ISOLATION OF A HERPESVIRUS FROM AN AMERICAN KESTREL WITH INCLUSION BODY DISEASE

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Abstract: A herpesvirus was isolated from the liver of a captive-bred American kestrel *(Falco sparverius)* which had died of inclusion body disease. Initial isolation was achieved in chicken embryo fibroblasts after three blind passages. Cell-adapted virus produced a distinct rounding of CEF cells within 24 to 48 h. Biologic and serologic tests suggested that the kestrel virus is similar to falcon herpesvirus and pigeon herpesvirus and is at least partially related to owl herpesvirus. However, serologic tests indicated that the kestrel herpesvirus is neither related to infectious laryngotracheitis virus nor to a herpesvirus from a psittacine bird; *(Eupsittula canicularis)* with Pacheco's parrot disease. This is the first report on the recovery of a herpesvirus similar to falcon herpesvirus from an American kestrel with naturally-occurring inclusion body disease, and on the serologic comparison between falcon herpesvirus and a psittacine herpesvirus.

INTRODUCTION

Inclusion body disease (IBD) of raptors is characterized by focal necrosis of hepatocytes and elements of the reticuloendothelial system and by intranuclear inclusions in cells of these tissues.[6] Herpesviruses have been isolated from a variety of falcons *(Falco mexicanus, F. chiquera, F. peregrinus)*[8] and from several owl species *(Bubo bubo, B. virginianus, Asio otus, Nyctea scandiaca)* with this disease.[2,3,12] The owl and falcon viruses reportedly are antigenically related to one another and to pigeon herpesvirus.[8,9] However, although the pathogenicity of owl and falcon herpesviruses is similar, the host range of pigeon herpesvirus seems to be different.[9]

Herpesviruses also have been recovered from psittacine birds with Pacheco's parrot disease, a condition very similar to IBD.[13,14] Although parakeets succumb to experimental infections of falcon herpesvirus[9] and pigeon herpesvirus,[15] the relationship of the falcon-owl-pigeon herpesvirus group to psittacine herpesviruses has not been investigated.

Experimental infections of kestrels by falcon herpesvirus have been recorded[8,9] but natural infections have not been reported. In a previous report we described the occurrence of inclusion body disease in an American kestrel[7] and we now report on the isolation of a herpesvirus from its tissue and on the relationship of this virus to several other avian herpesviruses.

MATERIALS AND METHODS

Virus Isolation and Propagation

Methods for cell culture propagation and virus isolation have been described

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previously.[11] Virus isolation was attempted using the liver from an American kestrel with naturally-occurring inclusion body disease. A 10% suspension of the liver was made in cell culture medium supplemented with the usual antibiotics and inoculated onto chicken embryo fibroblast (CEF) cell cultures prepared from specific-pathogen-free embryonated hens' eggs.

Viruses

The virus isolated from the American kestrel was designated "670". Falcon herpesvirus (FHV), owl herpesvirus (OHV), and pigeon herpesvirus (PHV) were obtained from C.J. Maré, University of Arizona, Tucson, Arizona. A herpesvirus isolated from a halfmoon conure *(Eupsittula canicularis)* with Pacheco's parrot disease was obtained from J.E. Pearson, Veterinary Services Laboratory, A.P.H.I.S., U.S.D.A., Ames, Iowa.

Virus Concentration and Purification

Cell-culture propagated virus was concentrated 20 times and partially purified by differential centrifugation using alternate centrifugation at 3,000 × g and 48,000 × g. The final virus pellet was resuspended in distilled water. The latter preparation was used for antiserum production and electron microscopy.

Fluorescent Antibody Test

The indirect fluorescent antibody test (IFAT) was carried out as previously described.[10] Cells (CEF) were grown on cell culture chamber slides, infected with virus at a 0.04 multiplicity of infection, incubated for 6-8 h and fixed for 10 min in acetone at room temperature. These slides were used in the IFAT with serial 2-fold dilutions of antisera and then applying the appropriate anti-IgG fluorescein isothiocyanate conjugates.[2]

Antisera

Antisera to "670"-Virus, PHV, OHV, and FHV were produced in specific-pathogen-free, white leghorn hens and New Zealand white rabbits. One ml of partially purified virus was inoculated intravenously twice (at 2-weekly intervals), and serum was collected 1 week after the last inoculation. Antiserum to ILT virus was obtained from M.L. Hofstad, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa. Antiserum to a herpesvirus from a halfmoon conure *(Eupsittula canicularis)* was obtained from J.E. Pearson, Veterinary Services Laboratory, A.P.H.I.S., U.S.D.A., Ames, Iowa. All antisera were absorbed with CEF cells prior to their use in the IFAT as described before.[10]

Electron Microscopy

a. **Negative Staining**
Negatively-stained preparations of partially purified virus were examined by electron microscopy using a previously described method.[11]

b. **Ultra-Thin Section**
The ultrastructure of virus-infected cells was examined by electron microscopy as described previously.[7] Ultra-thin sections were prepared from CEF which were scraped off growth surfaces of 75-cm² cell culture flasks 12-24 h post infection.

Ether Sensitivity

The sensitivity of virus to ether was tested as described by Maré and Graham.[8]

Light Microscopy

Infected CEF cells on cell culture chamber slides were fixed with Bouin's fixative and stained with hematoxylin and eosin using standard procedures.

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[2] Miles Laboratories, Inc., Elkhart, Indiana 46515, USA.

RESULTS

A suspension prepared from the liver of an American kestrel with inclusion body disease was inoculated onto CEF cell cultures in an attempt to isolate a virus. A cytopathic effect (CPE) of the CEF was not observed during two 8-day passages. On the fourth incubation day of the third passage, a CPE characterized by cell rounding was observed (Fig. 1). Intranuclear inclusions were observed in several affected cells (Fig. 2). On subsequent passaging a CPE developed in the cell culture within 24-48 h post inoculation. Low concentrations of this virus resulted in localized areas of CPE within 24-72 h and did not progress to involve unaffected cells, suggesting that secondary spread of the virus did not readily occur in the cell monolayer. The CPE observed with the kestrel virus "670" was very similar to that of FHV, OHV, and PHV in CEF cells.

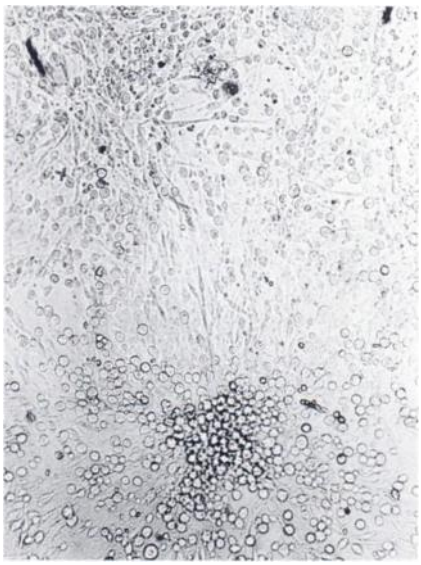

FIGURE 1. Cytopathic effect produced by "670"-virus in CEF cells. Note the normal spindle-shaped fibroblasts and the rounded affected cells. (unstained, phase contrast × 80).

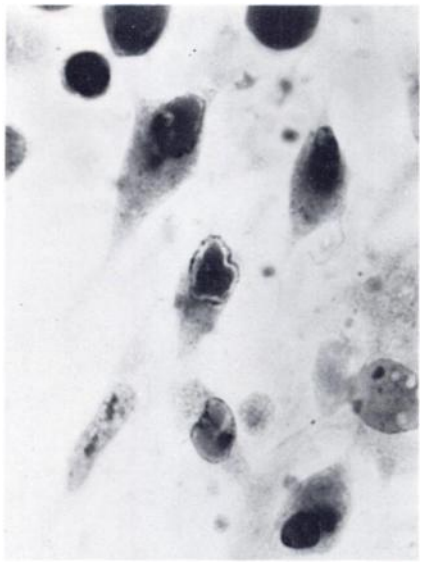

FIGURE 2. CEF cells 24 h after infection with "670"-virus. Note intranuclear inclusions and margination of chromatin in some cells. (H&E × 1360).

Electron microscopic examination of negatively-stained preparations of partially purified "670"-virus revealed typical herpesvirus nucleocapsids (Fig. 3). Enveloped herpesvirus virions were observed only in crude infected cell culture fluids (Fig. 4). Herpesvirus nucleocapsids, at various stages of maturation, were readily discerned in the nuclei of infected CEF by electronmicroscopic examination of ultra-thin sections (Fig. 5). Mature herpesvirus virions were observed in cytoplasmic vacuoles of infected CEF (Fig. 6). Ether treatment destroyed the infectivity of "670"-virus for CEF cultures.

Rabbits and chickens inoculated with partially purified "670"-virus did not produce significant levels of neutralizing antibodies since virus neutralization was detected with some serum samples diluted 1:4 but was absent at greater dilutions. However, specific antibodies were readily detectable by the IFAT (Fig.

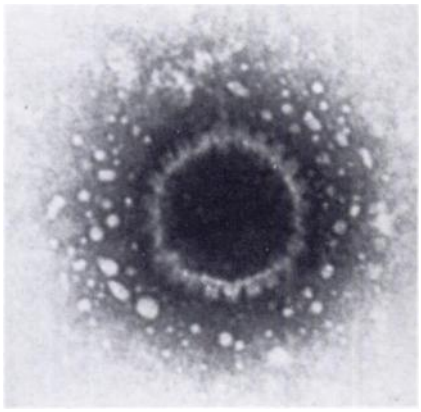

FIGURE 3. Electron micrograph of negatively-stained partially purified "670"-virus. Note herpesvirus-like, empty capsid with characteristic hollow capsomers. (× 217,300).

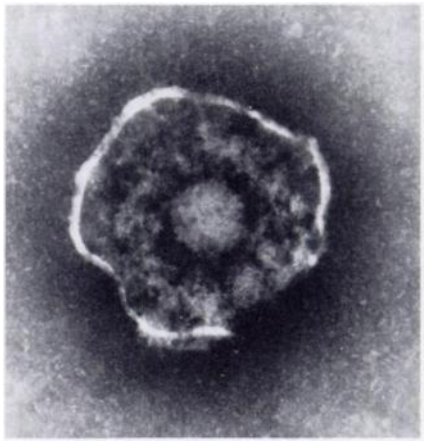

FIGURE 4. Electron micrograph of negatively-stained crude "670"-virus preparation. Note herpesvirus-like virion with a distinct envelope. (× 104,345).

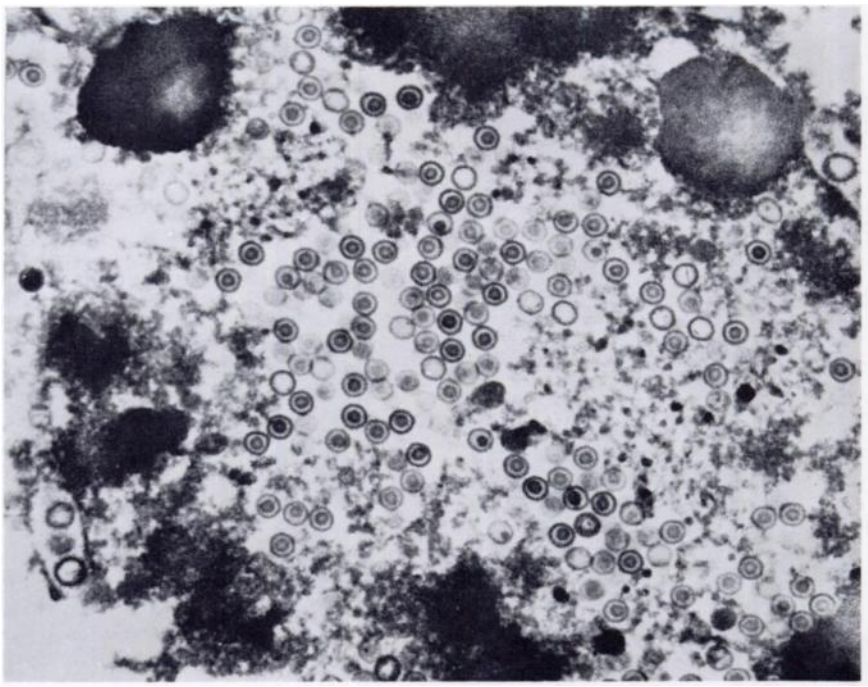

FIGURE 5. Herpesvirus-like capsids at various stages of maturation in the nucleus of a CEF cell 12 h after infection with "670"-virus. (× 42,395).

7). Nuclear and cytoplasmic fluorescence was observed and 6- to 8-hour infected CEF cells provided the best preparations for detecting antibodies by this method.

Comparative IFAT tests with "670"-virus, FHV, PHV, and OHV suggested that all these viruses were related (Table 1). There was a one way cross reaction between "670"-virus and OHV since "670"-virus antiserum reacted relatively poorly in OHV preparations. A similar result was noted when PHV and OHV were compared since PHV antiserum consistently had lower titers with OHV than with the other viruses. Antiserum to ILT virus was negative by IFAT using "670"-virus, OHV, FHV, and PHV preparations. Antiserum to the psittacine herpesvirus resulted in positive fluorescence in psittacine virus preparations but not with "670"-virus or FHV. Psittacine herpesvirus preparations resulted in fluorescence with homologous antiserum but not with "670"-virus, FHV, PHV, OHV, and ILT virus antisera.

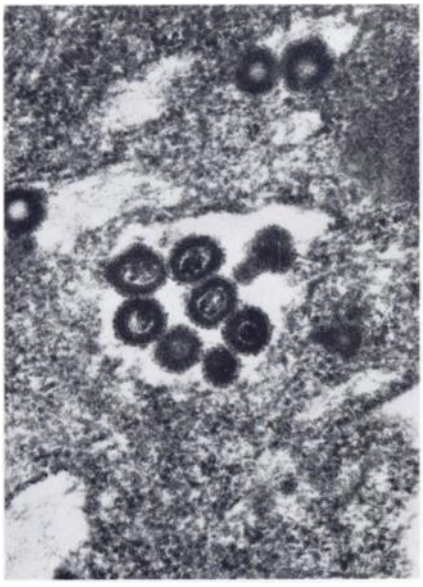

FIGURE 6. Herpesvirus-like virions in cytoplasmic vacuoles of a CEF cell 12 h after infection with "670"-virus. Note double-layered structure of virions. (× 53,699).

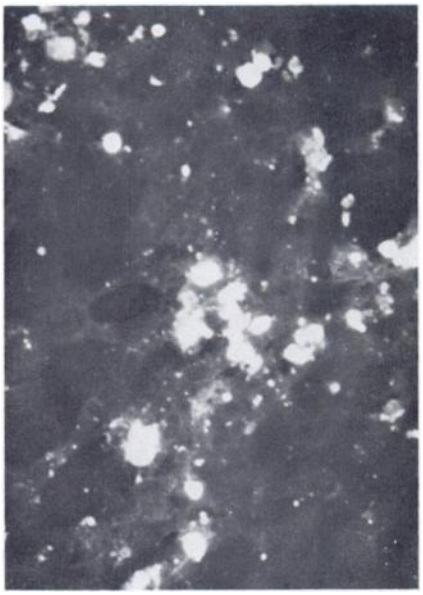

FIGURE 7. Immunofluorescence of "670"-virus-infected CEF cells 8 h post inoculation. (× 153).

DISCUSSION

It was concluded that the virus isolated from an American kestrel "670" with inclusion body disease was a herpesvirus based on morphologic, biologic and physical comparison with properties of this virus group.[1] The envelope of the kestrel virus seemed to be very fragile since only nucleocapsids were seen in partially purified preparations. However, enveloped virions were demonstrated in crude cell culture fluids and in the cytoplasm of infected cells. The presence of an envelope also was indicated by the ether sensitivity of the virus.

Results with the IFAT suggested that the kestrel virus is related closely to FHV

TABLE 1. Indirect fluorescent antibody test with selected avian herpesviruses.

Antiserum to	Reciprocal of Antibody Titer to Virus: "670"	FHV	PHV	OHV	Psittacine
"670"-Virus	320	320	320	20	0
FHV	640	320	640	320	0
PHV	160	160	160	40	0
OHV	640	640	640	640	0
ILT Virus	0	0	0	0	0
Psittacine Virus	0	0	N.D.*	N.D.*	1280

*ND: Not done

and PHV and at least partially related to OHV. Since the host range of some of these viruses differ,[8] one may also expect serologic differences, and perhaps antigenic analysis by IFAT lacks the specificity to distinguish between them.[5]

However, these viruses did not cross react by the IFAT with other avian herpesviruses (ILT virus and a psittacine herpesvirus). Additionally, Maré and Graham[8] reported similar conclusions based on virus neutralization tests. However, we were unable to detect significant levels of neutralizing antibody in the antisera tested. The latter may be explained by the method used for antiserum production since the partially purified virus (consisting mainly of naked capsids) used for inoculation may not have been elicited antibodies to envelope antigens which include the neutralizable antigens.[4] Maré and Graham[8] immunized laboratory animals with infected cell culture fluids and may well have been able to elicit antibodies to the neutralizable envelope antigens. However, some viruses do not elicit neutralizing antibodies, even in the natural host,[4] and perhaps "670"-virus is in the category.

It is interesting that although Pacheco's parrot disease is very similar to IBD of raptors, the two herpes viruses involved appear to be serologically distinct. Further study with several psittacine herpesvirus isolates is needed to confirm our observation.

The IFAT was a convenient and sensitive test for antibody to kestrel virus. The test is best done with cell cultures fixed 6-8 h post infection when adequate virus antigen is present and when a CPE, which contributes to nonspecific staining, is not evident.

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