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Genetic Analysis of a Cytomegalovirus-Like Agent Isolated from Human Brain

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An unusual cytomegalovirus (CMV, strain Colburn) isolated from brain biopsy of a boy with clinical encephalopathy was studied for genetic relatedness to human and simian CMV. Cross-examination of the purified viral DNA by DNA-DNA reassociation kinetics analyses showed more than 90% homology between Colburn virus and simian CMV (strain GR2757) and a lack of detectable homology between Colburn virus and human CMV (strains AD-169 and TW-87). Restriction endonuclease analysis of Colburn DNA showed some similarity of the DNA fragment pattern with that of simian CMV DNA, although the DNA fragment patterns were not identical, and showed no similarity to that of human CMV DNA. The molecular size and density of viral DNA were close to those of simian CMV DNA. The antigenic study, as performed by complement fixation and neutralization tests, showed strong cross-reactivity of Colburn virus to simian GR2757 virus. One-way cross-reaction of Colburn virus to several human CMV isolates (AD-169, Davis, and Town) was detected by complement fixation; this one-way cross-reaction was not obvious in a plaque neutralization test. It was concluded that Colburn is a simian CMV-related virus.

Cytomegaloviruses (CMV) are a group of viruses within the herpesvirus group (19) with distinctive morphological and biological characteristics. This group of viruses displays some species specificity both in vitro and in vivo. In cultures, the virus grows in a restricted range of host cells of homologus origin except for simian CMV, which will also grow in human fibroblasts, whereas human CMV only grows in fibroblasts of human origin. Although there is a great deal of structural and biological similarity among human CMV, mouse CMV, and simian CMV, there is, nevertheless, a lack of genetic relatedness among these groups as detected by nucleic acid hybridization (8). An unusual, CMV-like agent (Colburn strain) has been isolated from a child with encephalopathy (L. J. Charamella, R. B. Reynolds, R. B. Ch'ien, and C. A. Alford, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V373, p. 256). This virus can replicate in cell cultures of either simian or human origin and can induce a primary and persistent infection in marmosets experimentally (12). The appearance of the cytopathic effect of Colburn-infected human fibroblasts is similar to that of human CMV-infected cells, except that the process of cytopathic change is faster and virus yield is greater than that of any human CMV isolates. These unusual characteristics led us to examine the genetic relatedness of Colburn virus to human and simian CMV by nucleic acid hybridization and restriction endonuclease cleavage analysis. We report here that Colburn virus, which may possess pathological potential in humans, is genetically related to simian CMV.

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MATERIALS AND METHODS

Cells and viruses. The WI-38 strain of human fibroblast (Hayflick, obtained from American Tissue Culture Collection, CCL-75, passage 22-28) was used for the entire experiment. The Colburn virus used in this study was isolated in primary human embryonic kidney cells, at the University of Alabama, from the brain biopsy of a child with encephalopathy (Charmella et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V373, p. 256). The cytopathic effect appeared 3 days after inoculation and reached completion 4 weeks after infection. This strain of virus was subsequently passed on human embryonic kidney cells and then to human embryonic fibroblasts and was never subcultured in any simian-related cell culture. The Colburn virus was sent to the University of North Carolina for genetic analysis at passage 75. Also used in this study were five human cytomegalovirus strains (AD-169, Town, Davis, Esp, and TW-087) and a simian strain (GR2757) (2). The viruses were propagated by infection of WI-38 roller-bottle cultures at a multiplicity of infection of 1 to 2 PFU per cell and were harvested

from the extracellular fluid, as described before (6).

Purification of virus. The extracellular fluid of infected culture was clarified by centrifugation at 6,000 rpm in a Sorvall GSA rotor for 20 min. The cell-free virus particles in the supernatant were pelleted in a Spinco T-19 rotor. The virus was suspended and purified by sedimentation through a 10 to 50% sucrose gradient, followed by brief centrifugation through a preformed cesium chloride gradient (density, 1.16 to 1.375 g/cm^3), as previously described (6).

Preparation of ^{32}P-labeled viral DNA. For restriction enzyme digestion analysis, the viral DNA was labeled with ^{32}P. Twenty-four hours after infection, the medium of the infected-cell cultures was replaced with low-phosphate minimal essential medium (containing 10^{-5} M phosphate) supplemented with 5% dialyzed phosphate-free fetal calf serum and 30 μCi of carrier-free $^{32}P_i$ (New England Nuclear Corp.) per ml. The ^{32}P-labeled viral DNA was purified as described above except that the CsCl isopycnic purification step was reduced from two to one cycle.

Purification of enzymes. Restriction endonuclease *Hin*dIII was purified from *Haemophilus influenzae* by the method of Smith and Wilcox (16). Restriction endonuclease *Eco*RI was purified from *Escherichia coli* Ry-13 by the method of Greene et al. (4).

Kornberg DNA-dependent DNA polymerase was purified from *E. coli* B by the method of Jovin et al. (10). Sephadex G-100 gel filtration, rather than hydroxyapatite chromatography, was used to remove exonuclease III.

S1 enzyme (*Aspergillus oryzae*) was purified from Enzopharm (Enzyme Development Corp., Keyport, N.J.) by the method of Vogt (17).

Analysis of viral DNA with restriction enzymes. Purified ^{32}P-labeled viral DNA (20 μl, 10×10^3 cpm) in TBS (0.05 M Tris-hydrochloride, pH 7.4–0.15 M NaCl) with 0.01 M $MgCl_2$ and 0.006 M β-mercaptoethanol was digested with 5 μl of *Hin*dIII or *Eco*RI at 37°C for 20 h. After digestion, the samples were subjected to 1% agarose slab-gel electrophoresis in buffer E as described before (11). The gel was then vacuum-dried onto a Whatman filter paper. X-ray film (Kodak RP/R2) was used for autoradiography to trace the migration of DNA fragments in the agarose gel.

Radioisotopic labeling of viral DNA in vitro and DNA-DNA reassociation kinetic analyses. To obtain high-specific-activity viral DNA for DNA-DNA reassociation kinetic analyses, the viral DNA was labeled in vitro by Kornberg enzyme repair synthesis as described before (6, 8). The specific activities for AD-169 DNA and Colburn DNA were 1.7×10^6 and 2.6×10^6 cpm/μg, respectively. Ultrasonically fragmented DNA (0.02 μl, 3.4×10^4 to 5.2×10^4 cpm), cold purified viral DNA (3 μg), and calf thymus DNA (20 μg) were mixed and denatured at 103°C for 15 min in 0.8 ml of 0.01 M Tris-hydrochloride (pH 7.4)–0.0025 M EDTA. After rapid chilling in ice, the salt concentration of the reaction mixture was adjusted to 0.9 M NaCl. The sample was then divided and sealed in 100-μl pipettes. The hybridization was carried out in a 66°C water bath. After intervals of hybridization, the samples were tested for the kinetics of reassociation by single-strand-specific S1 enzyme differential digestion as previously described (8).

Preparation of hyperimmune serum and complement fixation test. Preparation of CMV hyperimmune sera with purified viruses, preparation of CF antigens, and the method employed for complement fixation were as described before (9). The plaque reduction neutralization test followed the method of Benyesh-Melnick (1), except that 0.5% agarose was used and the $NaHCO_3$ concentration was maintained at 0.22%.

RESULTS

Nucleic acid homology: (i) Colburn and human CMV. DNA-DNA reassociation kinetics analysis of tritiated human CMV (AD-169) DNA with the unlabeled DNA of Colburn and various human CMV isolates showed no obvious relatedness, i.e., less than 5% detectable homology between Colburn and human CMV (Fig. 1). The presence of 3 μg of unlabeled Colburn DNA did not accelerate the reassociation of tritiated AD-169 DNA. The addition of unlabeled DNAs from various human CMV strains (e.g., Davis, Esp, and TW-087) to the reaction did, however, accelerate the reassociation of the ^{3}H-labeled AD-169 DNA probe. This result indicates that Davis, Esp, and TW-087 share at least 80% homology with AD-169 DNA, whereas Colburn does not have detectable DNA homology with AD-169 human CMV. It is worth mentioning that the kind of testing system used in this experiment cannot detect less than 5% homology.

(ii) Colburn and simian CMV. Figure 2 shows the results of the reassociation kinetics analyses in which labeled simian CMV (strain GR2757) DNA was used in comparison with unlabeled Colburn or human CMV (strain TW-087) DNA; unlabeled simian GR2757 CMV DNA was also used for the homologous 100% control. The Colburn DNA did accelerate the reassociation of ^{3}H-labeled simian DMV DNA probe with kinetics that indicate more than 90% homology between these two viruses. As shown before (8), there is no evidence of detectable homology between simian CMV GR2757 DNA and human CMV TW-087 (a strain isolated from the human cervix) DNA.

Velocity sedimentation and density analysis of Colburn viral DNA. The velocity sedimentation analysis of viral DNA was carried out by release of the DNA from purified virus with sodium dodecyl sulfate and Pronase as described previously (6). In sucrose gradient centrifugation (Fig. 3A), the Colburn DNA cosedimented with simian GR2757 DNA, with a sedimentation value of about 56S (6). Cesium chloride centrifugation to equilibrium revealed that Colburn virus DNA possessed the same density as that of simian GR2757 CMV DNA, (1.710 g/cm^3; 6).

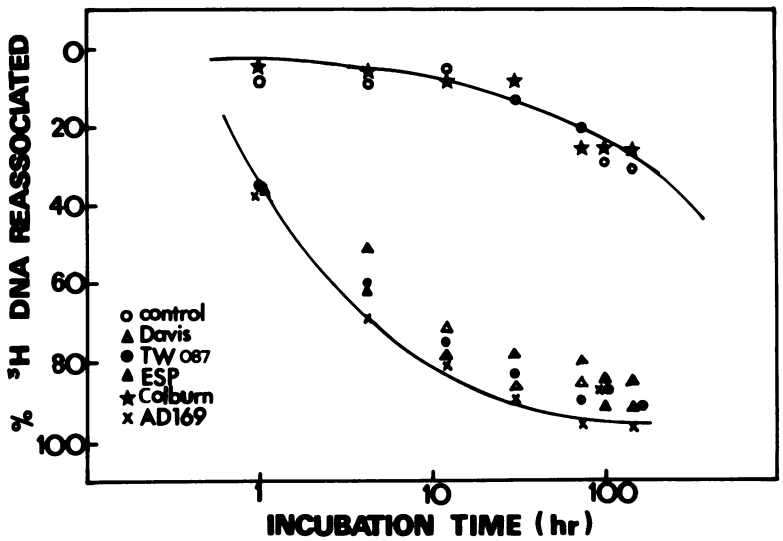

FIG. 1. *Reassociation kinetics analysis of tritiated AD-169 DNA with the cold DNA of Colburn and several human CMV strains. Sonically disrupted ^{3}H-labeled AD-169 DNA (0.02 μg, 3.4 × 10^4 cpm), calf thymus DNA (20 μg), and unlabeled viral DNA (3 μg) were mixed and denatured. The experiment was carried out as described in the text.*

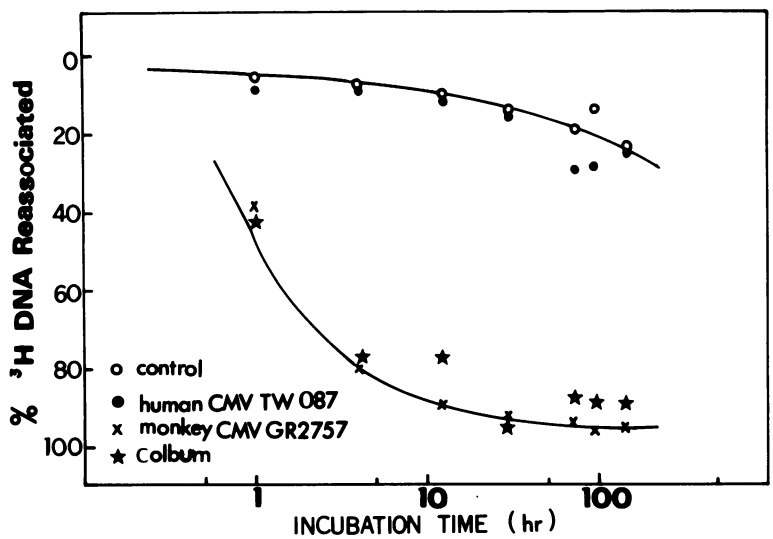

FIG. 2. *Reassociation kinetics analysis of tritiated Colburn DNA with the cold DNA of human CMV TW-087 and monkey CMV GR2757. The experiment was carried out as described in the text.*

Restriction enzyme analysis of viral DNA. Genetic relatedness of Colburn virus has also been approached by restriction endonuclease analysis of viral DNA. Two kinds of restriction endonucleases, *Eco*RI and *Hin*dIII, which recognize and cleave the specific base sequence (6, 15), were used for this study. Figure 4 shows the restriction enzyme fragment patterns of Colburn, simian CMV, and human CMV DNA. Coelectrophoresis of *Eco*RI or *Hin*dIII digests showed considerable matching of comigrating fragments between Colburn and simian GR2757 (gels 2 and 3 for *Eco*RI; gels 4 and 5 for *Hin*dIII), although they were not completely identical. Rare matching of comigrating fragments was found between Colburn and human Town CMV strains (see gels 2 and 8 for *Eco*RI and gels 5 and 6 for *Hin*dIII). The degree of similarity in the fragment patterns should somewhat reflect the degree of homology in DNA sequence be-

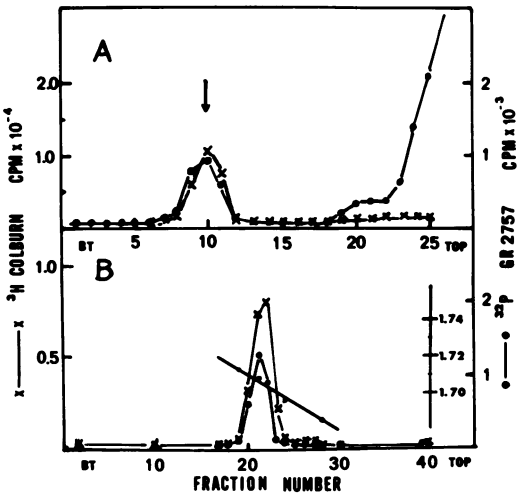

FIG. 3. *Velocity sedimentation and isopycnic centrifugation of Colburn and simian CMV DNA. (A) [³H]thymidine-labeled Colburn and ³²P-labeled GR-2757 viruses were released from virions by treatment with Pronase (2 mg/ml) and sodium dodecyl sulfate (1%) in TBS with 0.005 M EDTA. The DNA was then centrifuged through a 10 to 30% sucrose gradient in an SW41 rotor at 36,000 rpm for 3 h at 20°C. Arrow indicates the location of both Colburn and simian GR2757 DNA. (B) The viral DNA obtained from sucrose gradient was phenol extracted once and subjected to CsCl isopycnic centrifugation. Fractions were collected from the bottom of the tubes.*

tween two virus strains. A detailed comparison of DNA fragment patterns with several other human CMV isolates was published previously (7, 11).

Antigenic cross-reactivity. Guinea pig hyperimmune sera prepared against purified virions were used for antigentic cross-reaction study. As examined by complement fixation tests (Table 1), antihuman CMV sera (strains AD-169, Davis, and Town) did not react with simian GR2757 antigen but did react with Colburn antigen to a titer of 1:80 to 1:320. When simian GR2757 antiserum was used to test against Colburn antigen, a titer of 1:640, equal to that against its own antigen, was obtained. Antiserum of Colburn strain reacted with its own antigen and GR2757 to titers of 1:1,280 and 1:320, respectively, but did not react with human AD-169, Davis, and Town strain antigens. An unexplained one-way cross-reaction was obtained from the testing of Colburn antigen against antisera of AD-169, Davis, and Town human CMV isolates; titers of 1:80 to 1:320 were obtained. This kind of cross-reactivity was not obtained when anti-Colburn serum was tested against AD-169, Davis, and Town antigens. All sera tested failed to react with control WI-38 cell antigen when a 1:10 dilution of serum was used.

The Colburn antiserum did not neutralize

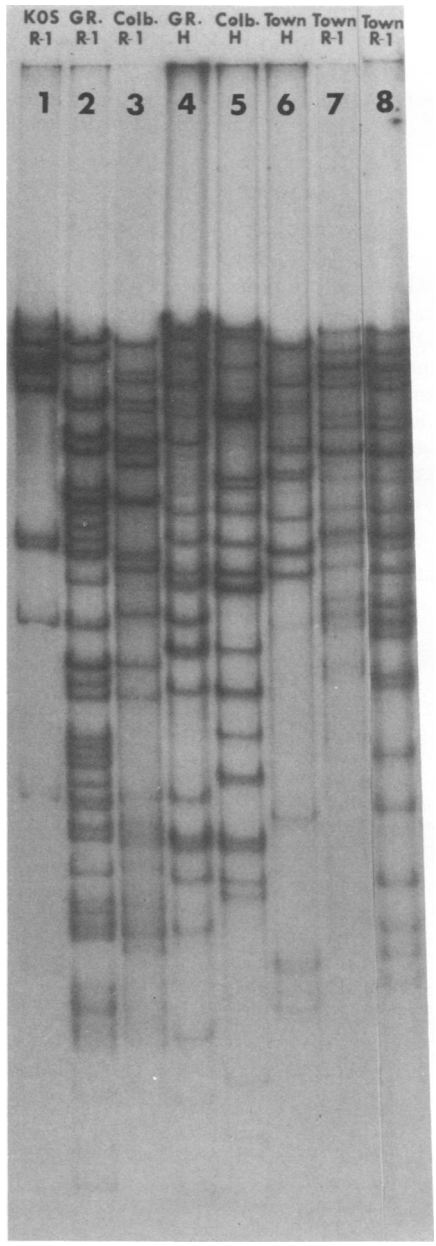

FIG. 4. *Comparison and analysis of Colburn, simian, and human CMV by cleavage of their DNA with restriction endonucleases EcoRI and HindIII. CMV DNA labeled with ³²P was purified, digested with either enzyme at 37°C for 20 h, and subjected to electrophoreses on 1% agarose. KOS, Strain of herpes simplex virus type 1; GR., simian CMV strain GR-2757; Colb., Colburn; R-1, EcoRI digested; H, HindIII digested.*

either AD-169 or Town strains of human CMV but did neutralize simian GR2757 to a titer of 1:320; it neutralized its own virus to the titer of 1:1,280 (Table 2). Conversely, antisera against human CMV did not show neutralization ability greater than 1:10 to Colburn and simian GR-2757 viruses. The kind of one-way cross-reactivity detected by the complement fixation test as mentioned above was not found in the plaque-reduction neutralization.

DISCUSSION

Genetic relatedness among DNA viruses can easily be resolved by nucleic acid hybridization and DNA restriction endonuclease analysis; commonly shared gene sequences or molecular arrangements can be detected. Although these techniques are powerful tools for genetic analysis, certain limitations do exist. Due to the specific activity of the radioactive DNA probe and the background radioactivity, genetic homology of less than 5% between both genomes is somewhat difficult to detect. With a genome size of 10^8 daltons, for example, 4% of a viral genome is enough to code for 10 to 15 proteins of average molecular size. The phenomenon of the one-way cross-reaction in complement fixation tests which contrasts with lack of detectable DNA homology and cross-neutralization activity between Colburn and human CMV might be explained by genome homology existing below the detectable level.

The close relation of Colburn virus to simian CMV was demonstrated by nucleic acid sequence homology, similarity in the size and density of viral DNA, similarity in viral antigenic structure, and ability of both viruses to grow in cells of either simian or human origin. As with Colburn, similar simian virus-related human infections have been described for the papovavirus system; several simian virus 40-related papovaviruses have also been isolated from human patients with progressive multifocal leukoencephalopathy (PML-DAR and JC viruses; 13, 15, 18) and from the urine of renal transplant patients (BK virus; 3). Remarkable nucleic acid

TABLE 1. *Comparative cross-reaction of Colburn, human, and simian CMV in complement fixation tests with hyperimmune sera*[a]

Antiserum	CF titer					
	Control WI-38[b, c]	Colburn[c]	GR2757[c]	AD-169[c]	Davis[c]	Town[c]
Guinea pig anti-Colburn	<1:10	1:1,280	1:320	<1:10	<1:10	<1:10
Guinea pig anti-GR2757	<1:10	1:640	1:640	<1:10	<1:10	<1:10
Guinea pig anti-AD-169	<1:10	1:320	<1:10	1:1,280	1:1,280	1:2,560
Guinea pig anti-Davis	<1:10	1:80	<1:10	1:640	1:1,280	1:640
Guinea pig anti-Town	<1:10	1:80	<1:10	1:640	1:1,280	1:2,560

[a] Two units of hemolysin, 2 U of complement, 2 U of antigen, and 2% sheep erythrocytes were used. CMV-infected WI-38 cells were washed with and suspended in Veronal-buffered saline (pH 7.8) to a concentration of 10^7 cells per ml. The suspension was sonically treated with a Branson Sonifier (model 200). Cell debris was removed by centrifugation at 8,000 rpm for 20 min; the supernatant, with a protein concentration of 0.4 mg/ml, was used as the complement fixation antigen.

[b] Uninfected WI-38 cell extract.

[c] Complement fixation antigen.

TABLE 2. *Comparative cross-reaction of Colburn, human, and simian CMV in plaque reduction neutralization tests*[a]

Antiserum	Neutralization titer			
	Colburn[b]	GR2757[b]	AD-169[b]	Town[b]
Guinea pig anti-Colburn	1:1,280[c]	1:320	<1:10	<1:10
Guinea pig anti-GR2757	1:320	1:640	<1:10	<1:10
Guinea pig anti-AD-169	<1:10	<1:10	1:640	1:320
Guinea pig anti-Town	<1:10	<1:10	1:320	1:1,280

[a] About 200 PFU of cell-free virus in 0.1 ml of minimal essential medium solution and 0.1 ml of various diluted sera were used for each reaction. Neutralization and absorption were both carried out at 37°C for 60 min.

[b] Virus.

[c] The titer was based on the reading obtained 3 weeks after infection. The end point was designated as 50% plaque reduction.

homology and high levels of T-antigen cross-reactivity have been demonstrated among these (13, 15). In addition, herpes B (herpes simiae) has been shown to be a simian virus that is lethal to humans.

The extent of involvement of Colburn and other simian or simian-related CMV in human disease is still unclear. But, so far, data obtained from Colburn alone pose the issue of the importance of simian-related CMV in public health and certainly merit a closer look at this group of viruses, both as potential human pathogens and as means of further understanding the infectious processes of other naturally occurring CMV.

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