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New Endogenous Herpesvirus of Guinea Pigs: Biological and Molecular Characterization

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Two known guinea pig herpesviruses, guinea pig cytomegalovirus (GPCMV) and guinea pig herpes-like virus (GPHLV), are well characterized. A third herpesvirus (GPXV) was originally isolated from leukocytes of healthy strain 2 guinea pigs. Growth of GPXV in guinea pig embryo fibroblastic cells produced a characteristic cytopathic effect. Electron microscopy of guinea pig cells infected with GPXV revealed the morphological development of a herpesvirus. Cross-neutralization tests and immunoferritin electron microscopy demonstrated that GPXV, GPCMV, and GPHLV were serologically distinct herpeviruses of guinea pigs. To confirm the distinction between these three herpesviruses, DNA genomes were compared by CsCl equilibrium buoyant density measurements and restriction endonuclease cleavage analysis. ^{32}P-labeled viral DNA was obtained from nucleocapsids isolated from virus-infected cells, and the buoyant density of GPXV DNA differed from that of GPCMV and GPHLV. Cleavage of viral DNAs with restriction endonucleases followed by gel electrophoresis revealed distinct patterns for each virus.

For over a century the guinea pig has been a useful experimental animal in several disciplines including virology, bacteriology, and immunology. Tissue culture cells derived from guinea pig embryos are convenient to prepare and can be experimentally infected with a variety of viral agents including human herpesviruses (6, 21). However, there are two well-investigated herpesviruses currently known to be endogenous only to this species.

Guinea pig cytomegalovirus (GPCMV), or caviid herpesvirus 1, closely mimics human CMV infection in humans and has proven to be a useful model for both transplacental transmission and congenital fetal infection with CMV (1, 2, 4, 12, 17, 18). The prototype strain of GPCMV (American Type Culture Collection 22122) was originally isolated by Hartley et al. from guinea pig submaxillary glands (9), yet the characteristic intranuclear inclusions present within GPCMV-infected salivary gland duct cells had been observed in guinea pigs by Jackson as early as 1920 (16).

In 1969 Hsiung and Kaplow reported a second guinea pig herpesvirus which was originally isolated from the renal tissue of a leukemic strain 2 guinea pig (13). Though a true herpesvirus (caviid herpesvirus 2), it retains a misnomer, guinea pig herpes-like virus (GPHLV). This virus is also endogenous, particularly to inbred strains of guinea pigs, and GPHLV can be isolated from leukocytes and various tissues in 80% of normal strain 2 and strain 13 animals by age 6 months (14). GPHLV possesses several biological properties which make it analogous to Epstein-Barr virus infection in humans (11); however, any relationship of GPHLV to neoplasia in guinea pigs remains elusive.

The purpose of this study was to characterize a third herpesvirus which was originally isolated from buffy coat of apparently healthy strain 2 guinea pigs and designated GPXV. Virus isolation and physical, biochemical, and serological characterization demonstrated GPXV to be a previously undescribed new herpesvirus endogenous to guinea pigs obtained from a variety of sources. Experimental infection of guinea pigs with tissue culture-passaged GPXV was undertaken to differentiate its pathogenesis from that of GPCMV and GPHLV, both of which have been under extensive investigation in our laboratory (1, 2, 7, 8, 12, 15, 22). These findings are reported in the present study.

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MATERIALS AND METHODS

Virus stocks, cell cultures, and maintenance media. The prototype strains of GPCMV (American Type Culture Collection 22122) and GPHLV (strain

LK 40), passaged in guinea pig embryo (GPE) cells, were used for comparison in these experiments (15, 22). GPE fibroblast cell cultures were prepared from 20- to 40-day-old Hartley guinea pig embryos; primary guinea pig or rabbit kidney cell cultures were prepared from 30- to 50-day-old virus-free rabbits or guinea pigs and maintained in minimal essential medium with Earle balanced salt solution containing 5% fetal bovine serum as described previously (10, 12, 14).

Source of animals and virus inoculation. Random-bred Hartley guinea pigs were purchased from CAMM Research Institute, Wayne, N.J. Inbred strain 2 guinea pigs were obtained and bred in our own facilities as described previously (1). Syrian hamsters were obtained from Charles River Laboratories, Wilmington, Mass. Experimentally infected guinea pigs and adult hamsters received 5 ml ($5 \times 10^{4.5}$ 50% tissue culture infective doses) and 2 ml ($2 \times 10^{5.5}$ 50% tissue culture infective doses) of stock GPXV intraperitoneally, respectively. Sampling of blood, tissues, and urine for virus isolation and neutralizing antibody determinations have been described previously (1).

Source of antisera and normal control sera. White New Zealand rabbits were obtained from Gloucester Laboratories, Chepachet, R.I., and were immunized against one of the guinea pig herpesviruses by multiple intraperitoneal and subcutaneous inoculations of one virus stock over several months. Freund adjuvant was included in the subcutaneous inoculations. Serum samples obtained from rabbits immunized with the new isolate (GPXV), GPCMV, or GPHLV were used for cross-neutralization testing and immunoferritin electron microscopy. Infectious bovine rhinotracheitis virus antisera were obtained from Colorado Serum Co., Denver, Colo. and as a gift of Frank Michalski. Rabbit anti-herpes simplex virus type 1 (MacIntyre) and anti-herpes simplex virus type 2 (MS) sera were purchased from Dako Laboratories, Copenhagen, Denmark. Human varicella-zoster virus, cytomegalovirus, and simian myxovirus antisera were also obtained commercially. Antiserum against Epstein-Barr virus was a gift of Warren Andiman. Parainfluenza 1 (C-39), parainfluenza 2 (Greer), and parainfluenza 3 (SF 4) antisera were obtained from the Center for Disease Control, Atlanta, Ga. For serological screening of human and 18 animal species to detect naturally occurring GPXV neutralizing antibody, pooled sera, commercially produced for use in forensic medicine, were obtained from Cappel Laboratories, Cochranville, Pa. Pooled rhesus monkey, African green monkey, chimpanzee, and rabbit sera were gifts of Norman Swack.

Light and electron microscopy; immunoferritin electron microscopy. Virus-infected and control GPE cell monolayers on cover slips and tissues fixed in Zenker acetic acid or Bouin solution were stained with hematoxylin and eosin, then examined for inclusions and pathological changes. Electron microscopic techniques for visualization of virus particles in cell cultures were as described previously (7, 8), except that after postfixation with osmium tetroxide the pellets were treated for 4 h with 0.5% uranyl acetate for en bloc staining. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 300 electron micrscope at 60 kV. Indirect immunoferritin electron microscopy was performed with each guinea pig herpesvirus and rabbit antiserum, using methods previously outlined (8).

Analysis of viral DNA. ^{32}P-labeled viral DNAs were prepared by a modification of that used for herpes simplex virus DNA (19, 23). Monolayer cultures (about 10^7 cells) of GPE cells were infected with an input multiplicity of infection of approximately 0.1. Cytopathic effect (CPE) was observed within 4 to 6 days. Two days before the development of extensive CPE, the medium was removed, and the cells were washed with phosphate-free saline. A 25-ml volume of Dulbecco-modified minimal essential medium without phosphate with 2% fetal bovine serum and with [^{32}P]phosphate (20 μCi/ml) was added, and the culture was incubated for 2 additional days. Cells were harvested and washed with phosphate-buffered saline. The cells were homogenized in 5.0 ml of 0.2% Triton X-100 in phosphate-buffered saline, and the nuclei were removed by centrifugation. Viral nucleocapsids were collected by centrifugation of the cytoplasmic extract at 30,000 rpm for 45 min at 5°C in a Spinco 40 rotor. The DNA was extracted from the nucleocapsids by treatment with sodium dodecyl sulfate and phenol as previously described (23). After ethanol precipitation, the DNA was stored in solution in 0.01 M Tris-hydrochloride (pH 8.0)-0.001 M EDTA at −20°C. Samples of DNA were analyzed by equilibrium density gradient centrifugation in CsCl. The gradient was determined refractometrically, and absolute densities were determined by reference to an internal standard of [^{14}C]DNA from *Enterobacter aerogenes* (1.717 g/cm^3).

Samples of DNA were digested with several restriction endonucleases and subjected to electrophoresis in gels of 0.7% agarose. The gel was dried, and contact autoradiographs (with intensifying screens) were prepared. The restriction endonucleases *Eco*RI, *Bgl*II, and *Bam*HI were prepared and used as previously described (20, 23). DNA from herpes simplex virus type 1 was included in some experiments to provide fragment size references.

RESULTS

Initial Isolation of GPXV. Thirty-two male strain 2 guinea pigs were obtained from the National Institutes of Health at age 3 months and were tested at age 6 months for virus in their blood by cocultivation of leukocytes from their buffy coats with GPE cells. Distinct CPE was observed at 3 weeks in inoculated cultures from four animals but not in control cultures. Initially, CPE was difficult to discern from spontaneous cellular degeneration. It became recognizable after a second passage in GPE cells and could be passaged to primary guinea pig kidney but not primary rabbit kidney cells. These cellular changes appeared to be different from those produced by known guinea pig viruses. Typical early focal CPE and more advanced diffuse CPE are shown in Fig. 1. After several passages in GPE cells and electron microscopic examination, it became apparent that a herpes-

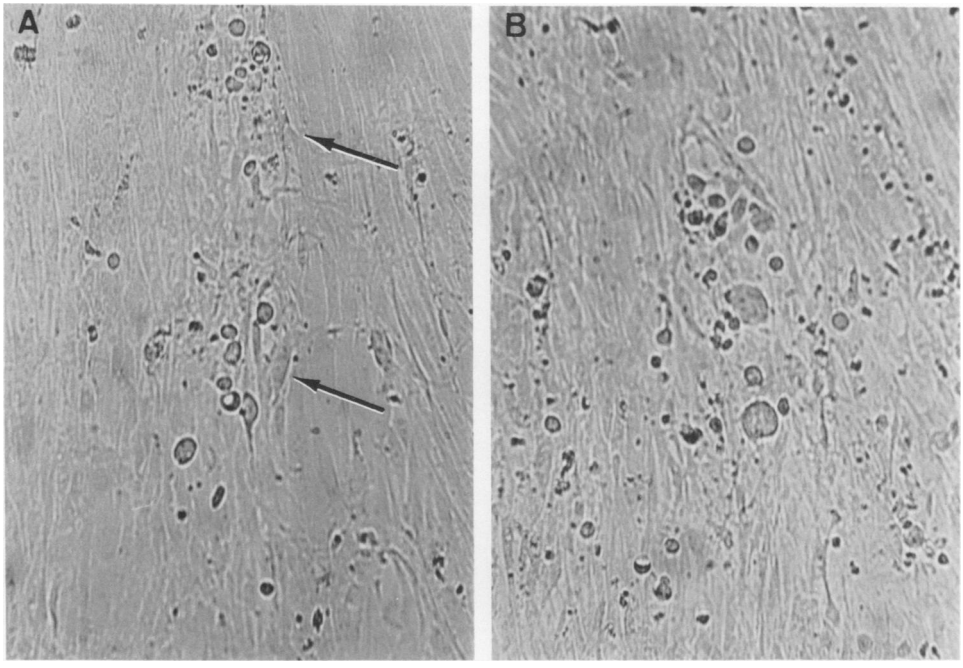

FIG. 1. *GPXV-induced CPE in GPE cells 13 days postinfection. (A) Early focal GPXV CPE (arrows) within an area of normal GPE cells. (B) More diffuse, advanced CPE in GPE cells (×100).*

virus had been isolated. This first isolate of GPXV was obtained from a strain 2 guinea pig with no evidence of active GPCMV or GPHLV infection. Stock virus was passaged exclusively in GPE cell tissue culture and stored at −70°C in 10% dimethyl sulfoxide. GPXV virus stocks were used at passage levels 6 to 10 with infectivity titers ranging from 10^3 to $10^{4.5}$ 50% tissue culture infective doses per 0.1 ml.

Characterization of GPXV. GPXV infectivity for GPE cells was destroyed after treatment with diethyl ether for 1 h. Infectivity titers were not diminished significantly after passage through a Millipore membrane filter with a limiting pore diameter of 300 nm but infectivity was lost after passage through a limiting pore diameter of 50 or 100 nm. GPXV-infected GPE cells did not cause hemadsorption of either rhesus monkey or guinea pig erythrocytes (10). Unlike GPCMV and GPHLV, plaques were not formed in GPE cells overlaid with 0.5% methyl cellulose. Treatment of GPE cells with heparin for 1 h before virus inoculation caused a 1-log drop in GPXV titer at a concentration of 50 U of heparin. Thus, GPXV is less sensitive than GPCMV to the effects of heparin pretreatment (3).

In hematoxylin–eosin-stained preparations of GPXV-infected cells, characteristic Cowdry type A intranuclear inclusions were observed (Fig. 2). Electron microscopy of GPXV-infected GPE cells revealed a morphological development characteristic of the herpesvirus group (Fig. 3). Intranuclear and intracytoplasmic inclusions consisting of aggregates of tubular material were consistently observed within infected cells, often in the region of nucleocapsid formation (Fig. 3B). This appeared to be a regular and consistent aspect of GPXV morphogenesis regardless of the level of passage in GPE cells. Neither intranuclear vacuoles containing enveloped virions nor dense bodies were observed. The former were frequently found in GPHLV-infected GPE cells, and the latter are characteristic of GPCMV-infected cells (7, 15).

Serological differentiation of GPXV, GPCMV, and GPHLV. Serological differences between GPXV, GPCMV, and GPHLV were demonstrated in cross-neutralization tests by inhibition of virus-induced CPE or plaque formation, and by indirect immunoferritin electron microscopy. There was no evidence of serological cross-reactivity between the three viruses by neutralization tests using antisera prepared in several animal species (Table 1). Immunoferritin electron microscopic studies using GPXV-infected cells demonstrated reactivity with homologous (Fig. 4A) but not heterologous antiserum (Fig. 4B). Extensive labeling of virus with ferritin was noted when homologous serum but not heterologous antiserum was used. Normal rabbit serum showed no reaction with the viruses tested. Results were in agreement with those

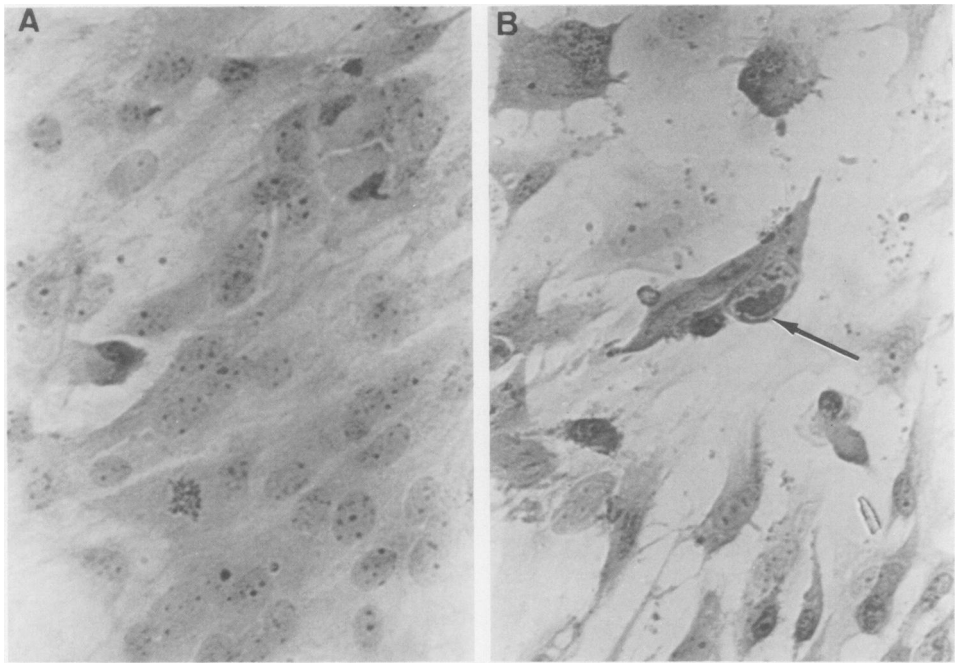

FIG. 2. *Hematoxylin–eosin-stained GPE cells. (A) Uninfected cells. (B) Cells 7 days postinfection with GPXV showing characteristic Cowdry type A intranuclear inclusion (arrow)* (×400).

obtained by cross-neutralization testing. GPXV was not neutralized by antisera to either infectious bovine rhinotracheitis virus or to any of the human herpesviruses and parainfluenza viruses noted above. Human sera, including convalescent sera obtained from 10 patients with known human herpesvirus infections and antibody responses, did not neutralize GPXV.

Sera obtained from 112 weanling and adult Hartley and strain 2 guinea pigs upon arrival, during quarantine, or after rearing in our animal facility at the West Haven VA Medical Center were tested for neutralizing antibodies to GPXV. The prevalence of infection, as demonstrated by the presence of neutralizing antibodies in a reciprocal titer of 2 or greater, was 38%. The range of antibody titers to GPXV in naturally infected guinea pigs was 2 to 64. Prevalence of infection appeared to increase with age of the animals tested.

Serological survey for naturally occurring GPXV antibody in other animal species. Sera obtained commercially and through other investigators acknowledged above were tested for naturally occurring neutralizing antibody to GPXV. Pooled sera obtained from humans, cattle, sheep, goats, horses, swine, cats, dogs, mice, rats, rabbits, rhesus monkeys, African green monkeys, chimpanzees, chickens, ducks, pigeons, and turkeys showed no neutralization of GPXV at dilutions of 1:2 and greater. Neutralizing antibody was only detected in pooled guinea pig serum obtained from the commercial source at a titer of 1:8.

Host range in vivo. Hartley guinea pigs inoculated intraperitoneally with $5 \times 10^{4.5}$ 50% tissue culture infective doses of GPXV demonstrated circulating virus in buffy coat cells 7 to 21 days after virus inoculation. Six weeks after inoculation, viremia decreased, and it usually became undetectable by cocultivation techniques within 12 weeks. Animals showed no apparent illness during the first month of infection as compared to controls inoculated with uninfected tissue culture. However, 6 to 11 weeks after inoculation, 50% of experimentally infected animals died. Specific histopathological findings were limited to focal hepatic necrosis, evident in animals found either dead or moribund. GPXV was isolated from liver, spleen, and/or thymus removed from two of three animals as well as from three of four surviving animals sacrificed 5 to 6 months after virus inoculation, but not from six control animals.

Adult Syrian hamsters inoculated with GPXV showed no ill effects and were sacrificed 6 to 9 months after virus inoculation. Buffy coat, urine, liver, spleen, thymus, lymph nodes, pancreas, salivary glands, kidneys, lungs, marrow, and brain were cocultivated in GPE cell tissue cul-

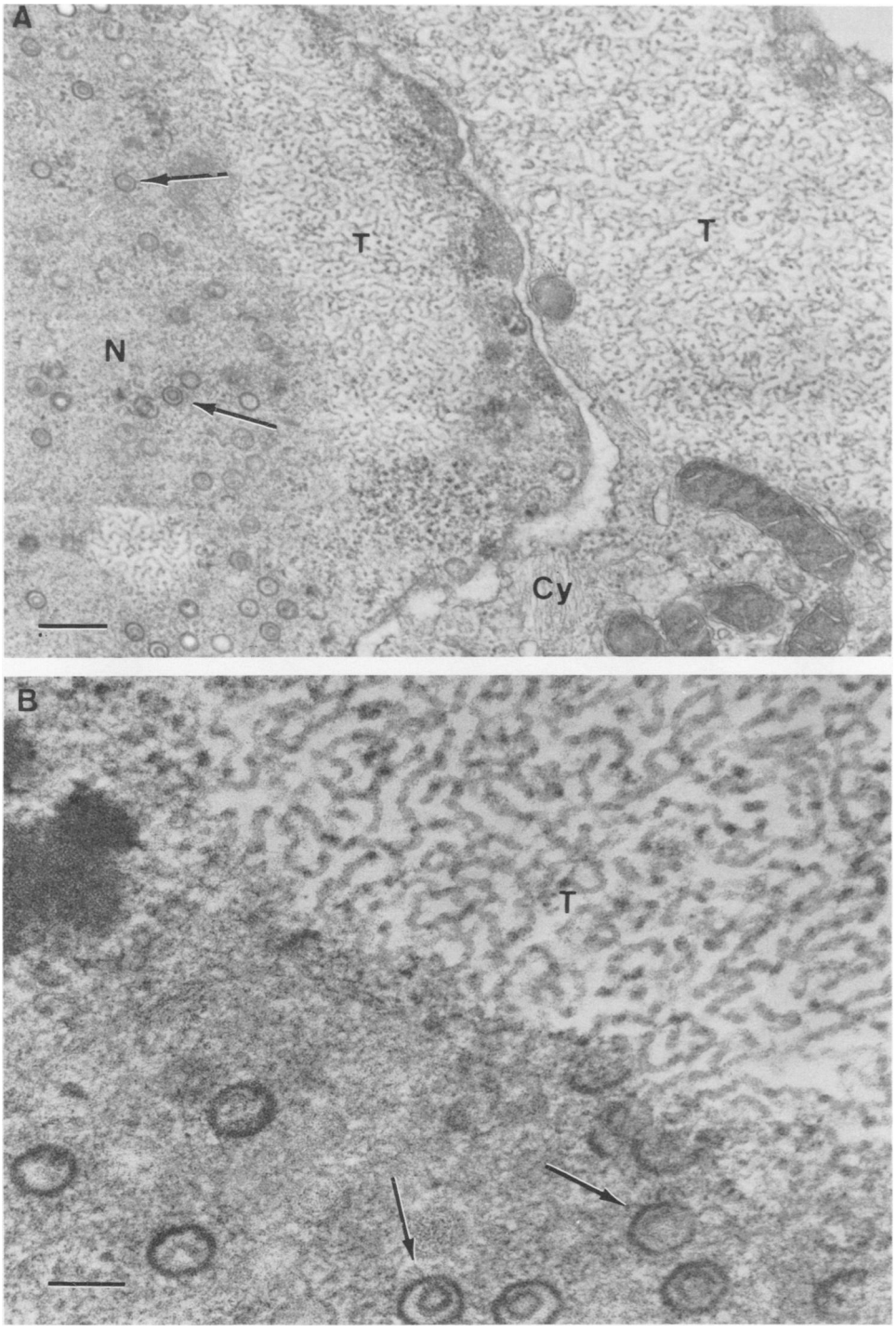

FIG. 3. *Transmission electron micrographs of GPXV-infected GPE cells, 7 days postinfection. (A) Developing intranuclear (N) nucleocapsids (arrows) are evident. Intranuclear and intracytoplasmic (Cy) tubular aggregates (T) are also seen. Bar, 300 nm. (B) GPXV nucleocapsid formation in area adjacent to an intranuclear tubular aggregate (T). Capsid and cores (arrows) are shown in several areas. Bar, 100 nm.*

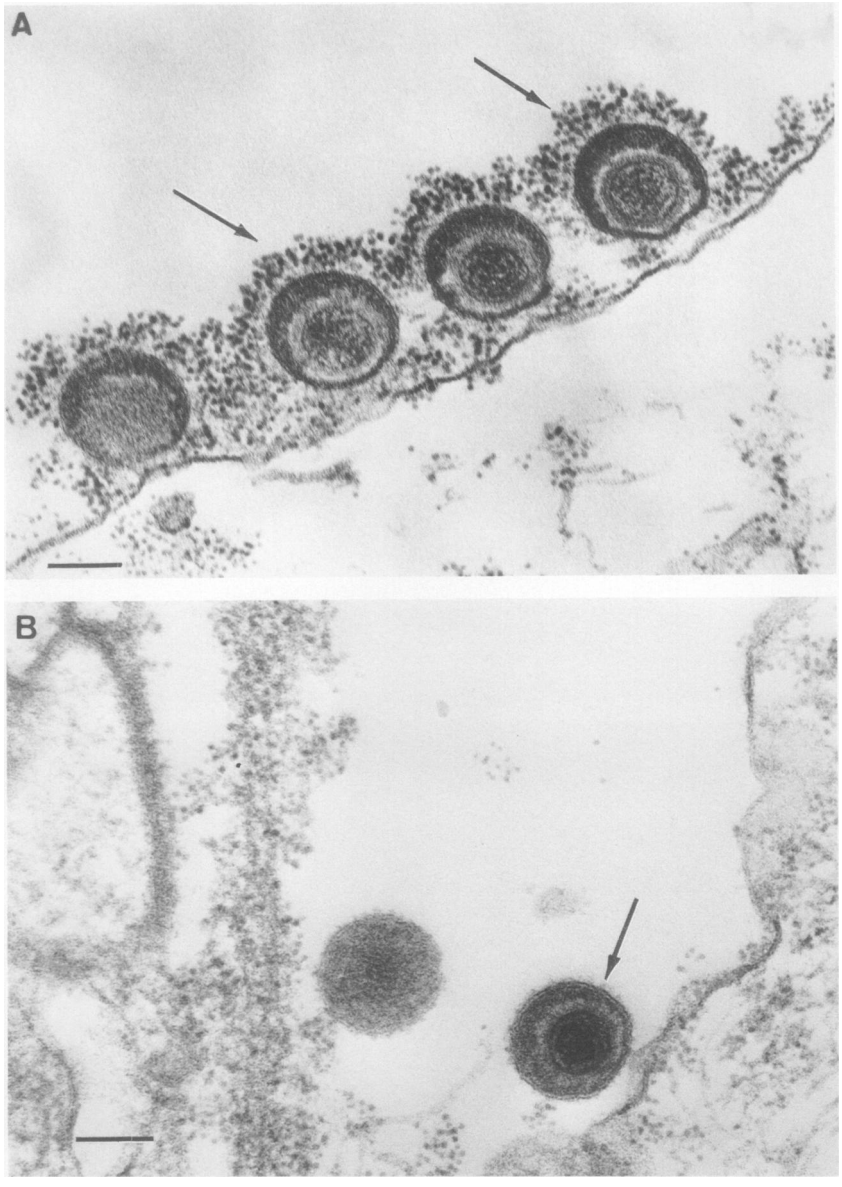

FIG. 4. *Indirect immunoferritin electron microscopy. GPXV-infected GPE cells were overlaid with rabbit antisera to GPXV, GPCMV, or GPHLV, washed, and overlaid with ferritin-labeled anti-rabbit immunoglobulin. (A) Four GPXV particles at cell surface are heavily labeled with ferritin after reaction with homologous (GPXV) antiserum (arrows). (B) GPXV particle (arrow) without ferritin label after reaction with heterologous (GPCMV) antiserum. Bar, 100 nm.*

ture, and no virus isolates were obtained.

Biochemical characterization of GPXV. DNA genomes of GPXV, GPCMV, and GPHLV were compared by CsCl equilibrium buoyant density measurements and restriction endonuclease cleavage analysis. Figure 5 shows the results of one such analysis. The internal density marker in each gradient was [^{14}C]DNA of *E. aerogenes* (1.717 g/cm^3). Although the measurement of buoyant density determinations in preparative CsCl gradients is not as precise as that obtained from analytical ultracentrifugation, these experiments demonstrated very clearly that each of the three viruses isolated from guinea pigs differs significantly from the others. The DNA of GPHLV was more dense than the

TABLE 1. *Serological differentiation of GPXV, GPCMV, and GPHLV by cross-neutralization tests*

Immunizing virus	Animal species immunized	No. of sera tested	Range of neutralizing antibody titers[a] against:		
			GPXV	GPCMV	GPHLV
GPXV	Guinea pig	4	2-16	<2	<2
	Rabbit	4	5-80	<2	<2
	Hamster	6	5-10	<2	<2
GPCMV	Guinea pig	3	<2	80	<2
	Rabbit	3	<2	5-10	<2
GPHLV	Rabbit	5	<2	<2	40-320

[a] Expressed as reciprocal of serum dilutions.

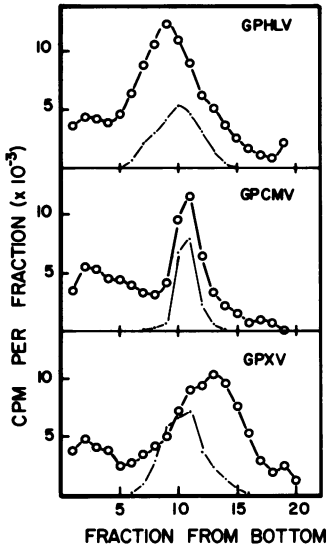

FIG. 5. *Equilibrium density gradient analysis of viral DNA in CsCl. The ^{32}P-labeled DNA used for restriction endonuclease cleavage site analysis shown in Fig. 6 was subjected to ultracentrifugation in CsCl in an SW50.1 rotor at 35,000 rpm at 20°C for 48 h. The tubes were dripped from the bottom, and the radioactivity of the fractions was determined. In each tube a standard of ^{14}C-labeled E. aerogenes DNA as a density marker was included.* (○) *Virus,* (.) *standard.*

internal marker; the DNA of GPCMV was of the same density as the marker; the DNA of GPXV was less dense than the internal marker. The distribution of the viral DNA in the CsCl gradients was the same shape and of the same degree of dispersity as the bacterial marker DNA. Cleavage of viral DNAs with restriction endonucleases *Bgl*II *Bam*HI, and *Eco*RI was performed after labeling of viral DNA with [^{32}P]phosphate and extraction from nucleocapsids. Contact autoradiographs prepared from dried agarose gels containing *Bam*HI and *Bgl*II digests are shown in Fig. 6. In Table 2 the differentiation of GPXV from GPCMV and GPHLV by biological, serological, and biochemical techniques is summarized.

DISCUSSION

An apparently new herpesvirus endogenous to guinea pigs has been isolated and characterized. Table 2 shows the major differences between GPXV and the two well-characterized endogenous herpesviruses of guinea pigs. GPXV was originally isolated from buffy coat cells which were cocultivated with GPE cells in an attempt to detect GPHLV in adult strain 2 guinea pigs. Neutralizing antibodies to GPXV were present in 38% of Hartley and strain 2 guinea pigs obtained from various sources. The virus belongs to the herpesvirus group by criteria of size, morphology, and biochemical characterization. Both cross-neutralization testing and immunoferritin electron microscopy showed GPXV to be sero-

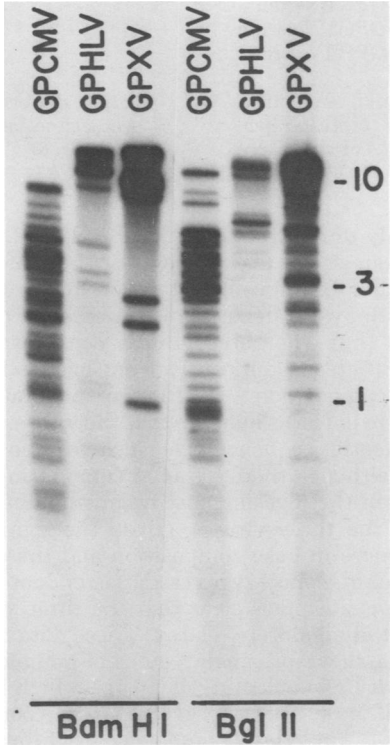

FIG. 6. *Contact autoradiographs of viral DNAs labeled in tissue culture with [^{32}P]phosphate and extracted from nucleocapsids. DNA was digested with the indicated endonuclease, BglII or BamHI. Unlabeled herpes simplex virus type 1 DNA fragments run in parallel tracks were used as size standards (megadaltons).*

TABLE 2. *Summary of GPXV, GPCMV, and GPHLV differentiation by biological, serological, and biochemical techniques*

Character	GPXV	GPCMV	GPHLV
Cytopathic effect in cell culture:			
Guinea pig embryo	+	+	+
Guinea pig kidney	+	−	+
Rabbit kidney	−	−	+
Ultrastructural development:			
Intranuclear and intracytoplasmic tubular aggregates	+	−	−
Dense bodies	−	+	−
Intranuclear vacuoles containing enveloped virions	−	−	+
Seroreactivity by neutralization testing and immunoferritin electron microscopy:			
Anti-GPXV serum	+	−	−
Anti-GPCMV serum	−	+	−
Anti-GPHLV serum	−	−	+
Restriction endonuclease cleavage patterns of viral DNA	Distinct patterns for each virus (see Fig. 6)		

logically unrelated to GPCMV or GPHLV, endogenous guinea pig herpesviruses both of which are under extensive investigation in our laboratory. Buoyant density measurements of the DNAs from the three viruses confirmed that they differed significantly from each other in base composition. Restriction endonuclease cleavage patterns indicate that the viruses differ in nucleotide sequence at most sites in the DNA. Thus, although there are a few comigrating fragments in the digests, most fragments differ between the three viruses. Given the significant differences in base composition and the extensive lack of homology in restriction endonuclease cleavage sites, it is clear that the three viruses are not at all closely related. These data do not exclude, however, small regions of homology or similarity of nucleotide sequence which might result if these viruses all evolved from a common ancestor. The relative homogeneity of the DNA profiles suggests that there was little defective virus DNA of different density in our preparations of labeled samples.

Preliminary investigation of GPXV pathogenesis in Hartley guinea pigs demonstrated a period of viremia beginning as early as 7 days after virus inoculation. Unlike experimental GPCMV infection, in which viremia is no longer readily detectable after 2 weeks (12), viremia was still evident 3 to 6 weeks after GPXV inoculation, generally becoming undetectable 12 weeks after experimental infection. In contrast, both natural and experimental GPHLV infection are characterized by continuous persistent virus infection of circulating leukocytes (14, 22).

Investigators working with herpesvirus infection of guinea pigs should be aware that this animal species can be infected with at least three endogenous guinea pig herpesviruses. Though they are readily distinguishable by standard neutralization tests, failure to recognize their presence could complicate the interpretation of experimental results. During experimental studies of human varicella-zoster virus pathogenesis in guinea pigs, Meyers et al. isolated an unidentifiable herpesvirus from the throat of a guinea pig (J. Infect. Dis., in press). Rabbit anti-GPCMV, anti-GPHLV, and anti-GPXV sera produced in our laboratory were forwarded to Meyers and tested against his herpesvirus isolate. GPXV antiserum, but not GPCMV or GPHLV antiserum, reacted with his isolate by immunofluorescence (personal communication). GPXV appears to have been isolated independently in each laboratory from the same animal species. Also, GPXV-neutralizing antibodies were detected only in pooled commercial guinea pig serum, but not in that of 18 other species whose sera were tested.

As previously reported, the guinea pig placenta is similar to that of humans, permitting the transplacental passage of herpesviruses to the fetus (2, 17, 18). Therefore, GPE cells prepared from fetal tissue are a potential source of contamination with endogenous herpesviruses. This may not be a common event, for in over 600 lots of GPE cell preparations from pregnant Hartley guinea pigs we have only occasionally isolated GPHLV and rarely isolated GPCMV from these cells. As yet, we have not recognized the presence of GPXV in such cell culture preparations.

Given our finding of a new herpesvirus in guinea pigs, we suggest that according to criteria of the International Committee on Taxonomy of Viruses (5), GPXV should be provisionally designated caviid herpesvirus 3.

We consider these findings of interest to virologists concerned with the biology of the herpes group of viruses. They may be of particular importance to those investigators utilizing either guinea pigs as animal models of virus infection, or cells derived from this species as a means for cultivating viruses.

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