SPV we isolated. We are considering the possibilities (i) that the agent is indigenous in the PK-15 cell line we obtained for these studies and (ii) that the agent is present in some lots of pancreas-derived trypsin to which our PK-15 cells were exposed. If the latter is correct, high-passage levels of stable swine cells (such as the PK-15 line) would be exposed repeatedly to swine agents during routine trypsinization for passage. The presence of virus in swine pancreas-derived trypsin would not be surprising because swine mycoplasma are common contaminants of cell cultures which are exposed to swine-derived trypsin (3). Now that we have developed a method for detecting SPV, we are examining trypsin and swine cells from many sources for the presence of this agent.

ACKNOWLEDGMENTS

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LITERATURE CITED


<table>
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<tr>
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<th>Size (nm)</th>
<th>Cubic symmetry</th>
<th>Filamentous forms</th>
<th>Nucleic acid type</th>
<th>Lipid (envelope)</th>
<th>Bouyant density</th>
<th>Assembly site</th>
<th>Growth in vitro</th>
<th>Natural host</th>
<th>Latent and chronic infections</th>
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<td>40</td>
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<td>N</td>
<td>+</td>
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<td>+</td>
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<td></td>
<td></td>
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<td>N</td>
<td>-</td>
<td>Man</td>
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<td>+</td>
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<td>1.33</td>
<td>N</td>
<td>-</td>
<td>Mouse</td>
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* Modified from Melnick (reference 11). Abbreviations: SPV = swine papovavirus; N = nucleus.

* Probable.


15. Registry of animal cell lines certified by the Cell Culture Collection Committee, American Type Culture Collection Cell Repository. 1964. Rockville, Md.


