Susceptibility of Guinea Pig Cell Cultures to Infection with Cell-Bound and Cell-Free Herpesvirus of Turkeys

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Cell-bound and cell-free herpesvirus of turkeys infected and replicated in guinea pig cell cultures as evidenced by cytopathic effects, intranuclear inclusions, and the presence of herpesvirus particles as seen by electron microscopy were studied in this investigation. Further evidence for the replication of herpesvirus of turkeys in cell cultures was determined by serum neutralization, complement fixation, and the fluorescent antibody tests.

Kawamura et al. (1970) first reported the isolation of a herpesvirus from turkeys (4). Later, Witter et al. (12) reported the isolation of a cell-associated herpesvirus from a flock of turkeys with a low incidence of lymphoid leukosis. Upon characterization, this herpesvirus from turkeys (HVT) was found to be distinguishable from other viruses previously isolated from turkeys (12). This strain was found to be antigenically related to the JM strain of type II leukosis (Marek’s Disease) virus (9) and was shown by Okazaki (6) to protect chickens to some degree against the development of type II lymphoid tumors.

Until recently it was thought that replication of HVT was specific to cells of the avian species (7). However, it was shown by Bedigian and Sevoian (1) and Elliot et al. (A. Y. Elliot et al., Amer. J. Vet. Res., in press) that HVT could infect and replicate in primary hamster kidney cell cultures. In our present study, attempts were made to infect primary guinea pig (GP) cell cultures with HVT to determine if these cells were susceptible to infection.

MATERIALS AND METHODS

Virus. HVT (FC-126) was isolated by Witter et al. (12) and has been propagated in this laboratory on duck embryo fibroblast (DEF) for approximately 10 passages. Cell-free HVT was prepared from DEF cell cultures by sonic treatment of the cell virus pools by using a Broswell sonicator at a power setting of 40 for 2 min. The sonicated treated material was then passed through a membrane filter (0.45 µm pore size; Millipore Corp.).

Preparation of GP cell culture. Primary GP cell cultures were prepared from 1- to 3-week-old animals. Kidneys were removed, minced, washed in pH 7.2 phosphate-buffered saline (PBS) containing antibiotic (100 U of penicillin, 0.1 mg of streptomycin, and 25 µg of mycostatin per ml), and trypsinized (0.25% trypsin) at room temperature until tubules could be seen under the microscope and then were collected (5). This was repeated until the miniced kidney was digested and no more cells could be obtained. The cells were spun at 800 × g for 5 min and the pellet was washed twice in PBS containing antibiotic as cited above. Finally, the pellet was suspended in disposable plastic Falcon petri dishes (60 by 15 mm) containing cover slips (11 by 22 mm) and placed at 37 °C under 5% CO2. When monolayers were formed, usually 4 to 5 days, the cells were inoculated with 8,000 plaque-forming units (PFU) of HVT virus per cc in trial 1 and 5,000 PFU per cc of cell-free HVT in trial 2. After infection, cultures were maintained in the above medium with 2% FCS.

Fluorescent antibody procedure. Antiser to HVT were prepared by inoculating HVT intravenously into 3-month-old New Zealand and Dutch Belted rabbits. Each rabbit received 5 to 7 weekly injections of 104 mean tissue culture infective doses of HVT-DEF antigen which was frozen and thawed three times. Blood was withdrawn by cardiac puncture 10 days after the last inoculation and the serum was collected. The serum was treated with saturated ammonium sulfate to precipitate out the gamma globulin fraction (2). The globulin fraction was then conjugated with fluorescein isothiocyanate according to the method of Cherry et al. (2). To reduce nonspecific fluorescence, the conjugate was adsorbed (three times) for 1 h at 4°C with tissue powder prepared from FCS and 10-day-old duck embryos. After each adsorption, the conjugate was centrifuged
at 10,000 x g for 30 min and the supernatant fluid was collected. After the last adsorption, the conjugate was divided into 0.5-cc samples and stored at −70 C until used.

When HVT-infected GP cell cultures began to show cytopathic effects (CPE), cover slips were removed, fixed in acetone for 2 min, and examined by using the direct fluorescent antibody method as described by Spencer et al. (10) with a Zeiss FA microscope with a 150-W high-pressure xenon bulb by using the following ultraviolet filters—BG 38, BL 12, and K 530. To determine the specificity of the conjugate, cover slips from control GP cell cultures, HVT-infected GP cell cultures treated first with unlabeled HVT-antisera, DEF cultures, and HVT-infected DEF cultures were treated with conjugate and examined.

**Cytopathic changes.** When cytopathic areas were visible on HVT-infected GP cell cultures, cover slips were removed, fixed in Bouins fixative, and stained with hematoxylin and eosin.

**Complement fixation.** The complement fixation (CF) test as described by Hamdy and Sevoian (3) was used to quantitate CF antigens in HVT-infected GP cell cultures.

**Serum neutralization test.** Cell-free HVT was prepared as previously described. The serum was first diluted 1 to 5 in PBS and then twofold dilutions were made. The various serum dilutions were mixed with an equal volume of filtered virus, allowed to stand at room temperature for 30 min, and then placed on chick kidney cultures. The neutralizing antibody titer was expressed as the highest serum dilution which

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**Fig. 1.** Cytopathic effects of sixth passage. HVT (trial 2) on guinea pig kidney cell cultures 4 days postinoculation. Notice rounded refractile cells. Unstained; x6.

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**Fig. 2.** Fifth passage guinea pig kidney cell culture infected with HVT 4 days after infection (trial 2). Note the inclusion bodies (arrows). H and E stained; x97.
caused a 50% reduction in virus titer. The same procedure was carried out by using a heterologous HVT antiserum (BS strain).

Treatment of infected cultures with 5-BUDR. DEF and GP monolayer cultures were inoculated with 0.5 cc of HVT-infected GP cell cultures. Each culture was treated with different concentrations (up to 20 μg) of 5-bromo-2' deoxyuridine (5-BUDR) in the medium. All cultures were examined for the presence of plaques 4 days postinoculation.

Virus isolations. One group of chickens was inoculated intraperitoneally with 0.2 cc of the control GP cell cultures and a second group with HVT-infected GP cell cultures (10th passage) containing 500 PFU per 0.2 cc. Chicks were kept 6 weeks in modified Horsfall-Bauer isolators after which kidneys were aseptically removed for reisolation of the virus in cell culture. Similar trials were carried out with DEF and DEF-HVT-infected cultures.

Electron microscopy. Preparations of second, third, and fifth passage HVT-infected and control GP cell cultures were scraped from petri dishes with a rubber policeman and fixed in 1% osmium tetroxide. The preparations were then dehydrated in graded alcohols, infiltrated, and embedded in Epon (Ernest Fullan Inc., N.Y.). After polymerization for 24 h at 61 C, the blocks were sectioned by using a Porter Blum-11 microtome. Sections were stained with uranyl acetate and lead citrate and were examined with a Phillips-200 electron microscope.

RESULTS

CPE. In trial 1, first passage GP cell cultures inoculated with 8,000 PFU of cell-bound HVT did not show any CPE. Seven days after infection, the infected GP cell cultures were trypsinized and collected in 2 cc of medium 199. A portion (0.5 cc) of the primary passage HVT-GP cell culture was placed on DEF to determine if the HVT was still active by exhibiting the characteristic CPE on fibroblast as described by Witter et al. The remainder (1.5 cc) of the infected GP cell cultures was transferred to new primary GP monolayers. The same procedure was followed for noninfected control GP cell cultures. After 4 days of the second passage, CPE, characteristic of HVT infection, was seen on DEF cultures, whereas CPE was not observed until 9 days on GP cell cultures. No CPE was observed on normal control GP cell cultures or DEF. The infected cells were transferred again to newly prepared GP monolayers and DEF; CPE was observed in third passage infected DEF 4 days postinfection, and at 7 days in GP cell cultures, but not in parallel control cultures. These cultures were passed seven times in GP cells with continued CPE characteristic of HVT infection. When the HVT-infected DEF cell cultures were replated onto new plates, only a few cells had settled and remained visible. Upon third passage of the HVT-infected DEF cell cultures, neither HVT-infected nor uninfected DEF cells were observed.

In trial 2 the same procedure was followed utilizing cell-free HVT. GP cell cultures inoculated with cell-free HVT did not show any cytopathic changes for three passages. Seven days after infection of the fourth passage, CPE

Fig. 3. Seventh passage HVT-infected cell culture (trial 2) stained by the direct fluorescent antibody method. ×400.
typical of HVT infection was observed. These cultures were passed seven times with continued CPE characteristic of HVT infection (Fig. 1). DEF cell cultures infected with cell-free HVT showed CPE typical of HVT infection 7 days after infection of the first passage. In all cases, when the HVT-GP inoculum was placed on DEF and newly prepared GP cell cultures, infection was observed 2 to 3 days earlier on the DEF cultures.

Cover slips from infected GP cultures stained with hematoxylin and eosin showed intranuclear inclusions which appeared spherical and were surrounded by a clear zone in the nucleoplasm (Fig. 2). No inclusions were observed in GP control cultures. Upon fluorescent microscope examination of infected GP cell cultures the nuclear fluorescences were predominantly observed with occasional cytoplasmic fluorescence (Fig. 3). Fluorescence was observed consistently in areas which showed CPE. Intranuclear fluorescence was also observed in some cells not associated with focal lesions. HVT-infected DEF also showed nuclear and cytoplasmic fluorescence, whereas noninfected GP and DEF cell cultures and HVT-infected GP cell cultures and HVT-infected GP cell cultures treated with unconjugated HVT antisera before staining were all negative.

**Complement fixation and serum neutralization.** CF antigens were demonstrated in both DEF and GP infected cultures using antisera prepared in rabbits. Specific CF antigen from various passages of HVT-GP cell cultures showed a CF titer of 1:16 to 1:32 (Table 1). Control antigens (normal DEF and GP cell cultures) did not fix complement with specific antisera prepared against HVT, nor did the normal control rabbit serum when tested with HVT-infected GP and DEF cell cultures. The CF test was considered positive when complement fixing activity was above 1:4.

The serum neutralization test demonstrated that HVT grown on GP cell cultures was neutralized by HVT antisera prepared in rabbits. A serum dilution of 1:20 and greater gave a 50% reduction in plaque counts in all trials tested (Table 1). Similar results were obtained using a heterologous HVT (BS strain) antisera.

**Effect of 5-BUdR on HVT replication.** Data is given in Table 2 in which DEF and GP cell cultures were infected and simultaneously treated with different concentrations of 5-BUdR. Concentrations of 5-BUdR of 10 μg/ml and more almost completely inhibited the development of plaques.

**Virus isolation.** HVT was resolated from

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**Table 1. Microscope and serological examination of HVT-infected (FC-126 strain) GP kidney**

<table>
<thead>
<tr>
<th>Passage</th>
<th>Primary plaques per plate in DEF</th>
<th>FA</th>
<th>SN*</th>
<th>CF</th>
<th>VI* on DEF</th>
<th>Primary plaques per plate in GP</th>
<th>Inoculum 1.5 cc/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 1*</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 2*</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 3*</td>
<td>70 +</td>
<td>1:20</td>
<td>1:16</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 4*</td>
<td>110 +</td>
<td>1:40*</td>
<td>ND</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 5*</td>
<td>150 +</td>
<td>1:40</td>
<td>1:32</td>
<td>210</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 6*</td>
<td>190 +</td>
<td>1:40</td>
<td>1:32</td>
<td>280</td>
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<tr>
<td>HVT 7*</td>
<td>220 +</td>
<td>1:40</td>
<td>1:32</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
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<td><strong>Trial II</strong></td>
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<td></td>
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</tr>
<tr>
<td>HVT 1*</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 2*</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 3*</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 4*</td>
<td>45 +</td>
<td>1:30</td>
<td>1:16</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 5*</td>
<td>170 +</td>
<td>1:30*</td>
<td>1:32</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 6*</td>
<td>175 +</td>
<td>1:30</td>
<td>1:32</td>
<td>125</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVT 7*</td>
<td>250 +</td>
<td>ND</td>
<td>ND</td>
<td>160</td>
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</tr>
</tbody>
</table>

* Controls were overlaid onto new primary cultures with each passage of infected GP cultures.

* SN, serum neutralization titer of HVT-antiserum against HVT from HVT-infected GP cell cultures.

* ND, not done.

* VI, isolation of HVT virus from infected GP cultures transferred onto DEF to determine if the viral agent is still active and to demonstrate differences, if any, from characteristic HVT infection on DEF.

* Serum neutralization test using heterologous antiserum to the FC-126 strain of HVT.

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**Table 2. Effect of 5-BUdR on replication of HVT in DEF and GP cell cultures**

<table>
<thead>
<tr>
<th>Culture</th>
<th>5-BUdR/ml of culture medium (μg)</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>DEF</td>
<td>135*</td>
</tr>
<tr>
<td>GP</td>
<td>65</td>
</tr>
</tbody>
</table>

*Counts represent the average number of plaques from six cultures.

**Table 3. HVT isolations from chickens**

<table>
<thead>
<tr>
<th>No. of chickens</th>
<th>Inoculum</th>
<th>Virus isolations</th>
<th>CF</th>
<th>FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>DEF</td>
<td>0/5</td>
<td>1:4</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>DEF-HVT</td>
<td>5/5</td>
<td>1:32</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>GP</td>
<td>0/5</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>GP-HVT</td>
<td>1/5</td>
<td>1:32</td>
<td>+</td>
</tr>
</tbody>
</table>

*Tenth passage HVT in DEF was used.*
kidneys of chickens inoculated with HVT-GP and DEF-HVT cell cultures: that the virus isolated was HVT is supported by CF and FA tests (Table 3).

**Electron microscopy.** Electron microscope examination of HVT-infected GP cell cultures showed herpesvirus type particles within the nucleus (Fig. 4), whereas viral particles were not observed in control GP cell cultures. The viral particles appeared as hexagonal shaped capsids. The electron lucent crosses as described by Nazerian were observed as well as naked virus particles and those which contained nucleoids.

**DISCUSSION**

It has been shown that HVT replicates in cells from mammalian species (1; A. Y. Elliot et al., Amer. J. Vet. Res., in press). Investigations on some viruses in vitro have shown that infection of the cell depends on whether the cell is in contact with another previously infected cell (11). Roizman (8) explained that the spread of infection by some herpesviruses was due to the cell fusion of virus-infected and uninfected cells. The fact that cell-free HVT is able to infect GP cell cultures gives evidence that the infection of the cell does not depend on the presence of previously infected cells. This virus was identified as HVT on the basis of CPE, type A intranuclear inclusion bodies, inhibition of plaque formation by 5-BUdR, and the presence in the nucleus of infected cells of naked and enveloped herpesvirus-type particles. Further evidence for HVT infection was given by the

![Electron micrograph of fifth passage HVT-infected guinea pig kidney cell cultures (trial 2) showing characteristic herpesvirus particles (arrows). Uranyl acetate and lead citrate. ×33,000.](http://iai.asm.org/)

**Fig. 4.** Electron micrograph of fifth passage HVT-infected guinea pig kidney cell cultures (trial 2) showing characteristic herpesvirus particles (arrows). Uranyl acetate and lead citrate. ×33,000.
demonstration of HVT-specific CF and immunofluorescent antigens in HVT-infected GP cell cultures. Furthermore, specific antisera to HVT as well as an heterologous antisera could neutralize the infectivity of HVT-infected cell cultures.

It was observed in all trials that DEF cell cultures infected with HVT showed CPE earlier than infected GP cell cultures. However, the increase in virus titer in HVT-infected GP cell cultures with each successive passage gave further evidence that GP cell cultures are susceptible to HVT infection and can support the growth of HVT.

That the morphological and immunological changes observed in HVT-infected GP cell cultures was due to a latent virus of guinea pigs is most unlikely, for control uninoculated guinea pig cultures passed in the same manner as infected cultures showed no CPE or the presence of virus particles upon electron microscope examination.

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LITERATURE CITED