1969

Persistent infections with hog cholera virus

William Lloyd Mengeling

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Microbiology Commons

Recommended Citation


https://lib.dr.iastate.edu/rtd/4131
PERSISTENT INFECTIONS WITH
HOG CHOLERA VIRUS

by

William Lloyd Mengeling, D. V. M.

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

Major Subject: Veterinary Microbiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
of Science and Technology
Ames, Iowa
1969
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td>Herpes Simplex Virus</td>
<td>2</td>
</tr>
<tr>
<td>Parainfluenza Virus Type 1</td>
<td>6</td>
</tr>
<tr>
<td>Parainfluenza Virus Type 2</td>
<td>7</td>
</tr>
<tr>
<td>Newcastle Disease Virus</td>
<td>7</td>
</tr>
<tr>
<td>Mumps Virus</td>
<td>8</td>
</tr>
<tr>
<td>Measles Virus</td>
<td>9</td>
</tr>
<tr>
<td>Eastern and Western Equine Encephalomyelitis Viruses</td>
<td>10</td>
</tr>
<tr>
<td>West Nile Virus</td>
<td>10</td>
</tr>
<tr>
<td>Lymphocytic Choriomeningitis Virus</td>
<td>10</td>
</tr>
<tr>
<td>Rubella Virus</td>
<td>11</td>
</tr>
<tr>
<td>Rauscher Murine Leukemia Virus</td>
<td>11</td>
</tr>
<tr>
<td>Polyoma Virus</td>
<td>12</td>
</tr>
<tr>
<td>Hog Cholera Virus</td>
<td>13</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>15</td>
</tr>
<tr>
<td>Viruses</td>
<td>15</td>
</tr>
<tr>
<td>Stock Cell Cultures</td>
<td>16</td>
</tr>
<tr>
<td>Persistently Infected Cell Cultures</td>
<td>17</td>
</tr>
<tr>
<td>Pigs</td>
<td>19</td>
</tr>
<tr>
<td>Antiserums</td>
<td>19</td>
</tr>
<tr>
<td>Fluorescent Antibody Technique</td>
<td>21</td>
</tr>
<tr>
<td>Virus Titrations</td>
<td>22</td>
</tr>
<tr>
<td>TABLE OF CONTENTS (Cont.)</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Test for Virus-Neutralizing Capacity of Anti-Hog Cholera Serum</td>
<td>27</td>
</tr>
<tr>
<td>Enumeration of Cells</td>
<td>28</td>
</tr>
<tr>
<td>Photomicrographs</td>
<td>28</td>
</tr>
<tr>
<td>Mechanisms for the Spread and Persistence of Hog Cholera Viral Infection in Cell Culture</td>
<td>28</td>
</tr>
<tr>
<td>Relative Yields of Hog Cholera Virus from Arbitrarily Selected Subcultures of Persistently Infected Cultures</td>
<td>29</td>
</tr>
<tr>
<td>Test for Attenuation of Virulence of Virus During Subculture of Persistently Infected Cultures</td>
<td>29</td>
</tr>
<tr>
<td>Susceptibility of Cells Persistently Infected with Hog Cholera Virus to Infection with Other Viruses</td>
<td>30</td>
</tr>
<tr>
<td>Enumeration of Chromosomes</td>
<td>30</td>
</tr>
<tr>
<td>Replication of Hog Cholera Virus</td>
<td>31</td>
</tr>
<tr>
<td>Effect of Persistent Infection on Cell Replication</td>
<td>34</td>
</tr>
<tr>
<td>Effect of Various Concentrations of Actinomycin D on Cell and Viral Replication</td>
<td>35</td>
</tr>
<tr>
<td>Percentage of Cells Infected in Persistently Infected Cultures</td>
<td>35</td>
</tr>
<tr>
<td>Antigenic Relationship between Strains Ames and 331 of Hog Cholera Virus</td>
<td>36</td>
</tr>
<tr>
<td>Resistance of PK-15 (Ames) and PK-15 (331) to Superinfection with Virulent Strains Ames and 331 of Hog Cholera Virus</td>
<td>37</td>
</tr>
<tr>
<td>Resistance of Pigs Infected with Strain 331 of Hog Cholera Virus to Superinfection with Strain Ames of Hog Cholera Virus</td>
<td>39</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Cont.)

RESULTS

General Observations

Mechanisms for the Spread and Persistence of Hog Cholera Infection in Cell Culture

Relative Yields of Hog Cholera Virus from Arbitrarily Selected Subcultures of Persistently Infected Cultures

Attenuation of Virulence of Virus During Subculture of Persistently Infected Cultures

Susceptibility of Cells Persistently Infected with Hog Cholera Virus to Infection with Other Viruses

Enumeration of Chromosomes

Replication of Hog Cholera Virus

Effect of Persistent Infection on Cell Replication

Effects of Various Concentrations of Actinomycin D on Cell and Viral Replication

Percentage of Cells Infected in Persistently Infected Cultures

Antigenic Relationship between Strains Ames and 331 of Hog Cholera Virus

Resistance of PK-15 (Ames) and PK-15 (331) to Superinfection with Virulent Strains Ames and 331 of Hog Cholera Virus

Resistance of Pigs Infected with Strain 331 of Hog Cholera Virus to Superinfection with Strain Ames of Hog Cholera Virus

DISCUSSION

SUMMARY

LITERATURE CITED

ACKNOWLEDGEMENTS
INTRODUCTION

The phenomenon of persistence or latency of animal viruses within their host cell is of wide biological and medical interest. Foremost is the fact that because viruses are not free-living, the carrier animal usually provides the means by which these agents are perpetuated and disseminated in nature (18, 35, 36).

With the development of techniques for routine propagation of animal cells in the laboratory it was possible to study persistent virus-cell relationships apart from the animal. This not only provided a readily available and economical source of host cells but also reduced the number of variables. As a result persistent infections of cell cultures have been established experimentally with a number of animal viruses and many of these studies are reviewed in the following section.

The primary objective of the study reported here was to characterize persistent infection of an established porcine kidney cell line with selected strains of hog cholera virus. Various properties of both virus and host cells were examined at intervals after the carrier state was induced.
REVIEW OF LITERATURE

Herpes Simplex Virus

In 1959 Wheeler and Canby (50) described the effect of antibody on the establishment of a carrier state in HeLa cell cultures infected with herpes simplex virus. They found that cultures nourished with medium that contained homologous neutralizing antibody survived, multiplied, and could be subcultured, apparently indefinitely, even though viral infection persisted. Removal of antibody resulted in rapid destruction of the culture.

Coleman and Jawetz (5), on the other hand, were able to establish a persistent infection of cell cultures with herpes simplex virus in the absence of homologous viral antibody in the medium. However, several conditions were necessary for initiation and maintenance of the carrier state. First, the type of cell and strain of virus were important. The carrier state occurred in Maben cells (derived from an adenocarcinoma of the lung) infected with the Z strain but not in L or HeLa cells infected with the Z strain or in Maben or L cells infected with the HF strain of virus. Second, persistent infection occurred only when the input multiplicity of infection was approximately $10^{-4}$. When the inoculum contained much more virus the cells were rapidly destroyed and with less virus a persistent infection failed to develop. Third, it was necessary to maintain infected cultures at $31\, ^\circ \text{C}$. Incubation at
34°C resulted in degeneration of the culture. Even at 31°C infected cultures did not appear healthy and could not be subcultured at regular intervals.

Clones of cells did not develop from single cells isolated from infected cultures. Continuous exposure of the infected culture to homologous viral antibody did not free the cells of infection.

Szántó (44) produced a carrier state for herpes simplex virus in both HeLa and Detroit 6 cell cultures by inclusion of human antiviral serum in the culture medium. Without antibody or with rabbit antiviral serum in the medium the cells were destroyed by the virus.

Glasgow and Habel (8) found that a persistent infection with herpes simplex virus could be established in the absence of viral antibody in a mouse embryo cell culture that was already persistently infected with polyoma virus. Cultures from which polyoma virus infection was eliminated were rapidly destroyed by herpes simplex virus except when endogenous interferon was added. Thus it appeared that the carrier state for herpes simplex virus was mediated by interferon. A genetic factor was also indicated since two additional polyoma-infected, mouse embryo cell cultures (derived from other mice) were readily destroyed by herpes simplex virus.

Hampar (9, 10, 11), Hampar and Copeland (12), and Hampar and Keehn (13) conducted a series of investigations of the persistence of herpes simplex virus in cell cultures. They
reported persistence of the virus in the absence of supportive measures such as maintenance of a low temperature of incubation (31°C) or addition of viral antibody or interferon to the culture medium. Infections were established in both the MAL and PEL lines of Chinese hamster cells. Cycles of cell destruction and regrowth were observed. At the end of a cycle of cell destruction less than 0.5% of the cells remained attached to the culture vessel. Fluorescent antibody staining indicated that only about 5% of the intact but detached cells contained viral antigen whereas almost all of the cell fragments contained antigen. The cytopathic effect was evidenced by the appearance of small syncytia followed by expanding necrotic lesions. Cultures were subsequently repopulated from the relatively few cells that remained attached to the culture vessel and from intact cells that had released from the vessel but later re-attached. It was suggested that proliferating cells were temporarily resistant to infection with virus.

It was found that a variant strain of herpes simplex virus was selected when homologous viral antibody was included in the medium of a carrier cell culture. The variant was evidenced by the nature of its cytopathic effect which, unlike the non-proliferative type of the original viral population, was associated with the formation of giant cells. The presence of the original, variant, or both populations of virus in a single culture was also indicated by the extent of cytopathology and the titer of virus detected in the antibody-containing nutrient
medium. The most severe cytopathology and highest titer of virus occurred when both populations were present. The least cytopathology and lowest titers of virus were associated with the presence of only the original population of virus and moderate cytopathology and titers were associated with the variant. The reason that more virus was detected in the medium from cultures infected with the variant was that the variant was more closely associated with cell debris and thus protected from antibody.

It was also found by neutralization kinetics that herpes simplex virus changed antigenically during persistent infection of cell cultures. The virus acquired new antigenic determinants with time while still retaining the determinants of its ancestry.

Blackmore and Morgan (3) were able to establish a temporary carrier state (approximately 50 days) for herpes simplex virus in L cells. During this interval cultures were resistant to superinfection by homologous virus but once persistent infection was no longer evidenced by isolation of virus, cultures were again susceptible. This indicates that elimination of virus from the carrier culture was not due to selection of a resistant population of cells. Because virus collected from carrier cultures had lost none of its virulence for L cells it was also apparent that persistent infection was not due to selection of an avirulent population of virus. Based on the identification of a viral interference factor, with several
of the properties of interferon, from carrier cultures and from cultures exposed to heat inactivated virus, it was postulated that autointerference was responsible for resistance and for the elimination of persistent infection.

Parainfluenza Virus Type 1

Ishida et al. (19) reported that HeLa cells infected with parainfluenza virus type 1 (hemadsorption virus type 2) produced viral antigens without any cytopathic effect and were serially subcultured like normal HeLa cells. By fluorescent antibody staining it was demonstrated that all cells contained viral antigen. Moreover, each cell contained inclusions and also acted as an infectious center. Carrier cultures were completely resistant to the cytopathic effect of Sendai and mumps viruses, but not at all to that of polio, adeno, and vaccinia viruses. Mitosis occurred normally and fluorescent cells containing viral antigen were seen in all stages of mitosis.

Miyamoto et al. (31, 32) demonstrated that the cloning efficiency of HeLa cells persistently infected with hemadsorption virus type 2 was the same as that of noninfected HeLa cells. For both types of cells the cloning efficiency was essentially 100%. They emphasized the perfect relationship between parainfluenza virus type 1 and HeLa cell genomes in the carrier culture. While each cell was infected and capable of transmitting the infection it was still able to divide and
form new clones of cells.

Homma et al. (17) were able to demonstrate a change in karyotype in a clonal strain of HeLa cells when the cells were infected with parainfluenza virus type 1.

Parainfluenza Virus Type 2

Fraser and Anderson (7) found that BHK 21 cells infected with parainfluenza virus type 2 became carriers of the virus. Infected cultures could be propagated without distinguishable alterations of their appearance when examined without fixation. Although all cells contained fluorescent antigen, less than 1% served as infectious centers. Inclusion of antiserum in the medium did not "cure" the infection. Cloning efficiency of infected cells was equal to that of noninfected cells and growth rates were similar. The number of apparent plaque-forming units of encephalomyocarditis, influenza A and polyoma viruses were slightly less on cultures persistently infected with parainfluenza virus but no significant difference in titer of vaccinia was observed between infected and noninfected cultures. Carrier cultures were not resistant to the homologous strain of virus. Addition of a high titer of parainfluenza virus type 2 to the culture resulted in destruction of the monolayer 4 days later.

Newcastle Disease Virus

Szántó et al. (45) developed sublines of HeLa cells persistently infected with both small and large plaque variants
of Newcastle disease virus. With either variant, sublines arose from the few cells that survived cytonecrosis following initial infection. Although both lines could be subcultured, the interval was longer for the culture infected with the large plaque variant. Morphologically, infected cells were similar to noninfected cells except that syncytia and inclusion bodies were observed in infected cultures. Persistent infection with Newcastle disease virus was also established in human diploid cells.

**Mumps Virus**

Walker and Hinze (48, 49) and Walker et al. (47) reported on carrier cultures of mumps virus in human conjunctiva cells. During persistent infection a variant strain of virus of reduced cytopathogenicity was selected and it was suggested that this was a major factor in the stability of the carrier state. Infectious virus was produced and most was extracellular. Approximately 50% of the cells contained inclusions and about 90% contained viral antigen as evidenced by immunofluorescence. In carrier cultures the antigen was observed as sharply circumscribed, discrete masses and thus differed from acute cytocidal infections in which antigen is distributed throughout the cytoplasm. Infected and noninfected cells multiplied at a similar rate. Transfer of virus through the medium was not necessary for maintenance of the carrier infection since infection.
persisted for 5 1/2 months during weekly subcultures in the presence of antibody. The results indicated that infection passes from mother to daughter cells during mitosis. Cloning efficiency of carrier cultures was similar to that of non-infected cultures. Of 269 clones from the carrier culture 262 contained viral antigen. Eight antigen-containing clones were grown into cultures, all of which produced infectious virus. Cultures from 2 clones that lacked viral antigen were susceptible to the cytopathic effects of mumps virus. In healthy, growing carrier cultures 0.1 to 1.0% of the cells adsorbed erythrocytes to their surfaces, suggesting that these cells were releasing virus. It was demonstrated that viral and cell multiplication were not closely linked. Each could proceed independently.

Measles Virus

Rustigian (39, 40, 41) observed that 1 to 3% of HeLa cells survived the cytopathic effect of infection with the Edmonston strain of measles virus. From these cells were established cultures that were carriers of measles virus and most of the cells contained viral antigen. By immunofluorescence it was observed that virus or viral subunits were transferred from mother to daughter cells during mitosis. Carrier cultures were resistant to superinfection by the same strain of measles virus but were susceptible to vaccinia, herpes simplex, and polio type 2 viruses. There was possibly a slight resistance
to a simian viral agent. Measles virus produced by the carrier culture was less cytopathic than the parent strain. Clones were obtained from infected cultures propagated in the presence of antibody in which only incomplete virus was synthesized.

Eastern and Western Equine Encephalomyelitis Viruses

Carrier cultures have been established with both Eastern, Bang et al. (1), and Western, Chambers (4), equine encephalomyelitis viruses. Cytopathology was observed and cultures spontaneously recovered from infection.

West Nile Virus

Jarman et al. (21) reported persistent infection of cultures of mouse fibroblasts with West Nile virus. Infected cells were subcultured routinely. Infection was evidenced by morphologic alterations which included increased cellular granularity, less distinct fibroblastic processes, and clumps of cells.

Lymphocytic Choriomeningitis Virus

The fact that lymphocytic choriomeningitis virus persists in the intact host makes it a particularly interesting virus to study in cell culture. Lehmann-Grube (23) described persistent infection of mouse L cells with lymphocytic choriomeningitis virus. The carrier state was readily established and virus was repeatedly demonstrated in the culture medium. The titer of extracellular virus ranged from $<10^2$ to $10^{8.55}$
In samples collected at different times after infection.
Interferon was not demonstrated and no resistance of carrier
cultures to vesicular stomatitis was found. However, carrier
cultures were resistant to infection with the same and a
closely related strain of lymphocytic choriomeningitis virus.

Rubella Virus

Rawls and Melnick (37) prepared persistently infected
cell cultures from tissues of infants with congenital rubella.
The growth rate of such cells was less than that of cells de­
rived from noninfected tissues. Infected cultures could not
be cured with rubella antibodies. They were resistant to
superinfection with vesicular stomatitis and herpes simplex
viruses but were susceptible to echovirus 11.

Rauscher Murine Leukemia Virus

Persistent infection with Rauscher murine leukemia virus
in cell cultures derived from tissues of mice has been observed
by Sinkovics and Howe (43), Hartley et al. (15), and Wright
and Lasfargues (51). More recently Duc-Nguyen et al. (6) re­
ported on persistent infection of cells derived from the kidney
of an adult rat that had been infected at birth with Rauscher
virus. In the latter study cells of the infected culture were
indistinguishable from those of noninfected cultures of rat
kidney. The presence of virus was confirmed by (i) demonstra­
tion of homologous complement-fixing antigen, (ii) electron
microscopy, (iii) recovery of virus from 5-month-old cultures, and (iv) production of the leukemogenic effects in rats and mice inoculated with infected cells. Interferon was not detected from the infected cultures.

Polyoma Virus

Most of the persistent viral infections reviewed thus far were characterized by replication of both virus and host cell. The same relationship was observed by Barski and Cornefert (2), Hare et al. (14), and Henle et al. (16) for polyoma virus and L cells. Recently, however, Kodama et al. (22) described a polyoma virus-L cell infection wherein certain nutritional factors were required for expression of the viral genome as infectious progeny virus. The authors stated that they were astonished to find that L cell cultures, which they believed were noninfected, released polyoma virus when calf serum in the nutrient medium was replaced by horse serum. A return to calf serum was followed by reversion to latency. A change from calf serum to either sheep, pig, or dog serum did not result in production of virus. Another interesting finding was that most of the cultures that were induced to produce polyoma virus by the addition of horse serum eventually lost their ability to produce virus without any further change in culture procedure.
Hog Cholera Virus

Because most strains of hog cholera virus are not cytopathogenic, persistent infections are readily established in cell lines that are susceptible to the virus. The number of susceptible lines, however, is limited. Loan and Gustafson (24, 25) demonstrated the persistence of hog cholera virus in a subculturable line of swine leukocytes they had developed. By inoculation of pigs, virulent virus was identified in culture medium collected on the 4th and 204th day after initial infection of the cells. During this interval cells were not subcultured but were maintained by changing the culture medium once or twice weekly. After 702 to 737 days of persistent infection, hog cholera virus was attenuated in virulence. They also found that virulent virus was able to replicate in leukocyte cultures that had been infected with attenuated virus 10 days before. In one experiment concomitant replication of virulent and attenuated virus was demonstrated. Carrier cultures were not freed of virus by inclusion of anti-hog cholera serum in the nutrient medium for 29 days. It is emphasized that the above experiments were conducted without subculturing the infected cells.

Sato et al. (42) reported that establishment of persistent infection of swine kidney cells was an effective way to attenuate virulence of the virus. The degree of attenuation was directly related to the duration of infection.
Mengeling et al. (28) reported that hog cholera viral antigens were observed only in the cytoplasm of persistently infected, porcine kidney cells of the PK-2a established cell line.

Izawa and Soekawa (20) established carrier cultures from kidney cells of pigs infected with the ALD strain of hog cholera virus. Infection persisted whether cells were subcultured or were maintained in the same vessel with periodic changes of medium. Infection also persisted in the presence of anti-hog cholera serum. By fluorescent antibody staining it was found that nearly the entire population of cells contained viral antigen in their cytoplasm. Virus was attenuated when infected cells were subcultured 80 times during an interval of 600 days but not when cells were maintained without subculture for the same interval. Quantitation of extracellular and cell-associated virus at several intervals during growth of infected cells indicated that most of the virus was extracellular.

Using 2 established porcine kidney cell cultures, Pirtle found that the cultures reacted differently to persistent infection with hog cholera virus. One culture underwent a change in its modal chromosome number and subtle changes in karyotype (33); whereas the second culture showed endoreduplication, a slow transition to a near-tetraploid population, anomalous mitosis, and chromosomal pulverization (34).
MATERIALS AND METHODS

Viruses

Hog cholera virus

Five strains of hog cholera virus, identified as 208, 289, 300, 331, and Ames, were used. Stock preparations of strains 208, 300, and 331 were serums from pigs given subcutaneous injections of tissues collected from pigs involved in herd epizootics of hog cholera in Tennessee, Indiana and Iowa respectively. The stock of strain Ames was also serum from an experimentally infected pig. This strain had been maintained in federal hog cholera research laboratories for more than 25 years by occasional passage in pigs. The stock preparation of strain 289 was a suspension of splenic tissue collected from a pig involved in a herd epizootic of hog cholera in Iowa.

The titers of virus stocks, expressed as plaque forming units/ml, were: 208 \( (1.7 \times 10^6) \), 289 \( (3.6 \times 10^3) \), 300 \( (1.5 \times 10^6) \), 331 \( (2.5 \times 10^5) \), and Ames \( (2.4 \times 10^6) \). Stock viruses were maintained at \(-70\) C in a mechanical freezer until needed.

Newcastle disease, parainfluenza 3, pseudorabies and vesicular stomatitis viruses

The GB strain of Newcastle disease virus and the SF4 strain of parainfluenza 3 virus were provided by Dr. G. H. Frank. The

\[1\text{Determined by fluorescence microplaque assay.}\]
Shope strain of pseudorabies virus was supplied by Dr. E. A. Carbrey and the Indiana strain of vesicular stomatitis virus was supplied by Dr. W. A. Malmquist. The viruses were from stocks maintained at the National Animal Disease Laboratory.

Each of the viruses was serially passed 10 times at 48 hour intervals in PK-15 cell cultures after it was received. Culture medium from the 10th passage was stored at -70 C in a mechanical freezer as stock virus. The titers of the stock viruses, expressed as median cell culture infective doses/ml, were: Newcastle disease virus ($10^{6.4}$), parainfluenza 3 virus ($10^{6.7}$), pseudorabies virus ($10^{8.4}$), and vesicular stomatitis virus ($10^{4.2}$).

Stock Cell Cultures

**Cells**

An established porcine kidney (PK-15) cell line (33) was continuously maintained in culture during the study to provide cells when needed for various experiments and for titrations of viruses.

**Nutrient medium**

Cells were nourished with Eagle's minimal essential medium containing nonessential amino acids, L-glutamine,¹ lactalbumin hydrolysate (0.5%), porcine serum (2%), sodium bicarbonate

¹Medium F-15, Grand Island Biological Co., Grand Island, New York.
(26.1 mM), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml).

Culture vessels

Stock cultures of PK-15 cells were routinely grown in 250 ml bottles; however, for certain experiments and for titrations of viruses, cells were seeded into other types of containers. These were 30 ml bottles, 50 mm Petri dishes, 15 X 125 mm tubes, and Leighton tubes with or without coverslips.

Subculture

Subcultures were made twice weekly at alternate 3- and 4-day intervals. Cells were dispersed with a solution of 0.05% trypsin (1:250) and 0.02% versene and divided at a ratio of 1:5, i.e., the number of cells from 1 culture vessel was sufficient to seed 5 additional vessels of the same size. Appropriate adjustments in cell number were made when cells were seeded into larger or smaller containers.

Persistently Infected Cell Cultures

Infection

Six 30 ml bottles were seeded with PK-15 cells. Forty-eight hours later 5 of the 6 cultures were each infected with a different strain of hog cholera virus (viz., 208, 289, 300,

\(^1\)Falcon Plastics, Los Angeles, California.
331, and Ames strains) by adding 2 ml of the appropriate virus stock to the nutrient medium. Unadsorbed virus was decanted 2 hours later and fresh medium was added.

**Propagation**

All 6 cultures (5 infected and 1 noninfected control) were subcultured 48 hours after infection. Thereafter, they were treated in parallel, with care to avoid either inadvertent infection of the control culture or cross-transfer of strains, and were nourished and subcultured as previously described for stock cultures of PK-15 cells. They were routinely grown in 30 ml bottles but were seeded into other types of culture vessels for certain experiments.

**Identification**

Persistently infected cultures of PK-15 cells were identified by the strain of hog cholera virus and by the number of subcultures following infection. For example, PK-15 (Ames) 5 designates the 5th subculture of PK-15 cells after infection with the Ames strain. Likewise, PK-15 (300) 65 designates the 65th subculture of PK-15 cells after infection with the 300 strain.

The noninfected control culture of PK-15 cells was identified in a similar manner to distinguish it from stock PK-15 cells and to emphasize the fact that it was subcultured and treated in parallel with cultures infected with the 5 strains of hog cholera virus. For example, PK-15 (Cont.) 12 designates
the 12th parallel subculture of noninfected PK-15 cells.

Pigs

Pigs were specific-pathogen-free and were either obtained by hysterectomy and deprived of colostrum or were first generation progeny from such pigs. They were of mixed breeding and most weighed between 30 and 60 lbs when first treated.

Antiserums

Each of the antiserums identified below was prepared in a specific-pathogen-free pig by subcutaneous injection(s) of virus(es). The total amount of virus contained in inoculums is presented when known. Serums were passed through bacteria-retaining filters and then refrigerated until needed.

Anti-hog cholera serums

Antiserum 8194 Pig 8194 was immunized with strain Ames that had been attenuated in virulence by 50 serial passages in PK-15 cell cultures. During the following 4 months, 32 additional injections of between $10^4$ and $10^6$ plaque-forming units of virulent strain Ames were administered. The pig was exsanguinated 17 days after the final injection.

Antiserum 11522 Pig 11522 was given $1.49 \times 10^4$ plaque-forming units of virus contained in 1 ml of nutrient medium from PK-15 (Ames) 65 and was exsanguinated 15 days later.

Antiserum 11845 Pig 11845 was given $3.6 \times 10^3$ plaque-
forming units of virus contained in 1 ml of nutrient medium from PK-15 (Ames) 80 and was exsanguinated 14 days later.

**Antiserum 10958** Pig 10958 was immunized with $5 \times 10^3$ plaque-forming units of hog cholera vaccine virus. Immunity was confirmed by challenge with $2.4 \times 10^6$ plaque-forming units of virulent strain Ames 3 weeks postvaccination. A mixture of nutrient mediums from the 14th subculture of all 5 persistently infected cultures (viz., 208, 289, 300, 331 and Ames) was administered 2 weeks postchallenge. Three and again 5 weeks postchallenge the pig was given a mixture of nutrient mediums from the 16th and 20th subcultures respectively of cultures persistently infected with strains 208, 289, 300, and 331. Between the 7th and 12th weeks postchallenge the pig was given 15 injections of between $10^3$ and $10^5$ plaque-forming units of virulent, stock strain 331. The pig was exsanguinated 6 days after the final injection.

**Antiserum 11524** Pig 11524 was given $9.5 \times 10^1$ plaque-forming units of virus contained in 1 ml of nutrient medium from PK-15 (331) 65 and was exsanguinated 15 days later.

**Antiserum 11844** Pig 11844 was given $8 \times 10^1$ plaque-forming units of virus contained in 1 ml of nutrient medium from PK-15 (331) 80 and was exsanguinated 14 days later.

---

1 Monovet, Affiliated Laboratories, White Hall, Illinois.
Polyvalent anti-Newcastle disease, parainfluenza 3, pseudorabies, vesicular stomatitis, and hog cholera serums

Antiserum 12389 Pig 12389 was given 0.5 ml of nutrient medium from PK-15 (Ames) 94 and 6 days later was given 0.5 ml of the stock of each of the following viruses: Newcastle disease, parainfluenza 3, pseudorabies, and vesicular stomatitis. During the following 2 months the pig was given 7 additional injections of pseudorabies virus, 8 injections of virulent stock strain Ames hog cholera virus, 8 injections of Newcastle disease and vesicular stomatitis viruses and 9 injections of parainfluenza 3 virus. Except for hog cholera virus, the injections consisted of nutrient medium from passages of the viruses in PK-15 cells.

Antiserum 12390 The pig was treated in the same manner as for production of antiserum 12389.

Fluorescent Antibody Technique

The methods used for preparation of fluorescent antibodies and staining cell cultures have been described (28, 30).

Identification of fluorescent antibody preparation

Preparations of fluorescent antibodies are hereafter referred to as conjugates and such conjugates are identified by the number of the antiserum used in their preparation.

One polyvalent conjugate was prepared from equal volumes of 2 polyvalent antiserums (12389 and 12390) and is identified as conjugate 12389-90. The presence of fluorescent antibodies
to each of the viruses used in the preparation of these antiseraums was confirmed by preliminary testing (Figures 1 and 2).

Virus Titrations

For both methods of titration presented below, dilutions of virus were made in Earle's balanced salt solution containing 2% porcine serum that had been examined and found free of neutralizing antibodies for hog cholera virus. Usually 10-fold dilutions were tested.

**Fluorescent microplaque assay of hog cholera virus**

Medium was aspirated from the required number of PK-15 cell cultures on coverslips in Leighton tubes. Each culture was inoculated with 0.2 ml of hog cholera virus and was then incubated for 2 hours at 37°C to allow virus adsorption. Usually 2 cultures were inoculated with each dilution of virus. Inoculum was redistributed over the cultures every 30 minutes. Following the adsorption period inoculum was decanted, cultures were rinsed with medium, 2 ml of fresh medium was added to each, and they were incubated at 37°C. The medium used here (i.e., overlay medium) was Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate, 10% porcine serum, and antibiotics. The serum portion of the medium routinely consisted of 9.5% nonimmune serum, 0.475% anti-hog cholera serum 10958, and 0.025% anti-hog cholera serum 8194. This combination of antiseraums, in the amounts
Figure 1. Fluorescent antibody staining of viral antigens within PK-15 cells with polyvalent conjugate 12389-90. Eighteen hours postinfection

A. Hog cholera virus X156
B. Parainfluenza 3 virus X312
C. Vesicular stomatitis virus X156
D. Newcastle disease virus X312
E. Pseudorabies virus X156
F. Noninfected PK-15 cells X312
Figure 2. Fluorescent antibody of viral antigens within PK-15 cells with polyvalent conjugate 12389-90. Eighteen hours postinfection X125

A. Parainfluenza 3 virus
B. Vesicular stomatitis virus
C. Newcastle disease virus
D. Hog cholera virus
indicated, neutralized extracellular progeny virus of all of the strains of hog cholera virus used in the study and thus prevented the formation of secondary foci of infection (27).

Cultures were incubated for 48 hours at 37 C after which they were processed for fluorescence microscopy and mounted on counting slides (27) for examination.

**Median cell culture infective doses**

Viruses other than hog cholera were titrated by determining the 50% endpoint of infectivity (38). Dilutions of Newcastle disease, vesicular stomatitis, and pseudorabies viruses were inoculated onto PK-15 cell cultures in 15 X 125 mm glass tubes. Infectivity was evidenced by cytopathology. Dilutions of parainfluenza 3 virus were inoculated onto PK-15 cell cultures on coverslips in Leighton tubes. The endpoint of infectivity was determined by fluorescent antibody staining.

**Test for Virus-Neutralizing Capacity of Anti-Hog Cholera Serum**

Serums were heat inactivated (56 C for 30 minutes) and then examined for antibody by mixing 1 ml of each of 2-fold dilutions of serum with a selected number of plaque-forming units of hog cholera virus contained in 1 ml of diluent. After mixtures were incubated for 1 hour at 37 C, 2 or more PK-15 cell cultures on coverslips were inoculated with each virus-serum mixture. The remainder of the procedure was as described
previously for determining plaque-forming units of hog cholera virus.

**Enumeration of Cells**

Cultures of PK-15 cells were dispersed with trypsin-versene just as for subculture and were vigorously pipetted to minimize the number of cell aggregates. Counts were made with an electronic counter.¹

**Photomicrographs**

Photomicrographs were taken with an automatic camera² and Kodak Tri-X film.

**Mechanisms for the Spread and Persistence of Hog Cholera Viral Infection in Cell Culture**

**Spread of virus from infected to noninfected cells**

Various concentrations of anti-hog cholera serum 8194 were included in the nutrient medium of PK-15 cell cultures on coverslips just after they were each infected with approximately 100 plaque-forming units of strain Ames of hog cholera virus. The medium was of the same composition as used for routine propagation of PK-15 cells except that the concentration of serum was increased from 2% to 10%. The concentration of 10% was maintained by increasing or decreasing the amount

¹Coulter Electronics, Hialeah, Florida.
²Orthomat, E. Leitz, Inc., Wetzlar, Germany.
of nonimmune serum in inverse proportion to the amount of antiserum 8194 present. All concentrations of antiserum 8194 used were sufficient to prevent formation of secondary foci (27). The relative extent of spread of virus to contiguous cells in the presence of a particular concentration of antiserum was determined by fluorescent antibody staining of the cultures 25.5 hours postinfection and counting the number of infected (fluorescent) cells/plaque. Counts of cells in small plaques were made directly, whereas counts were made from enlarged photomicrographs of larger plaques.

Transmission of virus from mother to daughter cells during mitosis

Persistently infected cultures were examined by immunofluorescence for cells in various stages of mitosis.

Relative Yields of Hog Cholera Virus from Arbitrarily Selected Subcultures of Persistently Infected Cultures

Virus titrations were made with nutrient medium from the 2nd, 10th, 36th, 43rd, 45th, 60th, 65th and 80th subcultures of persistently infected cultures to determine the relative and absolute yields of extracellular virus.

Test for Attenuation of Virulence of Virus During Subculture of Persistently Infected Cultures

Nutrient medium from the 2nd, 23rd, 43rd, 65th and 80th subcultures of persistently infected cultures was administered to pigs to determine virulence of the virus contained therein.
Each sample was administered to 1 or 2 pigs and each pig was given 1 ml of undiluted nutrient medium and placed in an individual isolation cage. The immunity of pigs that did not die after administration of the sample was challenged by subsequent administration of $2 \times 10^5$ or more plaque-forming units of stock strain Ames.

Susceptibility of Cells Persistently Infected with Hog Cholera Virus to Infection with Other Viruses

The 88th subcultures of persistently infected and parallel control cultures, all grown in 30 ml bottles, were challenged with Newcastle disease, pseudorabies, vesicular stomatitis, and parainfluenza 3 viruses. Forty-eight hours after subculture, 0.2 ml of challenge virus (from stock) was added to the nutrient medium and cultures were examined periodically during the following 72 hours.

The 88th subcultures of PK-15 (Ames) and PK-15 (Cont.), grown on coverslips in Leighton tubes, were challenged with the above viruses and photomicrographs were taken to demonstrate the similarities in cytopathology and distribution and amount of antigen of challenge virus in the cultures.

Enumeration of Chromosomes

Chromosomes of metaphase cells of the 75th subculture of each of the persistently infected cultures and the parallel control culture were prepared for counting and counted by
Dr. E. C. Pirtle of the National Animal Disease Laboratory, Ames, Iowa, by the method previously described (33). At least 12 metaphase cells were examined for each culture.

Replication of Hog Cholera Virus

Acute infection

Replication of strain Ames of hog cholera virus was followed by fluorescent antibody staining and by titration of progeny virus. PK-15 cells were infected 48 hours after subculture, at about the time a confluent monolayer was formed. The input multiplicity of infection was approximately 1 and cultures were exposed to the inoculum for 1 hour at 37 C. After infection, cultures were nourished with Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate, 10% nonimmune porcine serum and antibiotics. To minimize the effect of cooling of the culture on the synthesis of virus, the inoculum and medium used to rinse and nourish infected cultures were prewarmed to 37 C and all manipulations done at room temperature were completed as quickly as possible. Intervals, such as that of the latent period, were measured from the time cultures were first exposed to the virus.

Fluorescent antibody staining was done with cells grown on coverslips in Leighton tubes and infected with stock strain Ames. After infection, inoculum was decanted and cultures were rinsed twice with 2 ml of nutrient medium. Two ml of nutrient medium was then added to each culture and they were
incubated at 37 C. Cultures were processed for fluorescence microscopy at various times after infection.

For titration of progeny virus cells were grown in Leighton tubes without coverslips and infected with either stock strain Ames or a preparation of strain Ames that had been passed 27 times in PK-15 cells. After infection, inoculum was decanted and cultures were rinsed 7 times, each time with 5 ml of nutrient medium. Two ml of nutrient medium was then added to each culture and they were incubated at 37 C.

At selected times following the addition of nutrient medium, cultures were prepared for titration of virus in the following manner. Nutrient medium was collected from the cultures and centrifuged for 10 minutes at 2000 times gravity to sediment cells and debris. Each cell monolayer was rinsed 4 times, each time with 2 ml of nutrient medium, and then scraped into 2 ml of nutrient medium. Both supernatant fluids and cell suspensions were frozen at -20 C until all samples were collected. Cell suspensions were frozen and thawed an additional time before they were assayed for virus. From preliminary study it was known that the freezing and thawing of cells was as effective as ultrasonic disintegration for release of cell-associated virus and did not reduce the infectivity titer.
Persistent Infection

Growth curves for cells of persistently infected cultures PK-15 (Ames) and PK-15 (331) and virus replicated therein were determined in parallel. A selected number of cells was seeded into the required number of 30 ml culture bottles and the cultures were incubated at 37 C.

The "0 hour" sample was taken immediately after seeding by collecting the nutrient medium (with cells still in suspension) from 2 bottles. Cells were sedimented by centrifugation at 2000 times gravity for 10 minutes. Supernatant fluid was frozen at -20 C (extracellular virus) and cells were resuspended in fresh nutrient medium and also frozen at -20 C.

At each subsequent sample time, nutrient medium from 4 bottles was pooled and centrifuged at 2000 times gravity for 10 minutes. The supernatant fluid was frozen at -20 C. Cells of 2 of the 4 bottles were used to determine cell-associated virus. For this purpose, monolayers were each washed 5 times, each time with 5 ml of nutrient medium, and then scraped into 6 ml of fresh medium. Cell suspensions were pooled and frozen at -20 C. Cells of the remaining 2 bottles were dispersed intact with a trypsin-versene solution and counted.

Titrations of virus were made after cell samples were collected. Cell suspensions were frozen and thawed an additional time before they were titrated.

In some experiments only extracellular virus was sampled.
In 2 experiments a growth curve for cells of PK-15 (Cont.) was determined in parallel with those of PK-15 (Ames) and PK-15 (331). Like the persistently infected cultures, cells from 2 bottles were dispersed and counted at each sample time.

Effect of Persistent Infection on Cell Replication

The following experiments were done with cells of PK-15 (Ames), PK-15 (331), and PK-15 (Cont.).

Growth curves

Growth curves of cells were determined as described in the immediately preceding section.

Cloning efficacy

Petri dishes (50 mm) were each seeded with 50 cells in 6 ml of nutrient medium and were then incubated at 37 C in a humid atmosphere of 5% CO₂. Six days later the medium was decanted and the dishes were rinsed once with phosphate buffered saline. Clones of cells were fixed with methyl alcohol, stained with trypan blue, and counted.

Subculture twice weekly at a ratio of 1:10

Cells were routinely subcultured twice weekly at a ratio of 1:5; however, to determine the effect of persistent infection on cells under greater stress they were also temporarily subcultured twice weekly at a ratio of 1:10.
Effect of Various Concentrations of Actinomycin D\(^1\) on Cell and Viral Replication

A selected number of cells of the 108th and 109th subcultures of PK-15 (Ames) were seeded into 30 ml bottles in the presence of various concentrations of actinomycin D in the nutrient medium. At 24 hour intervals nutrient medium was collected from 2 bottles and centrifuged at 2000 times gravity for 10 minutes. The supernatant fluid was frozen at -20 C until assayed for virus. Cell monolayers of the same cultures were dispersed with a trypsin-versene solution and the cells were counted.

Percentage of Cells Infected in Persistently Infected Cultures

Percentage containing viral antigen

Cells of several subcultures of PK-15 (Ames), PK-15 (331), and PK-15 (Cont.) were seeded onto coverslips in Leighton tubes and examined by fluorescent microscopy at various times after seeding. Photomicrographs were taken of the 112th subcultures.

Percentage containing complete virus

Petri dishes (50 mm) were each seeded with 50 cells of the 101st subculture of PK-15 (331). Nutrient medium was the same as that used for routine propagation of cells except that for cloning the serum concentration was increased from 2% to

\(^1\) Actinomycin D was a gift from Dr. W. B. Gall, Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.
4% by the addition of antiserums 10958 (1.8%) and 8194 (0.2%). The antiserums were included to neutralize extracellular virus and thus prevent transfer of virus among clones of cells. Cells were incubated at 37 C in a humid atmosphere of 5% CO₂. Eight days after seeding, medium was aspirated from the cultures and a sterile, stainless steel cylinder was placed over each of several isolated clones. The base of each cylinder was "coated" with a thin layer of silicone grease to provide a seal between it and the surface of the Petri dish. Cells were dispersed by adding a trypsin-versene solution to the cylinder and incubating the cultures at 37 C for 30 minutes. Cells suspended in trypsin-versene were then added to fresh medium in another Petri dish. After 2 or 3 additional subcultures in Petri dishes, cells were transferred to 30 ml bottles. In this manner 10 sublines of PK-15 (331) were obtained, each from a single clone of PK-15 (331) 101. Nutrient medium from each of the sublines was tested for the presence of hog cholera virus.

Antigenic Relationship between Strains Ames and 331 of Hog Cholera Virus

Cross-neutralization

Antiserums 11522, 11524, 11844, and 11845 were each tested for neutralizing antibody against approximately 40 plaque-forming units of strains Ames and 331 of hog cholera virus. The viruses used were obtained from the 101st subcultures of
PK-15 (Ames) and PK-15 (331). The neutralization test procedure was described previously.

**Cross-fluorescence**

Cells infected with strains Ames and 331 of hog cholera virus were reacted with conjugates 10958 and 8194.

Resistance of PK-15 (Ames) and PK-15 (331) to Superinfection with Virulent Strains Ames and 331 of Hog Cholera Virus

**Inoculation of pigs**

Nutrient medium was aspirated from cells of the 70th subculture of each of the following cultures in 30 ml bottles: PK-15 (Ames), PK-15 (331), and PK-15 (Cont.). To each, 2.5 ml of virulent stock strain Ames was added. Viral inoculum was aspirated 5 1/2 hours later and each culture was rinsed 5 times, each time with 10 ml of nutrient medium. Six ml of fresh nutrient medium was added. The following day, and at alternate 3- and 4-day intervals thereafter for a total of 10 subcultures, cells were subcultured at a ratio of 1:5. Forty-eight hours after the last subculture, samples of nutrient medium were collected. Samples were also collected from the 80th subcultures of PK-15 (Ames), PK-15 (331), and PK-15 (Cont.) that had not been exposed to virulent virus at the 70th subculture. Each sample was administered to 1 or more pigs at a dose of 1 ml/pig.

The same general procedure as above was followed with the 96th subcultures of PK-15 (Ames), PK-15 (331) and PK-15
(Cont.) except that cells in one 30-ml bottle of each culture were exposed to virulent stock strain Ames and cells in a second bottle of each culture were exposed to virulent stock strain 331. Also, only 2 subcultures were made before samples were collected for testing in pigs.

Pigs that died after samples were administered were examined for macroscopic lesions indicative of hog cholera. Immunity of the remaining test pigs was challenged with $2 \times 10^5$ plaque-forming units of stock strain Ames, no less than 2 weeks after the sample was administered, to determine if they had become immune as a result of the sample inoculum.

**Titration of virus**

Nutrient medium was aspirated from the 96th subculture of one 30-ml bottle of PK-15 (Cont.) and two 30-ml bottles of PK-15 (331). The bottle of PK-15 (Cont.) and 1 bottle of PK-15 (331) were each exposed to 2.5 ml of a preparation of strain Ames that had been passed 27 times in PK-15 cells. The input multiplicity of infection was approximately 1. To the other bottle of PK-15 (331) was added 2.5 ml of nutrient medium as a sham inoculum. After 5 1/2 hours the inoculums were aspirated and each culture was washed 5 times, each time with 10 ml of nutrient medium, and 6 ml of medium was added. Eighteen and one-half hours later samples of nutrient medium were collected from each culture and titrated for virus.
Resistance of Pigs Infected with Strain 331 of Hog Cholera Virus to Superinfection with Strain Ames of Hog Cholera Virus

Although stocks of both strain Ames and strain 331 killed experimentally infected pigs, there was a relative difference in their virulence (29). Hog cholera susceptible pigs given strain 331 occasionally developed the chronic form of illness (characterized in part by a persistence of virus); whereas the same type of pigs given strain Ames almost invariably develop the acute form of illness. Consequently, it was possible to investigate the resistance of pigs, persistently infected with strain 331, to superinfection with the more virulent strain Ames. Two pigs were given only strain Ames, 2 pigs were given only strain 331, and 2 pigs were given strains Ames and 331 simultaneously. Five pigs were given strain 331 and 14 or more days later they were given strain Ames.
RESULTS

General Observations

Following their exposure to different strains of hog cholera virus cultures of PK-15 cells were maintained in parallel with a noninfected control culture for an interval of 63 weeks during which 125 subcultures were made. Although the study was terminated after the above interval, it is believed that infected as well as noninfected cultures could have been maintained indefinitely. Persistent infection was confirmed by repeated isolation and identification of hog cholera virus. Morphologic differences between persistently infected and noninfected cells were not detected.

Mechanisms for the Spread and Persistence of Hog Cholera Infection in Cell Culture

Spread of virus from infected to noninfected cells

Cell-to-medium-to-cell The extent of spread of viral infection in cell culture was inversely proportional to the concentration of antiserum in the nutrient medium (Table 1, Figure 3). Thus it was determined that the primary mechanism for dissemination of hog cholera virus from infected cells to noninfected cells was cell-to-medium-to-cell.

Cell-to-cell The direct transfer of virus from cell-to-cell without exposure to the medium was suggested by the observation that some spread of virus occurred even in the
Table 1. Relationship between plaque size and the concentration of anti-hog cholera serum 8194 in the medium

<table>
<thead>
<tr>
<th>Concentration of antiserum^a</th>
<th>Number of cells/plaque^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
<td>Average</td>
</tr>
<tr>
<td>10</td>
<td>4.0</td>
</tr>
<tr>
<td>0.1</td>
<td>36.4</td>
</tr>
<tr>
<td>0.05</td>
<td>38.1</td>
</tr>
<tr>
<td>0.025</td>
<td>43.0</td>
</tr>
<tr>
<td>0.0125</td>
<td>55.6</td>
</tr>
<tr>
<td>0.00625</td>
<td>71.6</td>
</tr>
</tbody>
</table>

^a Concentration of anti-hog cholera serum 8194 included in the nutrient medium of infected cell cultures. The neutralization titer of antiserum 8194 was 16,384.

^b For each antiserum concentration, cells were counted in 40 randomly selected plaques. The interval between initial cell infection and processing cultures for enumeration of cells/plaque was 25.5 hr.

The presence of medium that contained 10% antiserum 8194. However, the possibility that a few virions either escaped neutralization fortuitously or were refractory to neutralization could not be excluded.

Transmission of virus from mother to daughter cells during mitosis

Another possible means whereby hog cholera viral infection was spread and maintained in cell culture was mitosis of in-
Figure 3. Spread of hog cholera viral infection in PK-15 cells in the presence and absence of antiserum in the nutrient medium. Infected cells are demonstrated by fluorescent antibody staining with conjugate 8194

A. Fluorescent plaque developing in the presence of a 10\% concentration of antiserum 8194. 24 hours postinfection

B. Same as A except 48 hours postinfection

C. Fluorescent plaque developing in the presence of a 0.025\% concentration of antiserum 8194. 24 hours postinfection

D. Same as C except 48 hours postinfection

E. Fluorescent plaque developing in the absence of antiserum. 24 hours postinfection

F. Same as E except 48 hours postinfection
fected cells. By fluorescent antibody staining it was found that viral antigen was distributed bilaterally during mitosis (Figure 4) and it is probable that intracellular virus and/or viral genomes were distributed similarly.

Relative Yields of Hog Cholera Virus from Arbitrarily Selected Subcultures of Persistently Infected Cultures

Hog cholera virus was identified in all samples of nutrient medium collected from persistently infected cultures during the study. Titration of virus in selected samples allowed for the separation of cultures into 3 groups on the basis of their production of extracellular virus (Table 2, Figure 5). PK-15 (Ames), PK-15 (300), and PK-15 (208) yielded the most virus, PK-15 (289) was intermediate, and PK-15 (331) yielded the least virus.

Attenuation of Virulence of Virus During Subculture of Persistently Infected Cultures

All the strains of hog cholera virus collected from the 2nd subculture of persistently infected cultures were lethal for pigs (Table 3). However, a difference in virulence of strains was observed with virus collected from the 43rd subculture. Retention of virulence paralleled production of extracellular virus. That is, strains from higher yielding cultures, viz., PK-15 (Ames), PK-15 (300), and PK-15 (208), appeared to retain virulence longer than those from the intermediate and lower yielding cultures, viz., PK-15 (289) and
Figure 4. Distribution of hog cholera viral antigen during mitosis of PK-15 (Ames) 114. Fluorescent antibody staining with conjugate 8194. Late anaphase (arrow)
Table 2. Relative yields of hog cholera virus from arbitrarily selected subcultures of persistently infected cultures<sup>a</sup>

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number of subcultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>PK-15 (Ames)</td>
<td>1.78 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>PK-15 (300)</td>
<td>2.85 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>PK-15 (208)</td>
<td>3.56 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>PK-15 (289)</td>
<td>3.98 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>PK-15 (331)</td>
<td>1.65 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>PK-15 (Cont.)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Extracellular virus; expressed as plaque-forming units/ml of nutrient medium.
Figure 5. Relative yields of hog cholera virus from arbitrarily selected subcultures of persistently infected cultures
Table 3. Attenuation of virulence of virus during subculture of persistently infected cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number of subcultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>PK-15 (Ames)</td>
<td>2</td>
</tr>
<tr>
<td>PK-15 (300)</td>
<td>2</td>
</tr>
<tr>
<td>PK-15 (208)</td>
<td>2</td>
</tr>
<tr>
<td>PK-15 (289)</td>
<td>2</td>
</tr>
<tr>
<td>PK-15 (331)</td>
<td>2</td>
</tr>
</tbody>
</table>

aDenominator = number of pigs treated; numerator = number of pigs that died. Each pig was given, by subcutaneous injection, 1 ml of nutrient medium from the designated culture. All pigs that survived were found immune when their immunity was subsequently challenged with at least 10^5 plaque-forming units of virulent strain Ames.

PK-15 (331). By the 65th subculture all strains were sufficiently attenuated so that none of the experimentally infected pigs died.

Susceptibility of Cells Persistently Infected with Hog Cholera Virus to Infection with Other Viruses

No marked difference was observed in either the time of appearance or severity of the cytopathic effect of Newcastle disease, vesicular stomatitis, pseudorabies, and parainfluenza 3 viruses among previously noninfected cells, i.e., PK-15 (Cont.), and PK-15 cell cultures persistently infected with
the different strains of hog cholera virus. By fluorescent antibody staining it was found that neither the amount nor the distribution of intracellular antigens associated with the challenge viruses was affected by concomitant infection of the cells with hog cholera virus. To illustrate the above observations, culture PK-15 (Ames) 88 was arbitrarily selected for pictorial comparison with PK-15 (Cont.) 88 (Figures 6-21).

Moreover, the yields of Newcastle disease, vesicular stomatitis, and pseudorabies virus from PK-15 (Ames) 88 were similar to those from PK-15 (Cont.) 88 (Table 4).

Enumeration of Chromosomes

The modal number of chromosomes of cells of the 75th subculture of both persistently infected and noninfected cells was 36.

Replication of Hog Cholera Virus

**Acute infection with strain Ames**

Viral antigen was first demonstrated at the 4th hour postinfection by fluorescent antibody staining. The percentage of cells that contained viral antigen increased from less than 1% at 4 hours to more than 99% at 12 hours postinfection (Table 5, Figure 22).

Progeny virus was first detected at the 6th hour postinfection and increased progressively to the end of the experiment at 24 hours postinfection. The titer of extracellular
Figure 6. Cytopathic effect of Newcastle disease virus in PK-15 (Cont.) 88. Forty-six hours postinfection. Hematoxylin and eosin stain.

Figure 7. Cytopathic effect of Newcastle disease virus in PK-15 (Ames) 88. Forty-six hours postinfection. Hematoxylin and eosin stain.
Figure 8. Cytopathic effect of vesicular stomatitis virus in PK-15 (Cont.) 88. Twenty-two hours post-infection. Hematoxylin and eosin stain

Figure 9. Cytopathic effect of vesicular stomatitis virus in PK-15 (Ames) 88. Twenty-two hours post-infection. Hematoxylin and eosin stain
Figure 10. Cytopathic effect of pseudorabies virus in PK-15
(Cont.) 88. Twenty-two hours postinfection.
Hematoxylin and eosin stain

Figure 11. Cytopathic effect of pseudorabies virus in PK-15
(Ames) 88. Twenty-two hours postinfection.
Hematoxylin and eosin stain
Figure 12. Fluorescent antibody staining of PK-15 (Cont.)
88. Noninfected. Conjugate 12389-90

Figure 13. Fluorescent antibody staining of PK-15 (Ames)
88. Not superinfected. Conjugate 12389-90
Figure 14. Fluorescent antibody staining of PK-15 (Cont.) 88 infected with Newcastle disease virus. Twenty-two hours postinfection. Conjugate 12389-90. Only Newcastle disease viral antigen is present.

Figure 15. Fluorescent antibody staining of PK-15 (Ames) 88 superinfected with Newcastle disease virus. Twenty-two hours postinfection. Conjugate 12389-90. Both Newcastle disease and hog cholera viral antigens are present.
Figure 16. Fluorescent antibody staining of PK-15 (Cont.) 88 infected with vesicular stomatitis virus. Twenty-two hours postinfection. Conjugate 12389-90. Only vesicular stomatitis viral antigen is present.

Figure 17. Fluorescent antibody staining of PK-15 (Ames) 88 superinfected with vesicular stomatitis virus. Twenty-two hours postinfection. Conjugate 12389-90. Both vesicular stomatitis and hog cholera viral antigens are present.
Figure 18. Fluorescent antibody staining of PK-15 (Cont.)
88 infected with pseudorabies virus. Twenty-
two hours postinfection. Conjugate 12389-90. Only pseudorabies viral antigen is present

Figure 19. Fluorescent antibody staining of PK-15 (Ames)
88 superinfected with pseudorabies virus.
Twenty-two hours postinfection. Conjugate 12389-90. Both pseudorabies and hog cholera
viral antigens are present
Figure 20. Fluorescent antibody staining of PK-15 (Cont.) 88 infected with parainfluenza 3 virus. Twenty-two hours postinfection. Conjugate 12389-90. Only parainfluenza 3 viral antigen is present.

Figure 21. Fluorescent antibody staining of PK-15 (Ames) 88 superinfected with parainfluenza 3 virus. Twenty-two hours postinfection. Conjugate 12389-90. Both parainfluenza 3 and hog cholera viral antigens are present.
Table 4. Replication of Newcastle disease, vesicular stomatitis and pseudorabies viruses in PK-15 (Cont.) and PK-15 (Ames) cultures

<table>
<thead>
<tr>
<th>Superinfecting virus</th>
<th>Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PK-15 (Cont.) 88</td>
</tr>
<tr>
<td>Newcastle disease</td>
<td>$10^{6.9}$</td>
</tr>
<tr>
<td>Vesicular stomatitis</td>
<td>$10^{3.7}$</td>
</tr>
<tr>
<td>Pseudorabies</td>
<td>$10^{7.5}$</td>
</tr>
</tbody>
</table>

*Virus titer expressed as 50% cell culture infective doses/0.2 ml of nutrient medium from a culture infected 48 hours before.

virus exceeded that of cell associated virus (Figure 23).

**Persistent infections with strains Ames and 331**

Extracellular virus titers of PK-15 (Ames) and PK-15 (331) at the 90th, 96th, and 103rd subcultures are presented (Figures 24 and 25). At each subculture level more virus was present with PK-15 (Ames) than with PK-15 (331). With both persistently infected cultures titers were maximal or near maximal by 24 hours after subculture, but thereafter were more stable with PK-15 (Ames). That is, the extracellular virus titer of PK-15 (Ames) was relatively constant between 24 and 144 hours after subculture whereas a general decrease in titer was observed during the same interval with PK-15 (331).

Extracellular and cell-associated virus titers are compared
Table 5. Appearance of viral antigen in PK-15 cells acutely infected with strain Ames of hog cholera virus

<table>
<thead>
<tr>
<th>Hours after infection</th>
<th>Percent of cells containing viral antigen&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>5.5</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>6.5</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup>Estimated from fluorescent antibody-stained cultures.

<sup>b</sup>Occasional dull-fluorescent cell.
Figure 22. Appearance of viral antigen in PK-15 cells at various times after exposure to strain Ames of hog cholera virus. Fluorescent antibody staining with conjugate 8194

A. 5 hours postinfection
B. 6 hours postinfection
C. 7 hours postinfection
D. 8 hours postinfection
E. 12 hours postinfection
F. 24 hours postinfection
Figure 23. Replication of strain Ames of hog cholera virus in cell culture. Acute infection
STRAIN AMES (CCA)

- Extracellular Virus
- Cell-Associated Virus

HOURS AFTER INFECTION

LOG_{10} PFU/CULTURE

<1 4 8 12 16 20 24
Figure 24. Titers of virus in the nutrient medium (extracellular virus) of PK-15 (Ames) at various times after subculture of cells.
Graph showing the number of plaque forming units per culture over time for different subcultures.
Figure 25. Titers of virus in the nutrient medium (extracellular virus) of PK-15 (331) at various times after subculture of cells
with each other and with cell replication in the following figures: Figure 26, PK-15 (Ames) 103; Figure 27, PK-15 (331) 96; Figure 28, PK-15 (331) 103. The titer of cell-associated virus reached its peak at about the same time as the maximum number of cells was observed, i.e., 72 to 120 hours following subculture. Early, the titer of extracellular virus exceeded that of cell-associated virus but by 72 to 96 hours after subculture this relationship was reversed.

Effect of Persistent Infection on Cell Replication

Growth curves

Growth curves for cells of PK-15 (Ames), PK-15 (331), and PK-15 (Cont.) were similar in both the 96th and 103rd subcultures (Figures 29 and 30). However, slightly greater numbers of cells were observed in PK-15 (Cont.) than in either PK-15 (Ames) or PK-15 (331) during the logarithmic stage of cell replication.

Cloning efficacy

Cloning efficacy was slightly, and in one instance (subculture 101) considerably, better with persistently infected than it was with noninfected cells (Table 6).

Subculture twice weekly at a ratio of 1:10

After 9 subcultures only a few groups of cells were observed in culture vessels and the experiment was terminated. None of the cultures, i.e., PK-15 (Ames), PK-15 (331), and
Figure 26. Extracellular and cell-associated virus titers and cell numbers at various times after subculture of PK-15 (Ames) 103
CELLS EXTRACELLULAR VIRUS CELL-ASSOCIATED VIRUS

HOURS AFTER SUBCULTURE

CELLS/CULTURE(×10⁵)

Log₁₀ PLAQUE FORMING UNITS/CULTURE

0 15 24 48 72 96 120 144 168
Figure 27. Extracellular and cell-associated virus titers and cell numbers at various times after subculture of PK-15 (331) 96
Figure 28. Extracellular and cell-associated virus titers and cell numbers at various times after subculture of PK-15 (331) 103
Figure 29. Replication of cells of the 96th subcultures of PK-15 (Cont.), PK-15 (Ames), and PK-15 (331)
Figure 30. Replication of cells of the 103rd subcultures of PK-15 (Cont.), PK-15 (Ames), and PK-15 (331)
Table 6. Efficacy of cloning of cells from PK-15 (Cont.), PK-15 (Ames), and PK-15 (331)

<table>
<thead>
<tr>
<th>Culture</th>
<th>Age of culture (hours)</th>
<th>No. of plates seeded</th>
<th>Clones of cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cloning efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK-15 (Cont.)</td>
<td>94</td>
<td>48</td>
<td>25-44</td>
<td>36.0</td>
</tr>
<tr>
<td>PK-15 (Ames)</td>
<td>94</td>
<td>48</td>
<td>28-45</td>
<td>37.6</td>
</tr>
<tr>
<td>PK-15 (331)</td>
<td>94</td>
<td>48</td>
<td>27-48</td>
<td>39.4</td>
</tr>
<tr>
<td>PK-15 (Cont.)</td>
<td>101</td>
<td>72</td>
<td>20-40</td>
<td>32.6</td>
</tr>
<tr>
<td>PK-15 (Ames)</td>
<td>101</td>
<td>72</td>
<td>26-48</td>
<td>33.3</td>
</tr>
<tr>
<td>PK-15 (331)</td>
<td>101</td>
<td>72</td>
<td>36-56</td>
<td>46.5</td>
</tr>
<tr>
<td>PK-15 (Ames)</td>
<td>105</td>
<td>96</td>
<td>24-41</td>
<td>30.1</td>
</tr>
<tr>
<td>PK-15 (331)</td>
<td>105</td>
<td>96</td>
<td>28-44</td>
<td>33.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Plates were seeded with 50 cells each.

<sup>b</sup>Hours after subculture that cells were dispersed for cloning.

PK-15 (Cont.), was able to maintain itself under conditions of the experiment, although slightly greater numbers of cells were consistently observed in subcultures of PK-15 (331).

**Effects of Various Concentrations of Actinomycin D on Cell and Viral Replication**

Concentrations of actinomycin D that prevented cell replication (0.5 and 0.05 μg/ml) also inhibited viral replication
Concentrations of actinomycin D that reduced the rate of cell replication (0.00417 and 0.005 µg/ml) likewise seemed to reduce the rate of viral replication, but perhaps to a lesser extent. For example, in experiment I the virus-to-cell ratio at 72 hours after subculture was 0.0087 in the absence of actinomycin D and 0.0389 in the presence of 0.005 µg of actinomycin D/ml of culture medium.

Percentage of Cells Infected in Persistently Infected Cultures

**Percentage containing viral antigen**

By fluorescent antibody staining viral antigen was demonstrated in all cells of PK-15 (Ames) 112 at 22, 44, 72, and 96 hours after subculture. The less intense fluorescence observed with cells of PK-15 (331) 112, particularly at 72 and 96 hours, made recognition of infected cells more difficult. However, it appeared that compared to PK-15 (Cont.) 112 at the same intervals, all cells of PK-15 (331) 112 contained antigen as evidenced by a low intensity of fluorescence. The asynchrony of antigen production was especially obvious with cells of PK-15 (331) 112. In most areas of the culture a few cells were conspicuous by their more intense fluorescence (Figure 32).

**Percentage capable of producing infectious virus**

Infectious virus was produced by all 10 cloned sublines of PK-15 (331) 101, although quantitative differences were
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Concentration of actinomycin D in culture medium (µg/ml)</th>
<th>Hours after subculture</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cells</td>
<td>Virus</td>
<td>Cells</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>3.0 X 10^5 6.8 X 10^3</td>
<td>9.0 X 10^5 2.7 X 10^4</td>
<td>1.95 X 10^6 1.7 X 10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>1.9 X 10^5 2.5 X 10^3</td>
<td>3.0 X 10^5 1.3 X 10^4</td>
<td>3.6 X 10^5 1.4 X 10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.4 X 10^5 2.5 X 10^3</td>
<td>7.8 X 10^4 1.9 X 10^3</td>
<td>3.7 X 10^4 7.0 X 10^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.2 X 10^5 5.7 X 10^2</td>
<td>9.1 X 10^4 1.1 X 10^2</td>
<td>3.6 X 10^4 5.0 X 10^1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>3.0 X 10^5 1.0 X 10^4</td>
<td>9.7 X 10^5 8.3 X 10^3</td>
<td>2.3 X 10^6 1.1 X 10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00026</td>
<td>2.4 X 10^5 1.1 X 10^4</td>
<td>1.0 X 10^6 1.2 X 10^4</td>
<td>2.1 X 10^6 8.1 X 10^3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00104</td>
<td>2.9 X 10^5 8.5 X 10^3</td>
<td>8.8 X 10^5 1.1 X 10^4</td>
<td>1.7 X 10^6 7.8 X 10^3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00417</td>
<td>1.8 X 10^5 8.7 X 10^3</td>
<td>4.2 X 10^5 1.4 X 10^4</td>
<td>5.4 X 10^5 7.0 X 10^3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In experiment I each culture was seeded with 6.0 X 10^5 cells of PK-15 (Ames) 108; in experiment II each culture was seeded with 5.0 X 10^5 cells of PK-15 (Ames) 109. In experiment II extracellular virus was titrated at 0 hour. The titer was 8.3 X 10^2 plaque-forming units/ml of culture medium.*

*Total cells/culture.*

*Virus titer expressed as plaque-forming units/ml of culture medium.*
Figure 31. Effects of various concentrations of actinomycin D on cell and viral replication of PK-15 (Ames)

A. Experiment I; cell replication
B. Experiment I; viral replication
C. Experiment II; cell replication
D. Experiment II; viral replication
CONCENTRATION OF ACTINOMYCIN D (μg/ml of culture medium)  

**A**  
CONCENTRATION OF ACTINOMYCIN D (μg/ml of culture medium)  
- 0.00 
- 0.005 
- 0.05 

**B**  
CONCENTRATION OF ACTINOMYCIN D (μg/ml of culture medium)  
- 0.00 
- 0.025 
- 0.5 

**C**  
CONCENTRATION OF ACTINOMYCIN D (μg/ml of culture medium)  
- 0.0025 
- 0.00025 
- 0.00017 

**D**  
CONCENTRATION OF ACTINOMYCIN D (μg/ml of culture medium)  
- 0.00 
- 0.00025 
- 0.000025 
- 0.0000025
Figure 32. Cultures PK-15 (Ames) 112, PK-15 (331) 112, and PK-15 (Cont.) 112 at various times after subculture. Fluorescent antibody staining. Conjugate 8194

A,B,C. Cultures PK-15 (Ames), PK-15 (331), and PK-15 (Cont.), respectively, 22 hours after subculture

D,E,F. Same as above except 44 hours after subculture

G,H,I. Same as above except 70 hours after subculture

J,K,L. Same as above except 96 hours after subculture
Antigenic Relationship between Strains Ames and 331 of Hog Cholera Virus

Cross neutralization

Both strains were antigenically similar by cross neutralization (Table 9).

Cross fluorescence

Strain specificity was not demonstrated by cross fluorescence. The most intense fluorescence was observed with the homologous Ames virus-serum system, whereas the least intense fluorescence was seen with the homologous 331 virus-serum system (Table 10).

Resistance of PK-15 (Ames) and PK-15 (331) to Superinfection with Virulent Strains Ames and 331 of Hog Cholera Virus

Inoculation of pigs

The 70th and 96th subcultures of PK-15 (331) were susceptible to superinfection with virulent stock strain Ames; whereas, PK-15 (331) 96 was resistant to superinfection with the homologous strain (Table 11).

The 96th subculture of PK-15 (Ames) resisted superinfection with virulent strain 331. The 70th but not the 96th subculture of PK-15 (Ames) also resisted superinfection with the homologous strain. The possible effect of numbers of subcultures on the latter results will be discussed.
Table 8. Yield of virus from 10 clones sublines established from PK-15 (331) 101

<table>
<thead>
<tr>
<th>Cloned sublines</th>
<th>Extracellular virus titer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.4 X 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>1.9 X 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>9.4 X 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td>1.1 X 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>E</td>
<td>8.6 X 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>1.3 X 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>G</td>
<td>1.4 X 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>H</td>
<td>2.6 X 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>1.8 X 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>J</td>
<td>8.6 X 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Titer 24 hours after subculture.

**Titration of virus**

Although it was clearly demonstrated above that virulent strain Ames was able to replicate in PK-15 (331), a partial resistance of this culture was revealed by comparing the total yield of virus from PK-15 (331) and PK-15 (Cont.) after infection with the virulent strain (Table 12).
Table 9. Cross neutralization of strains Ames and 331 of hog cholera virus

<table>
<thead>
<tr>
<th>Antiserums</th>
<th>Strain of virus</th>
<th>Dilution of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>11522 (anti-Ames)</td>
<td>Ames</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>331</td>
<td>1.0</td>
</tr>
<tr>
<td>11524 (anti-331)</td>
<td>Ames</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>331</td>
<td>0</td>
</tr>
<tr>
<td>11845 (anti-Ames)</td>
<td>Ames</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>331</td>
<td>0</td>
</tr>
<tr>
<td>11844 (anti-331)</td>
<td>Ames</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>331</td>
<td>0</td>
</tr>
</tbody>
</table>

*aFrom PK-15 (Ames) 101 and PK-15 (331) 101; diluted to contain approximately 40 plaque-forming units/culture.

bAverage plaque count of 2 replicate cultures.

Table 10. Cross fluorescence of strains Ames and 331 of hog cholera virus

<table>
<thead>
<tr>
<th>Strains of virus</th>
<th>Conjugatesa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8198</td>
</tr>
<tr>
<td>Ames</td>
<td>++++b</td>
</tr>
<tr>
<td>331</td>
<td>+++</td>
</tr>
</tbody>
</table>

aConjugate 8198 was prepared from serum collected from a pig given only strain Ames. Conjugate 10958 was prepared from serum collected from a pig given several strains of hog cholera virus (including Ames and 331) early in the course of hyperimmunization but only strain 331 (15 injections) during the 6 weeks prior to exsanguination.

bIntensity of fluorescence.
Table 11. Resistance of PK-15 (Ames) and PK-15 (331) to superinfection with strains Ames and 331

<table>
<thead>
<tr>
<th>Culture</th>
<th>Super-infecting virus</th>
<th>Number of subsequent passages</th>
<th>Result of inoculation of pigs&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Primary inoculation&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Challenge of immunity&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK-15(Cont.)70</td>
<td>-</td>
<td>10</td>
<td>0/2</td>
<td>10/10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2/2</td>
</tr>
<tr>
<td>PK-15(Cont.)70</td>
<td>Ames</td>
<td>10</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>PK-15(Ames)70</td>
<td>-</td>
<td>10</td>
<td>0/2</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>PK-15(331)70</td>
<td>-</td>
<td>10</td>
<td>0/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>PK-15(331)70</td>
<td>Ames</td>
<td>10</td>
<td>3/4</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>PK-15(Cont.)96</td>
<td>-</td>
<td>2</td>
<td>0/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>PK-15(Cont.)96</td>
<td>Ames</td>
<td>2</td>
<td>2/2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PK-15(Cont.)96</td>
<td>331</td>
<td>2</td>
<td>2/2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PK-15(Ames)96</td>
<td>-</td>
<td>2</td>
<td>0/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>PK-15(Ames)96</td>
<td>Ames</td>
<td>2</td>
<td>2/2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PK-15(Ames)96</td>
<td>331</td>
<td>2</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>PK-15(331)96</td>
<td>-</td>
<td>2</td>
<td>0/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>PK-15(331)96</td>
<td>Ames</td>
<td>2</td>
<td>2/2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PK-15(331)96</td>
<td>331</td>
<td>2</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Virulent stock virus.

<sup>b</sup> Denominator = number of pigs treated; numerator = number of pigs that died following treatment.

<sup>c</sup> Each pig was given 1 ml of nutrient medium from the designated culture.

<sup>d</sup> Immunity of pigs that survived the primary inoculation was challenged by subcutaneous injection of at least 2 X 10<sup>5</sup> plaque forming units of virulent stock strain Ames.

<sup>e</sup> Virus from this culture was administered alone and in mixtures with attenuated virus from PK-15 (Ames) 80 and PK-15 (331) 80. See Materials and Methods for details.
Table 12. Resistance of PK-15 (331) 96 to superinfection with virulent strain Ames

<table>
<thead>
<tr>
<th>Culture</th>
<th>Infection with virulent strain Ames</th>
<th>Titer of virus(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK-15 (Cont.) 96</td>
<td>Yes</td>
<td>10^{4.8}</td>
</tr>
<tr>
<td>PK-15 (331) 96</td>
<td>Yes</td>
<td>10^{4.0}</td>
</tr>
<tr>
<td>PK-15 (331) 96</td>
<td>No</td>
<td>10^{3.6}</td>
</tr>
</tbody>
</table>

\(a\) Titer of virus/ml of nutrient medium 24 hours after infection with virulent strain Ames.

Resistance of Pigs Infected with Strain 331 of Hog Cholera Virus to Superinfection with Strain Ames of Hog Cholera Virus

Pigs 11061, 11866, and 11869, already infected with strain 331, lived longer after administration of highly virulent strain Ames than did pigs 11060 and 11064, infected only with strain Ames (Table 13).

A complete resistance of pigs infected with strain 331 to superinfection with strain Ames seemed unlikely from the observed increase in body temperatures of pigs 11868 and 11869 after administration of strain Ames (Figures 33 and 34). On the other hand, under similar circumstances, no early increase in body temperature occurred with pig 11866 (Figure 35). Body temperatures of pigs 11065 and 11061 were not taken.
Table 13. Resistance of pigs infected with strain 331 of hog cholera virus to superinfection with strain Ames of hog cholera virus

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Pig number</th>
<th>Days from administration of virus strain to death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Strain 331</td>
</tr>
<tr>
<td>50 plaque-forming units (PFU) of strain Ames</td>
<td>11060</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>11064</td>
<td>--</td>
</tr>
<tr>
<td>5000 PFU of strain 331</td>
<td>11066</td>
<td>22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>11058</td>
<td>42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50 PFU of strain Ames and simultaneously 5000 PFU of strain 331</td>
<td>11062</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>11063</td>
<td>9</td>
</tr>
<tr>
<td>5000 PFU of strain 331 and, 14 days later, 50 PFU of strain Ames</td>
<td>11065&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>11061</td>
<td>39</td>
</tr>
<tr>
<td>100 PFU of strain 331 and, 19 days later, 200,000 PFU of strain Ames</td>
<td>11868&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29</td>
</tr>
<tr>
<td>10 PFU of strain 331 and, 19 days later, 200,000 PFU of strain Ames</td>
<td>11866&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>11869&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39</td>
</tr>
</tbody>
</table>

<sup>a</sup>Developed the chronic clinical form of hog cholera and was killed 6 weeks postinoculation.

<sup>b</sup>Strain 331 was detected in the blood of these pigs in a titer of 10<sup>2</sup> or more just prior to administration of strain Ames.
Figure 33. Body temperature of pig 11868. Infected with strain 331 of hog cholera virus and 19 days later superinfected with strain Ames of hog cholera virus.
STRAIN AMES ADMINISTERED

DIED 29th DAY

TEMPERATURE (°F)

DAYS AFTER INOCULATION OF STRAIN 331 OF HOG CHOLERA VIRUS
Figure 34. Body temperature of pig 11869. Infected with strain 331 of hog cholera virus and 19 days later superinfected with strain Ames of hog cholera virus
DIE D 39th DAY

STRAIN AMES ADMINISTERED

TEMPERATURE (F)

DAYS AFTER INOCULATION OF STRAIN 331 OF NCG CHOLERA VIRUS
Figure 35. Body temperature of pig 11866. Infected with strain 331 of hog cholera virus and 19 days later superinfected with strain Ames of hog cholera virus.
STRAIN AMES ADMINISTERED

DAYS AFTER INOCULATION OF STRAIN 331 OF HOG CHOLERA VIRUS

TEMPERATURE (°F)

102 103 104 105 106 107 108

12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44

DIED 43rd DAY
DISCUSSION

In 1964, Walker (46) provided a comprehensive review of current knowledge concerning the carrier state of viruses in cell culture. From this review, he posed several questions, the answers to which he believed would provide a good understanding of the carrier state for a particular virus-cell system. These were: 1) must antibody or other anti-viral factors be supplied in the culture medium to maintain equilibrium in the carrier culture? 2) Can cultures be freed of virus (cured) by serial cultivation in a medium containing antiviral antibody, or can virus-free clones readily be obtained by cloning under antibody? 3) Is the culture resistant to superinfection by the carrier virus and is it resistant to challenge by other viruses? 4) What fraction of the cell population is infected under the conditions in which the carrier state is most stable? 5) Do infected cells divide and grow into infected clones? And 6) can interfering factors be demonstrated in the carrier culture?

Data obtained during the present study provide answers to most of the above questions as they pertain to the hog cholera virus-PK-15 cell system. First, antibody or other antiviral factors were not necessary to maintain the carrier state. It was possible to propagate and repeatedly subculture carrier cultures in parallel with the noninfected control culture. The innocuousness of hog cholera virus for its host
cell was further indicated by several experiments and observations. Microscopic examination of infected and noninfected cells revealed no morphological differences. The similarity between the growth rate of persistently infected and noninfected cultures was apparent by visual comparison and by growth curves established for cells of PK-15 (Ames), PK-15 (331) and PK-15 (Cont.). The lack of measurable cytopathology was also indicated by the finding that the cloning efficacy of carrier culture PK-15 (Ames) and PK-15 (331) was as good or better than PK-15 (Cont.) at the same subculture level. It is obvious from these findings that if a susceptible culture were exposed to hog cholera virus, it would become a carrier of the virus and would go unnoticed unless tests specific for virus or viral antigen were used for its detection. A striking difference exists, therefore, between the marked virulence of nonattenuated hog cholera virus, such as strain Ames, for the pig and its lack of virulence in cell culture even after a long association.

The fact that hog cholera virus can persist in cultures nourished with medium containing specific neutralizing antibody has been well documented previously (25) and, consequently, this facet was not investigated. Whether any individual cells of a culture can be cured of infection by antibody is perhaps open to question. However, this seems unlikely since all ten sublines, obtained from PK-15 (331) by cloning of cells in the presence of antibody, were carriers
of the virus. That is, if any of the 10 cells from which the sublines originated was cured of infection, the resulting line would have been free of infection. Carrier culture PK-15 (331) was selected for cloning because its low virus yield and low intensity of fluorescence suggested that of all the carrier cultures, it would be most likely to contain some cells without the potential for producing infectious virus.

It was possible to superinfect carrier cultures with hog cholera virus even though all the cells of the culture were apparently already infected with the same virus. Nevertheless, depending on the viral strain involved, a variable degree of resistance to superinfection was observed. As evidenced by pig inoculations (Table 11) carrier culture PK-15 (331) was superinfected with strain Ames but not the homologous strain 331. The possibility that all carrier cultures would resist superinfection with the homologous strain but not the heterologous strain was excluded by the coincident observation that PK-15 (Ames) was probably also superinfected with strain Ames (at least in 1 of 2 experiments) but not with strain 331. Rather, it appeared that strain Ames was simply better able to superinfect carrier cultures.

Two possibilities have been considered to explain the fact that superinfection of carrier culture PK-15 (Ames) with strain Ames was confirmed in only 1 of 2 experiments. Challenge virus was identified after 2 subcultures of the carrier culture following its exposure to the challenge virus (one
ill experiment) but not after 10 subcultures under otherwise similar conditions (another experiment). Although it is possible that challenge virus identified after 2 subcultures could have been residual inoculum, the dilution and time factors involved make this doubtful. Perhaps superinfection occurred in both experiments but during the longer interval of 10 subcultures (5 weeks) the challenge virus was gradually eliminated because it could not compete for replicative sites with the cell-culture-adapted, attenuated form of the same strain also present in the culture.

Fluorescent antibody staining indicated that all of the cells of a carrier culture contained viral antigen. Also, the establishment of 10 sublines of PK-15 (331), all of which were infected with hog cholera virus, indicated that at least most of the cells were capable of producing complete virus.

There is no question that infected cells undergo mitotic division. During mitosis viral antigen was demonstrated in both daughter cells by fluorescent antibody staining.

No evidence was obtained for either direct or interferon-mediated interference in carrier cultures to infection with other viruses. Vesicular stomatitis, pseudorabies, Newcastle disease, and parainfluenza 3 viruses all produced cytopathology in carrier cultures that was indistinguishable from that seen in the parallel control cultures. The lack of interference was also indicated by the finding that pseudorabies, vesicular stomatitis, and Newcastle disease viruses grew to essentially
the same titer in carrier cultures as they did in noninfected control cultures. The failure of hog cholera virus to stimulate production of interferon or interferon-like substances in acutely infected PK-15 cells has been observed also (26).

Walker (46) recognized 4 general types of carrier cultures depending on the mechanism by which cells are protected from the cytopathic effects of the virus. Protection of cells by either genetic resistance, anti-viral factors in the medium (usually antibody), or interferon provides the basis for 3 of the types. A common characteristic of each of the 3 is that during a stable state of persistent infection only a small percentage of the cells in a culture are infected. Therefore, none of these applies to persistent infection with hog cholera virus in which apparently all of the cells are infected. A 4th type of carrier culture is protected by some sort of intracellular regulation of infection. Also included here would be cultures infected with viruses apparently lacking cytopathogenicity. This 4th type has the following general characteristics. Neither antibody nor other anti-viral factors need be supplemented in the culture medium to maintain an equilibrium. The culture is not cured by addition of antiserum to the medium. Few clones of uninfected cells can be obtained by cloning in an antibody-containing medium. The culture is resistant to superinfection by the homologous virus and may show some resistance to related viruses. There is probably little resistance to unrelated viruses. Uninfected
clones, if obtained, are no more resistant than the original line of cells from which the culture was started. All or a large percentage of the cells are infected when the culture is stable. Infected cells divide and grow into infected colonies. Most of the clones of cells obtained from the carrier culture are infected.

In general, the above characteristics of the 4th type of carrier culture parallel those described here for the carrier state of hog cholera virus in PK-15 cells. Somewhat similar virus-cell carrier systems reported previously are mumps virus in human conjunctival cells (47, 49) and measles virus in HeLa cells (40).

A carrier culture is actually a two-part system encompassing properties of both virus and host cell. Thus far, properties of persistently infected cells have been discussed and compared with those of noninfected cells.

Although no evidence was obtained to indicate that persistent infection with hog cholera virus significantly altered either the phenotype or genotype of the infected cell, a change in the virus was obvious by its attenuation in virulence. The length of time required for attenuation was but one of several observations that revealed differences among the strains used in the study.

Studies with PK-15 (Ames) and PK-15 (331) revealed that viral replication was related to but did not directly parallel cell replication. A logarithmic increase in virus occurred
within the first 24 hours after cells were subcultured and during this interval most of the progeny virus was released into the medium. These findings are similar to those obtained when PK-15 cells were acutely infected with strain Ames of hog cholera virus. Although the number of cells per culture increased more than tenfold during the next 72 to 96 hours, the titer of extracellular virus either remained relatively unchanged, as with PK-15 (Ames), or decreased, as with PK-15 (331). On the other hand, the titer of cell-associated virus was more closely related to the number of cells and reached a maximum from 72 to 120 hours after cells were subcultured. Since the nutrient medium of a culture was neither changed nor supplemented during the experiments, signs of cell degeneration and the decrease in the number of cells/culture, always seen by 168 hours after subculture of both infected or non-infected cultures, were attributed to depletion of nutrients and accumulation of metabolic byproducts.

The lesser yield and quicker attenuation of strains 289 and 331 in cell culture suggest a causal relationship between replicative power and virulence. Although this relationship may have been merely coincidental, it allows speculation as to the past history of the less virulent strains. Such a relationship could have been the result of prior adaptation of these strains to a nonswine host, thereby reducing both their ability to replicate in porcine tissues and their ability to produce disease. Whether adaptation to other hosts occurs
in nature is unknown but rabbit-adapted strains of hog cholera virus have been introduced and widely used in the field as vaccines.

Antigenic similarity among strains was confirmed by the fact that after attenuation in cell culture strains 208, 289, 300, and j31 as well as Ames protected pigs against subsequent inoculation of virulent stock strain Ames. It had been speculated previously that strain 331 might be poorly immunogenic because it occasionally produced a chronic illness and persisted in the tissues of experimentally infected pigs (29). However, when attenuated in virulence, strain 331 was as effective in inducing immunity as were the other strains tested.

Neither cross-neutralization nor cross-fluorescence revealed any marked antigenic differences between strains Ames and 331. Nonetheless, when propagated in cell culture, these strains differed in that cells infected with strain 331 contained generally less viral antigen reactive with fluorescent antibodies than did cells infected with strain Ames. This was true for both acutely and persistently infected cells and paralleled the lesser yield of infectious virus from cells persistently infected with strain 331. In addition, it has been noted that to limit spread of infection to contiguous cells, a greater concentration of anti-Ames serum is required in the nutrient medium of cultures infected with strain 331 than in cultures infected with homologous strain Ames (Figure 36). Perhaps a lesser avidity of anti-Ames serum for strain 331
Figure 36. Relative plaque size of strains Ames and 331 in the presence of 0.025% antiserum 8194 (anti-Ames)

A. Strain Ames

B. Strain 331. Note less intense fluorescence of this strain. Some spread of virus may occur directly from one cell to another cell several cell diameters away through cytoplasmic projections (arrows)
increased the likelihood of progeny virus reaching susceptible cells before irreversible neutralization occurred.

Pigs already infected with strain 331 were apparently susceptible to superinfection with strain Ames but as in cell culture there appeared to be some resistance. Resistance was probably mediated both by direct interference with the replication of strain Ames, as demonstrated in cell culture, and by the presence of anti-viral factors produced in response to infection with strain 331.
SUMMARY

Persistent infection of a porcine kidney (PK-15) cell line was established with each of 5 strains of hog cholera virus. Both infected cultures and a noninfected control culture were maintained by parallel treatment for more than 1 year during which time they were subcultured twice weekly.

Although infective virus was repeatedly recovered from the carrier cultures and viral antigen was demonstrated by fluorescent antibody staining, the virus had no apparent effect on either phenotypic or genotypic properties of the host cell. On the other hand, a change in all 5 strains of hog cholera virus was evidenced by their attenuation in virulence during subculture of persistently infected cells.

The carrier state was characterized by: 1) the absence of virus-induced cytopathology or morphologic changes in infected cells, 2) the persistence of infection in most and probably all the cells in a culture, 3) the bilateral distribution of viral antigen during mitosis of infected cells, 4) a cloning efficacy of infected cells as good or better than that of noninfected cells, 5) a growth rate of infected cells similar to that observed with noninfected cells, 6) some resistance to superinfection with the same virus but little or no resistance to superinfection with vesicular stomatitis, Newcastle, parainfluenza 3, or pseudorabies viruses, 7) an apparent relationship between growth curves of virus and host
The titer of extracellular virus was maximal or near maximal within 24 hours after subculture of persistently infected cells, whereas, the number of cells and the titer of cell-associated virus did not reach a maximum level until several days later. Concentrations of actinomycin-D that inhibited replication of persistently infected cells also inhibited replication of the virus.

Strains of hog cholera virus differed in both their ability to replicate in persistently infected cells and the time required for their attenuation in virulence for pigs. A direct relationship between these 2 properties was suggested by the fact that the lower yielding strains were also apparently attenuated in less time.

Certain pigs that were persistently infected with a moderately virulent strain of hog cholera virus had some resistance to superinfection with a highly virulent strain. Nonetheless, death of such pigs was hastened by the administration of the more virulent strain.


ACKNOWLEDGEMENTS

I wish to thank the following people for their contributions to the study reported here:

Dr. R. A. Packer for his guidance and suggestions and for the many hours he spent reviewing this thesis.

Drs. M. R. Zinober and E. C. Pirtle of the hog cholera research project of the National Animal Disease Laboratory for their interest and suggestions and for creating an atmosphere wherein research was both interesting and enjoyable.

Other members of my graduate committee, Drs. M. L. Kaeberle, L. O. Mott, F. K. Ramsey, and C. L. Tipton for their time and interest.

I also wish to acknowledge the assistance of the following people:

Mrs. L. H. Drake for technical services.

Mr. R. M. Glazier and his staff for the excellent photographs.

Mrs. M. J. Eernisse for typing the first draft of the thesis; Mrs. S. D. Hulse for typing the final draft of the thesis.

Dr. P. J. Matthews and his staff for providing and caring for the experimental animals.

To my wife Barbara, I express my appreciation for reasons too numerous to list.

This study was conducted under P.L. 85-507.