Isolation and Characterization of a Herpesvirus from Leukemic Guinea Pigs

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A guinea pig herpesvirus (GPHV) has consistently been isolated from leukemic lymphoblasts of strain-2 guinea pigs. GPHV is serologically related to the guinea pig herpes-like virus isolated by Hsiung and Kaplow. The virions of GPHV consist of an icosahedral capsid containing a dense nucleoprotein core enclosed in a double-layered membrane. The average diameters of GPHV virion and capsid were 166 and 101 nm, respectively. Studies on the morphogenesis of GPHV revealed that, as in other herpesvirus infections, only the naked capsids with or without the nucleoprotein core were found in the infected cell nuclei; it was also learned that the virion acquired its envelope by budding from the nuclear membrane of the infected cells. However, GPHV-infected cell nuclei also contained dense fibrous rods, resembling nucleoprotein core outside the capsids, and tubules resembling viral core protein. The capsids were often embedded in dense granular antigen. GPHV deoxyribonucleic acid (DNA) has a density of 1.716 g/ml in cesium chloride compared to herpes simplex virus DNA (ρ = 1.728 g/ml) and cellular DNA (ρ = 1.700 g/ml).

Opler's observation (18) of C-type particles in lymphoblasts of leukemic guinea pigs suggested a viral etiology for guinea pig leukemia. Nadel et al. (14), and more recently Feldman and Gross (6), also observed such particles in leukemic, but not in normal, guinea pigs. However, no one has successfully demonstrated that these C-type particles are the leukemogenic agent in guinea pigs. We also studied the role of viral agents in guinea pig leukemia and consistently found C-type particles budding from the endoplasmic reticulum of leukemic lymphoblasts. However, we were unable to produce this agent in tissue culture in sufficient quantity to characterize its biological and biophysical properties. During this investigation, we consistently noted the activation of a second virus in leukemic lymphoblasts cultured in vitro. This agent has the properties of herpesvirus. A similar virus has been isolated from normal and leukemic strain-2 guinea pigs by Hsiung and Kaplow (9) and more recently from other strains of guinea pigs as well by Bhatt et al. (2). This report describes some morphological and biochemical characteristics of this guinea pig herpesvirus (GPHV) which was isolated from leukemic strain-2 guinea pigs.

MATERIALS AND METHODS

Guinea pigs. All the strain-2 guinea pigs (both infected and uninfected) used in these experiments were kindly supplied by S. Opler of Stanford University. Leukemia in these animals is maintained by inoculating tissues of infected animals (spleen, blood, tumors, etc.) into healthy animals. No infectious leukemogenic agent has yet been isolated from these leukemic guinea pigs. Infected animals develop leukemia within 2 to 3 weeks after inoculation. The animals in terminal phase of leukemia were exsanguinated by cardiac puncture. Gross and histopathology of the disease have been described by Opler (19). Hartley and English short-hair strains of guinea pigs were purchased locally.

Cell culture. Usually, circulating lymphoblasts or lymphoblasts from a local tumor, mesenteric lymphoid tumors, and spleen of leukemic guinea pigs were placed in tissue culture. Modified Eagle medium containing inactivated fetal calf serum (10 to 20%), tryptose phosphate broth (10%), penicillin (200 IU/ml), and streptomycin (200 μg/ml) was used. The medium was normally changed twice a week or as needed. Guinea pig embryo cultures (GE) were prepared from 1- to 1.5-month-old embryos by using the usual procedure of cell culture.

Labeling of infected cells. Monolayers of cultures were infected with GPHV. When the initial cytopathic changes became evident (usually 6 to 8 days after infection), the old medium was replaced with medium containing ³H-thymidine (specific activity, 25 Ci/mmol; 20 μCl/ml) and dialyzed fetal calf serum (10%). The fresh medium was incubated for 48 to 72 hr until all of the cells rounded up, at which time the medium and the cells were harvested and processed for virus purification as well as for the isolation of intracellular DNA.
Virus purification. The culture medium was freed from cells and subcellular debris by two cycles of centrifugation in an RC-2B Sorvall centrifuge (10,000 rev/min for 10 min in an SS 34 rotor). Virus was concentrated twice at the interface of 70 and 20% sucrose and finally purified by density-gradient centrifugation in a preformed linear 70 to 20% sucrose gradient (15). After centrifugation, the density, radioactivity, and infectivity of each fraction were analyzed, and peak fractions were examined by negative staining and thin sectioning for the presence of virus particles.

Isolation and analysis of viral and cell DNA. Deoxyribonucleic acid (DNA) from infected cells and purified virus was isolated and analyzed in a cesium chloride gradient as described by others (21, 25). The cells or fractions containing virus were lysed with sodium dodecyl sulfate (3%), dialyzed, and treated with self-digested Pronase (200 μg/ml). DNA so isolated was mixed with concentrated cesium chloride to make a solution with a density of 1.700 g/ml and centrifuged at 35,000 rev/min for 72 hr at 20 C in an SW 50 rotor. Fractions were collected and analyzed for density and radioactivity.

Electron microscopy. Small pieces of selected tissues, cultured cells, or pelleted virus materials were immediately fixed in 2% glutaraldehyde in phosphate buffer (pH 7.2 to 7.4) for 30 to 60 min, washed in three changes of phosphate buffer for 1 min each, and treated with 1% osmium tetroxide in phosphate buffer (pH 7.2 to 7.4) for 30 min. The tissue was then rinsed in Ringer’s solution and block-stained for 30 min in 1% aqueous uranyl acetate brought to pH 4.9 to 5.0 with NaOH. All of the foregoing solutions were made slightly hypertonic with sucrose. Dehydration was accomplished with the usual ethanol series. The tissue blocks were infiltrated first with a 1:1 mixture of absolute ethanol and embedding medium and then embedded in UNOX epoxy (Spurr). For negative staining, virus particles were fixed on carbon-coated grids and stained with 1% phosphotungstic acid.

RESULTS

Isolation of the GPHV. Spleen cells, lymphoid tumors, local tumors, and circulating leukemic lymphoblasts were placed in culture in modified Eagle medium containing inactivated fetal calf serum (10 to 20%) and 10% tryptose phosphate broth. The medium was changed twice a week. In such cultures, most of the cells remained in suspension, and over 80% were dead in 72 hr as determined by dye exclusion. Some fibroblasts adhered to the bottom of the dishes in about a week. By the second week, a few foci of clumped cells appeared in culture. Finally, all of the cells would round up and die around the fourth or fifth week of culture. It is at this time that the herpes-like virus (GPHV) can be first seen in the electron microscope. The results were the same if a feeder layer of GE culture was used for these leukemic lymphoblasts.

Morphology. The morphology of GPHV was studied both by negative staining of intact virions isolated from sucrose-density gradients as well as by positive staining of a thin section of virions and infected cells. It was clear that the tissue culture-activated GPHV was completely different from mature and immature C-type particles observed in vivo in leukemic lymphoblasts by Opfer (18), Nadel et al. (14), Feldman and Gross (6), and by us (Fig. 1). These C-type viruses with an average diameter of 101 nm bud from the endoplasmic reticulum into the cytoplasmic cisternae. GPHV on the other hand has the morphological characteristics of a herpesvirus (Fig. 4–9; Table 1). Mature GPHV consists of an icosaheiral capsid containing a dense nucleoprotein core surrounded by a double-layered membrane. The outer membrane often extends with the formation of a small tail. Mature GPHV is spheroidal and has an average diameter of 166 nm. The icosaheiral capsid is about 101 nm. There is a dense material between the capsid and the outer envelope (Fig. 5 and 7). Negative staining of purified virions and capsids also revealed the same structural components. Large virion-like structures containing several (as many as eight) capsids (Fig. 12) and naked capsids were often found in some preparations of purified virion isolated from sucrose gradients (Fig. 9).

Biological and serological properties. Once the virus is activated it can be passed serially in normal guinea pig embryonic culture. It usually takes 7 to 14 days to produce visible cytopathic effect (CPE; Fig. 2 and 3). CPE consists of infected cells which become rounded, refractile, and loose and which often clump together. In GE cells, the maximum titer is around 10^4 infectious units as determined by end point dilution in tissue culture. This titer is 50- to 100-fold over the input virus. Freezing and thawing once reduced the infectivity at least 10-fold. The infectivity of GPHV is also sensitive to ether treatment. CPE in GE cultures after infection was enhanced by incubation in reduced temperature (33 C), in acidic culture medium, and in medium deficient in arginine. All of these conditions have been found to activate the EB virus in Burkitt’s lymphoma cells (7, 9). GPHV also causes CPE in primary rabbit kidney cells and Vero monkey kidney cells and is serologically related to GPHLV (9) as determined by neutralization test and by immunofluorescence technique. Virus neutralization test was carried out on guinea pig embryo cultures as described by Bhatt et al. (2); virus titer was reduced 1,000-fold by the homologous antiserum and the antisera against GPHLV provided by G. D. Hsiung.

Morphogenesis. Morphological development of subviral components and assembly of these com-
Fig. 1. Immature C-type particles in leukemic lymphoblasts budding from endoplasmic reticulum. $\times 84,000$.

Fig. 2. Uninfected guinea pig embryo culture. $\times 125$.

Fig. 3. Guinea pig embryo culture infected with GPHV. Cytopathic effect was evident 7 days postinoculation. $\times 125$. 
FIG. 4. Thin section of GPHV present outside of the infected cells. X 63,000.

FIG. 5. Guinea pig herpesvirus. Note the membranous tail. X 113,000.

FIG. 6. Guinea pig herpesvirus. Note the segmentation of nucleoid. X 113,000.

FIG. 7. Guinea pig herpesvirus. Note the eccentric position of the capsid and presence of dense material between the capsid and envelope. X 84,000.

FIG. 8. Guinea pig herpesvirus enclosed in a pinocytic vacuole on the cell membrane. X 113,000.

FIG. 9. Negatively stained naked capsids without any envelope isolated from sucrose gradient. X 84,000.
TABLE 1. Morphological characteristics of guinea pig herpesvirus (GPHV) and its components

<table>
<thead>
<tr>
<th>Component</th>
<th>Avg diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPHV virion</td>
<td>166 ± 15</td>
</tr>
<tr>
<td>Capsid</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>Electron-dense nucleoid inside the capsid</td>
<td>50-70</td>
</tr>
<tr>
<td>Electron-lucent core inside the capsid</td>
<td>71.5 ± 5</td>
</tr>
<tr>
<td>Intranuclear tubules</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>Intranuclear fibrous rods resembling viral nucleoprotein</td>
<td>12 or 24</td>
</tr>
</tbody>
</table>

Components into morphologically mature virions were studied in GE cell monolayers grown over a plastic sheet and infected with GPHV. When infected foci became evident, the cells were fixed and embedded; specific foci of cells were selected under the light microscope, sectioned either in parallel or vertical plane to the monolayer, and examined with the electron microscope. In this way, cells in the same foci at different stages of infection could be observed.

In general, the morphological development of GPHV followed closely that of other herpesviruses. The nuclear phase of GPHV consisted of naked icosahedral capsids, most of which were empty (Fig. 10). The electron-dense material, when found inside the capsids, appeared in different stages of condensation: ring form or dense nucleoid (Fig. 10 and 13). Some capsids possibly contained only core protein as apparent from the lack of electron density (Fig. 13). These structures representing different stages in the development of GPHV capsids were similar to those found in other herpesvirus infection (3, 4). Also found inside the nucleus were dense, rod-like structures as reported in some herpesvirus-infected cells (4). The rods resembled DNA protein core and were often found in close association with capsids (Fig. 13). Inside the nucleus, capsids were often found embedded in a spherical electron-dense material (Fig. 11) which was similar to intranuclear granular antigens in herpesvirus-infected cells (16, 23). In later stages of infection, these intranuclear structures possibly disintegrate into smaller pieces containing one or more capsids and are released into the medium after lysis of infected cells. This could explain the origin of some large virus-like particles containing multiple capsids found in the preparation of purified virions (Fig. 12). This dense intranuclear granular antigen is present also around single capsids, is often eccentric in arrangement, and resembles closely the dense material often found between the capsid and the envelope (Fig. 4, 5, 7). A similar dense material between the envelope and the capsids in the herpesvirus associated with frog renal adenocarcinoma has been reported (24). Tubular structures which have the same density and diameter as the empty core proteins found inside the capsids can also be seen in the nucleus (Fig. 14). Such tubules have been found in guinea pig cytomegalovirus infection (13). Complete virions were not found in the nucleus; however, cytoplasmic invagination containing matured virions was observed inside the nucleus (Fig. 15). The virions acquire their envelope during budding from the nuclear membrane (Fig. 16) as has been reported by others (4). Matured virions were found in close association with the plasma membrane and outside the cell membrane (Fig. 8), but very few could be located in the cytoplasmic vacuoles.

Purification of virions and characterization of viral genome. The morphology and the maturation of the virions suggest that this agent is a herpes-type virus. Next, experiments were designed to purify the virus and characterize the viral genome. Cells were infected and labeled with 3H-thymidine (3H-TdR) when foci became visible. After labeling for 72 hr, the medium was harvested. The virus was concentrated on 2 M sucrose and purified on a sucrose-density gradient (20 to 70%) as described above. A sample of each fraction was counted, and the fractions containing the peak of radioactivity were examined by negative staining for virus particles. The infectivity in the peak fractions of the gradient was around 10^4.5 units/ml and coincided with the peak of radioactivity. The recovery of the total infectious virus in the sucrose gradient was only 10 to 15% of the total infectious units used in the purification procedure. DNA was isolated from purified GPHV and was found to be heavier than GE cell DNA (ρ = 1.700 g/ml). GPHV DNA had a density of 1.716 g/ml in cesium chloride (Fig. 17).

When DNA was isolated from infected cells after labeling with 3H-TdR, as above, the newly synthesized viral DNA also had a peak of 1.716 g/ml (Fig. 18). 14C-TdR-labeled DNA of HeLa cells infected with herpes simplex virus was used as a marker in the same gradient. HeLa cell DNA and HSV DNA had a density of 1.700 and 1.728 g/ml, respectively, in cesium chloride (20). 3H-labeled DNA isolated from purified GPHV and 14C-labeled DNA isolated from GPHV-infected cells had the same density if run in the same cesium chloride gradient. Further proof that the nucleic acid in GPHV virion is DNA was demonstrated by its deoxyribonuclease sensitivity, resistance to ribonuclease, and resistance to alkali.
FIG. 10. GPHV capsids in the infected nucleus. ×84,000.

FIG. 11. Multiple capsids embedded in granular antigen in the nuclei. Also note single capsids surrounded by the granular antigen. ×63,000.

FIG. 12. Negatively stained virus-like particles containing multiple capsids isolated from sucrose gradients. ×84,000.
FIG. 13. Section of an infected nuclei. X84,000. (a) Fibrous rod resembling viral nucleoprotein. (b) Ring form of nucleoprotein inside the capsid. (c) Capsids containing protein core only. (d) Section of a dense nucleoprotein core. (e) Sections of tubules.

FIG. 14. Section of masses of tubules found in infected nuclei. X84,000.
FIG. 15. Invagination cytoplasmic vacuole containing matured GPHV inside the nucleus. Nucleus (N), Cy (cytoplasm), INM (inner nuclear membrane), ONM (outer nuclear membrane). X84,000.

Fig. 16. Budding GPHV from nuclear membrane. X84,000.

FIG. 17. Cesium chloride equilibrium centrifugation of 3H-labeled guinea pig herpesvirus DNA (a) along with 14C-labeled guinea pig embryo cell DNA (b).

DISCUSSION

Characteristics and morphogenesis of GPHV. GPHV reported in this paper is closely related, if not identical, to GPHLV isolated by Hsiung and Kaplow (9) and Bhatt et al. (2) and is, therefore, different from guinea pig cytomegalovirus. The morphological and biochemical data indicate that GPHV has the characteristics of a herpesvirus. It is an enveloped virus with a icosahedral capsid which contains a nucleoprotein core. Its nucleic acid is double-stranded DNA. The assembly of capsids and nucleoprotein takes place in the cell nucleus, and the outer envelope is acquired during budding from the nuclear membrane. The virion is released in the medium by reverse pinocytosis or by lysis of cell. The large numbers of empty capsids in the nucleus, the large number of fibrous structures resembling DNA-containing protein core, and the paucity...
of complete virions either in the cytoplasm or outside the cell indicate a possible defect in the assembly process.

**Relationship of GPHV with guinea pig leukemia.**

Two different viruses have, therefore, been found in lymphoblasts of strain-2 leukemic guinea pigs. (i) A C-type virus particle has been observed in vivo, but usually not in vitro (Fig. 1). (ii) GPHV, a herpes-type DNA virus, is isolated from guinea pig cells only in vitro culture and not in vivo.

It is possible that guinea pig leukemia is caused by one of these two viruses or by the interaction of both viruses or by none. Wepsic et al. (27) and Sarma et al. (22) have suggested that certain forms of guinea pig leukemia may not possibly be caused by an infectious virus. Studies by Opler (18), Nadel et al. (14), and Feldman and Gross (6), on the other hand, suggest that a C-type virus is the cause of the guinea pig leukemia even though physicochemical characterization of this virion and its definitive role in guinea pig leukemia remains to be done. Their strongest argument, that such C particles are present only in leukemic guinea pigs, has been weakened by the recent finding of C-type particles resembling GPPV-Opler in germinal centers of lymph nodes of Hartley guinea pigs, a strain not susceptible to leukemia (11). However, the presence of C-type particles in nonleukemic guinea pigs does not rule out its etiological role in guinea pig leukemia. GPHV, on the other hand, has now been isolated from leukemic and nonleukemic strain-2 guinea pigs (2, 9). Preliminary data suggest that GPHV, when inoculated directly, fail to induce leukemia in strain-2 or Hartley guinea pigs (*unpublished data*). Inability to induce immunity against a similar herpesvirus isolated by Bhatt et al. (2) indicated the tolerance of guinea pigs to GPHV antigen, suggesting either congenital or hereditary transmission of GPHV. The characteristics of GPHV are very similar to those of EB virus (EBV) found in Burkitt's lymphoma. EBV is also activated in lymphoblasts only in tissue culture and not found in lymphoblastic cells in vivo (5). The conditions of activation for EBV and GPHV are very similar (7, 8). The density of GPHV DNA is similar to that of EBV DNA (26) which is also similar to cytomegalovirus DNA. The infection of EBV is also widespread and not limited to persons with Burkitt's lymphoma. Herpes-type viruses have been found as the causal agent in lymphoma in monkeys (12) and Marek's disease in chickens (17). Possible role of GPHV in inducing guinea pig leukemia has recently been suggested (10). Whether GPHV alone or in association with a C-type virus has any etiological role in guinea pig leukemia remains to be established.

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**LITERATURE CITED**


