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Cytomegalovirus Isolation from a Chimpanzee with Acute Demyelinating Disease After Inoculation of Multiple Sclerosis Brain Cells

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A strain of cytomegalovirus (CMV) was isolated during the third subcultivation of explants from the left frontal lobe of a chimpanzee that developed paralysis more than 3 years after intracerebral inoculation at birth with brain cell cultures derived from a patient with multiple sclerosis. Another strain of CMV was also isolated from a lymph node culture taken from the same chimp. The isolates, designated MZM-13 and MZM-14, produced a cytopathic effect characteristic for CMV when inoculated into brain, ganglion, or fibroblast cultures of human or simian origin. Infected cells contained characteristic Cowdry A intranuclear as well as intracytoplasmic inclusion bodies, and 100-nm spherical herpes-like virus particles were detected by electron microscopy in the nucleus and cytoplasm of infected cells. Virus was further identified as CMV with convalescent human anti-CMV serum. Complement-fixing antibody to CMV was present at a titer of 1:32 when the acutely ill chimpanzee was sacrificed. No antibody was detected at birth or at 1 or 2 years of age. A newborn chimpanzee inoculated intracerebrally with MZM-13 developed clinically asymptomatic lesions in the central nervous system characterized by acute and chronic inflammation and degeneration of myelin in cranial and spinal nerve roots. Restriction endonuclease analysis of viral deoxyribonucleic acid isolated from these two viruses indicated that MZM-13 and MZM-14 are identical and are closely related to chimpanzee CMV. No similarity in restriction endonuclease fragment patterns was found between MZM virus and the Towne and Clegg strains of human CMV.

An acute demyelinating disease developed in a chimpanzee more than three years after intracerebral inoculation, at birth, of brain cell cultures derived from a patient with multiple sclerosis (12). The central nervous system (CNS) lesions in the chimpanzee were primarily localized in the spinal cord at root entry zones and were characterized by demyelination and regeneration of myelin by Schwann cells. Virological studies performed on the CNS and other tissues obtained at necropsy of the chimpanzee are detailed in this paper.

MATERIALS AND METHODS

The acutely ill, paralyzed chimpanzee was sacrificed by exsanguination from the femoral artery. Heparinized blood was removed and stored at 4°C for immediate virological studies. Serum was stored at −20°C for subsequent serological analysis. Cerebrospinal fluid was removed before death and stored at −70°C for virological and serological studies.

Autopsy material. One lymph node and multiple areas of brain and spinal cord were aseptically removed. Portions were immersed in 10% neutral-buffered Formalin and 2% glutaraldehyde for histological and electron microscopic observation, respectively. Tissue was explanted and subcultivated as previously described (4, 18) and also cocultivated with African green monkey kidney (CV_1) cells and two lines of human fibroblast cells (WI-38 and HeLu 2000). The remaining CNS tissue was immediately frozen at −90°C.

Virus isolation attempts. Chimpanzee brain cells and cocultivations of chimpanzee brain and indicator cell cultures were observed daily for a cytopathic effect (CPE) and were also tested for the ability of cells to hemadsorb guinea pig and monkey erythrocytes at 4°C (9).

Indicator cells (mentioned above) in T25 plastic flasks (Costar, Cambridge, Mass.) were inoculated with 0.5 ml of chimpanzee cerebrospinal fluid or 0.5 ml of a 1:5 dilution of fresh heparinized blood. After 1 h of adsorption at 37°C, the cultures were refed with Eagle minimum essential medium supplemented with 2% fetal bovine serum and maintained at 37°C while being monitored for CPE. In addition, 1.5×10^6 chimpanzee brain cells in the second subcultivation and 6×10^6 brain cells in the seventh subcultivation were released with trypsin and in the presence of lysolecithin, fused with equivalent numbers of CV_1 and HeLu cells (5), and observed for CPE.

When CPE developed, the cells were scraped into the tissue culture medium from the same flask, and 1.0 ml was transferred to a T25 flask of normal chippanzee or human brain cell cultures. In some experiments, scraped cells were subjected to three cycles of freezing and thawing or sonication before inoculation. After 1 h of adsorption at both 33 and 37°C, cultures were refed with Eagle minimum essential medium with fetal bovine serum, incubated at 33 and 37°C, and monitored for CPE. When CPE developed, the cells and surrounding medium were harvested and stored at −190°C (stock virus).

From pooled fragments of fresh chimpanzee CNS tissue, 10% homogenates were prepared in Eagle minimum essential medium, and 0.02 ml of a clarified suspension was immediately inoculated into one litter each of newborn ICR mice and BALB/c mice, into four young adult nude mice, and into one litter of newborn Syrian hamsters. Animals were observed for 6 to 8 weeks. In addition, between postinoculation days 21 and 26, two to three asymptomatic mice were sacrificed (5), and 0.02 ml of a clarified suspension of a 10% brain homogenate was inoculated into newborn ICR and BALB/c mice (blind passage). After 6 to 8 weeks, animals were sacrificed and perfused with phosphate-buffered saline, followed by 10% neutral-buffered Formalin, and the brains were fixed for histopathological examination (5). Inoculated hamsters were observed for 6 months and similarly examined.

Four 9-day-old embryonated hen's eggs (Spafas, Spiglerville, Pa.) were inoculated in the amniotic cavity with 0.2 ml of homogenate from chimpanzee brain. Inoculated eggs were incubated at 35.5°C for 5 days and then placed at 4°C overnight. The next day, the allantoic and amniotic fluids were harvested and tested for hemagglutination with chicken erythrocytes at 4°C, as previously described (9).

Virus identification. Normal chimpanzee and human brain cells were grown on glass cover slips in 60-mm plastic petri dishes and incubated with stock virus. When CPE developed, the cultures were fixed in Bouin solution and stained with hematoxylin and eosin for histopathological examination (11). Other cultures with CPE were fixed in cold acetone and stained by indirect immunofluorescence (6) with a 1:10 dilution of rabbit anti-herpes simplex virus types 1 and 2 (neutralizing antibody titer 1:80), convalescent human cytomegalovirus (CMV) and varicella-zoster virus sera (immunofluorescence titer 1:64), and a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G or fluorescein isothiocynate-conjugated rabbit anti-human immunoglobulin G (Cappel Laboratories, Downington, Pa.). Uninfected cells were stained with the same sera.

Electron microscopy. Monolayer cultures of normal human brain cells in 30-mm plastic petri dishes were infected with the isolates as described above. When CPE developed, cultures were fixed in 3% glutaraldehyde and 1% OsO_4 in PBS. After fixation, cells were stained with uranyl acetate, gradually dehydrated with ethanol, and embedded in a thin layer of Epon 812. Thin sections of cells were then stained with lead citrate (16) and examined with a Hitachi HS-8 electron microscope.

Host cell range for virus isolate. A 0.5-ml volume of an undiluted suspension of stock isolates or the Towne strain of human CMV was inoculated onto monolayer cultures of normal human brain cells, normal human trigeminal ganglion cells, human fibroblasts (HeLu 2000), normal chimpanzee and trigeminal ganglion cells, and both primary African green monkey testicles and fetal lung cells (DBS-FCL-1) (Flow Laboratories, Rockville, Md.). In addition, human CMV was titrated on both human and simian CNS and fibroblast cells and on normal chimpanzee CNS and fibroblast cells. All cell lines were incubated at both 33 and 37°C and observed for CPE.

Serum removed from the chimpanzee at birth, before inoculation with multiple sclerosis brain cells, and at various intervals afterwards, was tested for complement-fixing antibody to CMV (15). (CMV [AD169] and control complement-fixing antigen were obtained from Flow Laboratories.)

Transmission studies. One newborn chimpanzee (designated 4X78) was inoculated into the right frontal lobe with 0.5 ml of stock isolate designated MZM-13 virus. Although the animal was neurologically asymptomatic, it developed an upper respiratory infection after 4 months. The chimpanzee was treated immediately but was found dead in its cage 3 days later. Tissue was removed for pathological and virological studies.

Restriction endonuclease analysis of viral DNA. Two strains of chimpanzee CMV (isolated from throat swabs from 2 of 21 asymptomatic chimpanzees in captivity at the Southwest Research Foundation in San Antonio, Texas, using fetal chimpanzee lung cells that had been maintained in vitro for 24 subcultivations before inoculation), the Towne and Clegg strains of human CMV (8), and the MZM-13 and MZM-14 isolates were propagated in WI-38 cells in a Wheaton roller bottle. Viral deoxyribonucleic acid (DNA) labeled with ^{32}P was purified from extracellular culture fluid as described previously (8). Four kinds of restriction endonuclease, *Eco*RI, *Hin*dIII, *Bam*I, and *Xba*I, which recognize and cleave the specific base sequence (7, 13, 19), were used for this study. Purified ^{32}P-labeled viral DNA (20 μl, 2×10 to 2.5×10^4 cpm) in Tris-buffered saline [0.05 M tris(hydroxymethyl)-aminomethane-hydrochloride, pH 7.4, and 0.15 M NaCl] with 0.01 M $MgCl_2$ and 0.006 M β-mercaptoethanol was digested with 5 μl of enzyme for 20 h at 37°C. After digestion, the samples were subjected to electrophoresis in a 1% agarose gel (8). The gel was then vacuum-dried onto Whatman filter paper. X-ray film (Kodax Pr/R2) was used for autoradiography to trace the migration of the DNA fragment in the agarose gel.

RESULTS

Tissue from one lymph node and 17 areas of the CNS from the paralyzed chimpanzee were explanted and maintained in vitro. CPE developed after the third subcultivation of both the lymph node (MZM-14) cells and the cells derived from the left frontal lobe (MZM-13). CPE was characterized by the gradual development of foci of enlarged dark cells. These foci spread slowly while new ones developed. Transfer of infection to normal human or normal chimp brain cell cultures was successful only with scraped cells, not with medium. Continued passage of the isolated virus(es) resulted in a reduction of the incubation period to 5 days. No CPE developed in cultures derived from other areas of the brain or spinal cord even after 10 to 14 subcultivations. All animals inoculated with homogenate of fresh chimpanzee CNS tissue remained asymptomatic, and no CNS lesions were found by histopathological examination. No virus-specific changes developed in embryonated hen's eggs inoculated with any chimpanzee tissue. No CPE developed in culture inoculated with chimpanzee blood and CSF.

Identification of the isolate. Infection of human brain cell cultures with both the MZM-13 and MZM-14 isolates produced typical Cowdry A intranuclear inclusions (Fig. 1) as well as perinuclear cytoplasmic inclusions. Ultrastructural examination of infected cells revealed both naked and enveloped 100-nm spherical particles in the nucleus and cytoplasm (Fig. 2), further identified as CMV by indirect immunofluorescence with human CMV convalescent serum (Fig. 3). No virus-specific immunofluorescence was detected when anti-varicella-zoster virus sera or rabbit hyperimmune sera to herpes simplex virus type 1 or 2 was applied to infected cells or when human CMV convalescent serum was applied to uninfected brain cells.

The MZM-13 and MZM-14 viruses replicated in human fibroblasts and in ganglion and brain cells (Table 1); they also produced CPE in simian ganglion and brain cells, but not in simian fibroblasts. The Towne strain of human CMV replicated in human fibroblasts and ganglion cells and produced CPE in human brain cells; it also replicated in simian brain and ganglion cells and produced CPE in simian fibroblasts. The MZM-13 and MZM-14 isolates initially replicated better at 33°C ($10^{3.8}$ and $10^{2.8}$ 50% tissue culture infective doses per ml, respectively) than at 36 to 37°C ($10^{1.3}$ 50% tissue culture infective doses per ml each) in human brain cells. How-

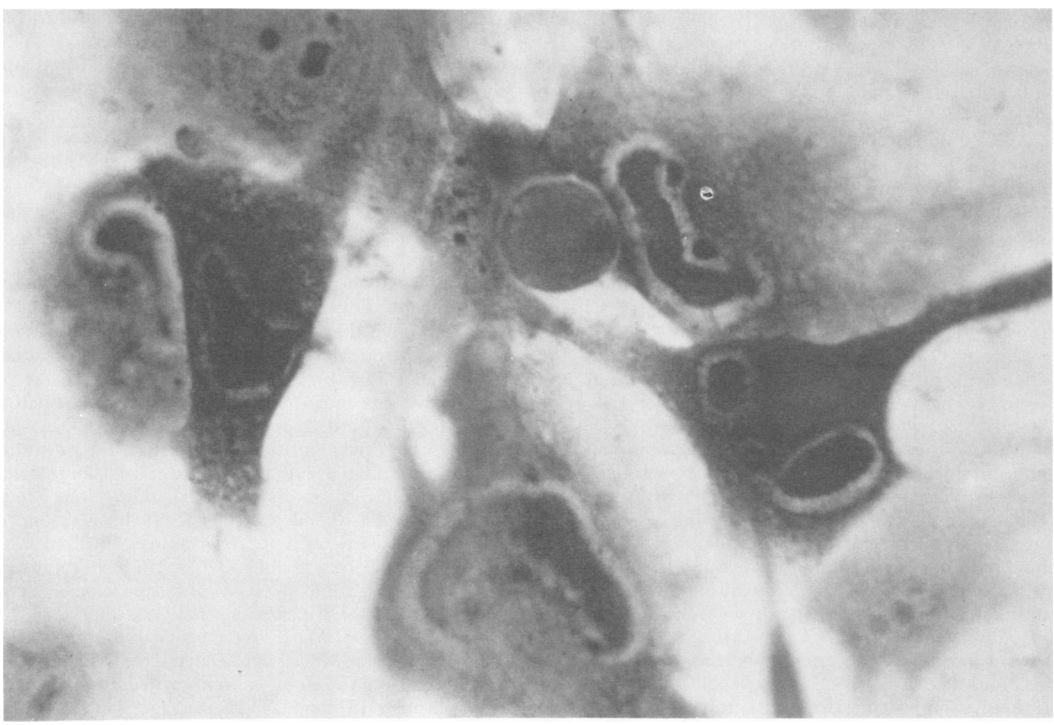

FIG. 1. *MZM-13-infected chimpanzee brain cell culture; Cowdry A and intracytoplasmic inclusion bodies present. Hematoxylin-eosin. ×225.*

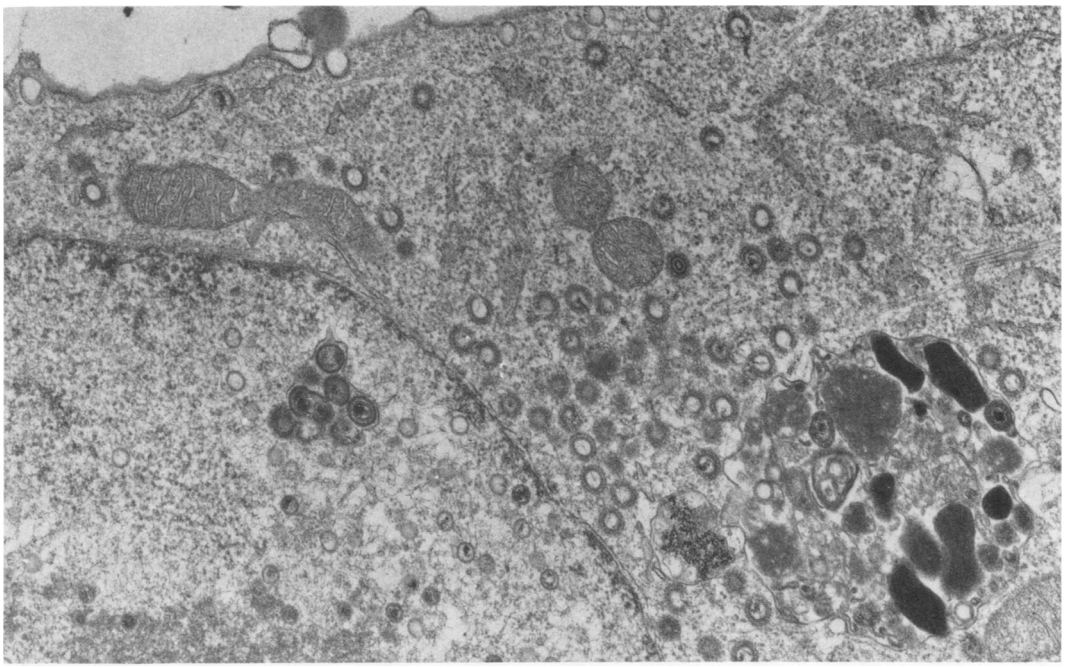

FIG. 2. *MZM-13-infected chimpanzee brain cell culture. Naked and enveloped virus particles seen in nucleus and cytoplasm. ×60,000.*

ever, after serial subcultivation of the MZM isolates in human fibroblasts at 36°C, virus replication increased (data not shown).

Anti-CMV complement-fixing antibody was present at a titer of 1:32 in serum obtained at necropsy. No antibody was detected in chimpanzee sera obtained at birth, before inoculation of multiple sclerosis brain cells, or at 1 or 2 years of age.

Necropsy of chimpanzee 4X78, which was used for transmission studies, revealed an acute bacterial pneumonia. CNS lesions were abundant and consisted of chronic meningitis and multiple glial nodules widely distributed in the brain stem, cortex, white matter, optic nerves (Fig. 4A), and chiasm. Infiltration of a spinal nerve root and the trigeminal nerve roots was associated with degeneration of myelin and some fragmentation of axis cylinders (Fig. 4B). The trigeminal ganglion contained minimal round-cell infiltration. No inclusion bodies were seen. A Gram stain for bacteria was negative. Indirect immunofluorescence of brain tissue did not reveal CMV, and no CPE developed after inoculation of a 10% brain tissue homogenate onto human and chimpanzee fibroblast cells.

Genetic relatedness of the two MZM isolates was studied by restriction enzyme fragmentation patterns of the MZM-13 and MZM-14 isolates with the Towne strain of human CMV. Identical fragment patterns were found in MZM-13 and MZM-14 (Fig. 5A). This result indicates that the viruses isolated from the chimpanzee lymph node and brain are identical.

Comigrating fragment patterns also indicated considerable homology between MZM-13 and both strains of chimpanzee CMV (Fig. 5B). Comigrating fragments rarely matched between MZM-13 and the two strains of human CMV (Fig. 5B).

DISCUSSION

A strain of CMV was isolated during the third subcultivation of explants from the left frontal lobe of a chimpanzee that had developed paralysis more than 3 years after intracerebral inoculation at birth with multiple sclerosis brain cell cultures. Another strain of CMV was also isolated from a lymph node tissue culture derived from the same animal. The isolates, designated MZM-13 and MZM-14, produced a CPE characteristic for CMV when inoculated into brain, ganglion, or fibroblast cultures of human or simian origin. Infected cells contained characteristic Cowdry A intranuclear as well as intracytoplasmic inclusion bodies, and 100-nm spherical herpes-like virus particles were detected by electron microscopy in the nucleus and cytoplasm of infected cells. Virus was further identified as CMV with convalescent human anti-CMV se-

FIG. 3. *MZM-13-infected chimpanzee brain cell culture. Indirect immunofluorescence with CMV human convalescent serum. ×225.*

TABLE 1. *Sensitivity of human and simian cell cultures to the MZM-13 and MZM-14 chimpanzee virus isolates and the Towne strain of human CMV*[a]

Virus	Human cells				Simian cells			
	HeLu 2000	Foreskin fibroblasts	Normal brain	Normal ganglion	Monkey lung fibroblasts	Monkey testicle fibroblasts	Normal chimp brain	Normal chimp ganglia
MZM-13 (brain isolate)	+	+	+	+	−	−	+	+
MZM-14 (lymph node isolate)	+	+	+	+	−	−	+	+
Human CMV (Towne)	$10^{6.3}$	+	+	$10^{5.3}$	+	+	$10^{4.8}$	$10^{5.3}$

[a] + and − indicate presence and absence of CPE, respectively. Data are expressed as 50% tissue culture infective doses per milliliter.

rum. No virus was isolated from cultures initiated from tissue of any other CNS area of the diseased chimpanzee, including the spinal cord, where histological examination revealed demyelinative lesions. No pathological lesions were found in that area of the explanted left frontal lobe from which CMV was eventually isolated.

Spontaneous paralysis with demyelination has never occurred in any chimpanzee in the large colony at the Southwest Research Foundation in San Antonio. The fact that virus was isolated only after prolonged subcultivation of brain cells suggests that CMV was latent in the brain tissue. CMV may have been transmitted subclinically from other chimpanzees in the primate colony with the resultant establishment of latency. The

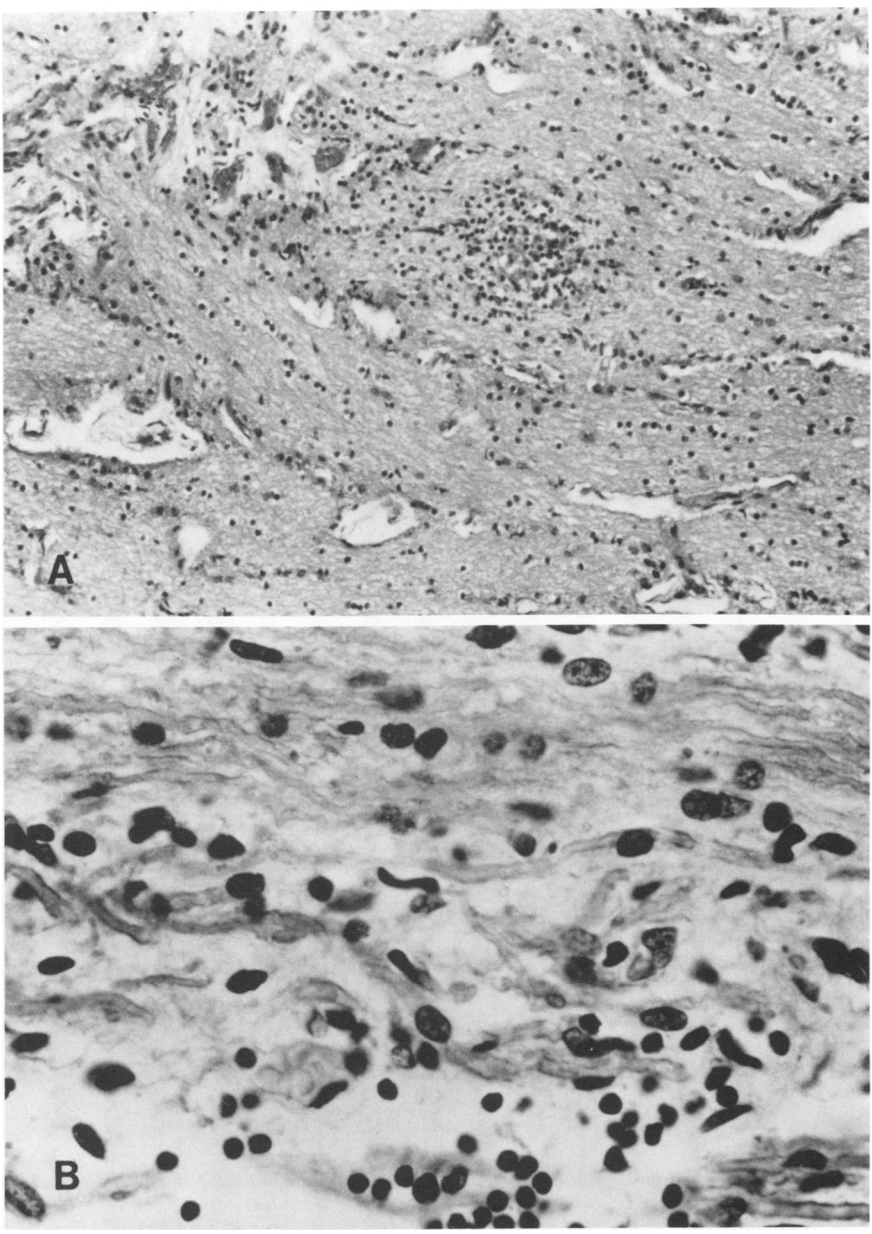

FIG. 4. *(A) Optic nerve of chimpanzee 4X78 used for transmission study. Note glial nodule consisting of a mixture of lymphocytes, astrocytes and microgliocytes. Hematoxylin-eosin. ×225. (B) Trigeminal nerve root from chimpanzee 4X78 used for transmission study. Note myelin degeneration with lymphocytic infiltration. Luxol fast blue-cresyl violet. ×400.*

fact that CMV was isolated twice from throat swabs of asymptomatic chimpanzees in the Southwest Research Foundation indicates that the chimpanzee colony harbors CMV. Although the prevalence of antibodies to CMV in chimpanzees there is not known, the seroconversion from negative to positive in the sick chimpanzee from which MZM virus was isolated is of interest. The latency of CMV in chimpanzees in captivity is unproven but has been estimated at 25% on the basis of the presence of inclusion bodies in the salivary gland, adrenal gland, and myocardium (17). In another, more extensive study, CMV was not recovered from multiple

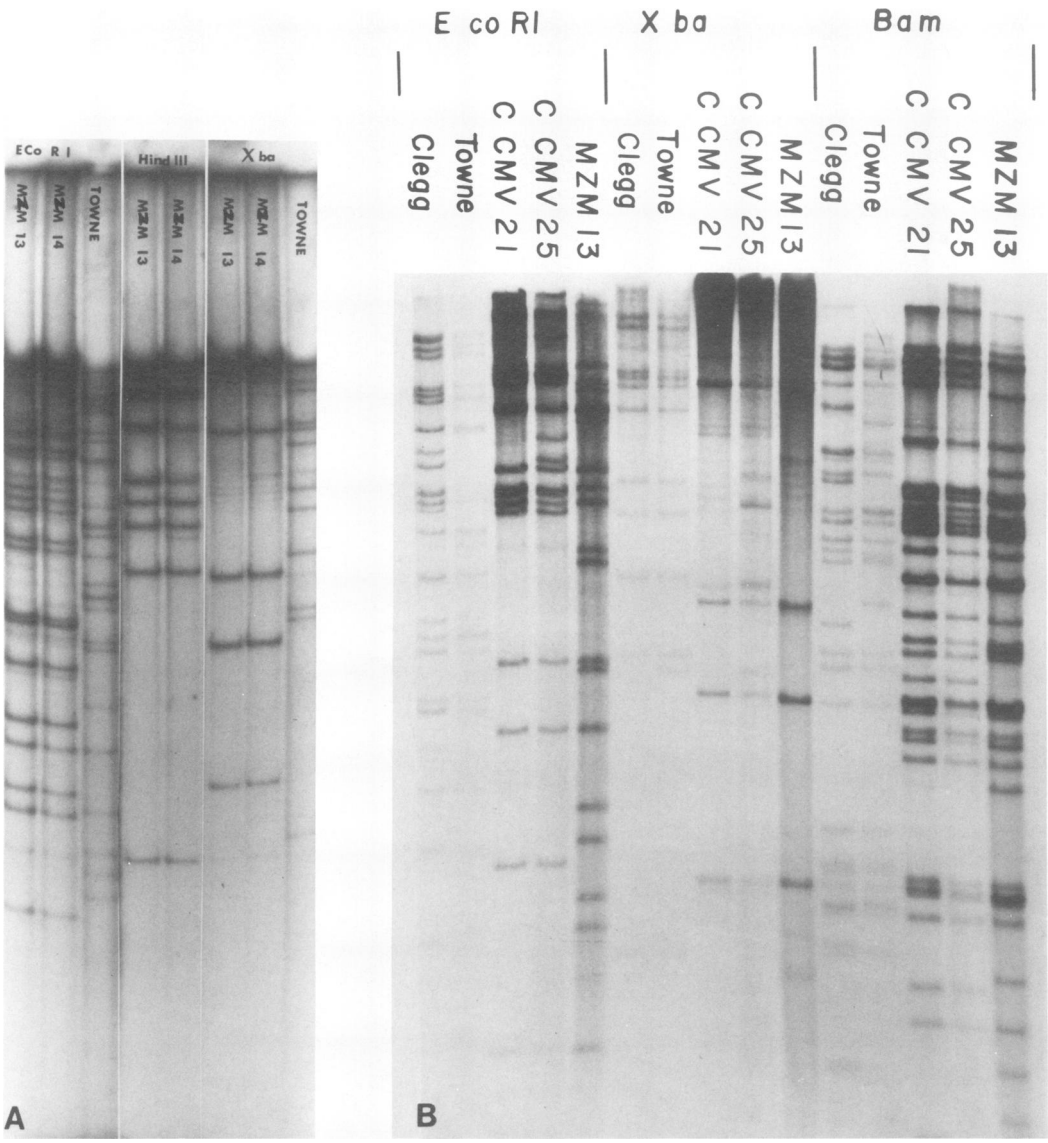

FIG. 5. *(A) Comparison and analysis of MZM-13 and MZM-14 isolates with human CMV (Towne strain) by cleavage of their DNAs with restriction endonucleases EcoRI, HindIII, and XbaI. (B) Comparison and analysis of MZM-13 with two strains of chimpanzee CMV and two strains of human CMV (Towne and Clegg) by cleavage of their DNAs with restriction endonucleases EcoRI, XbaI, and BamI. Viral DNA labeled with ^{32}P was purified and subjected to restriction endonuclease digestion as described in the text. The migration of DNA in agarose gel is from top to bottom.*

tissue specimens (including the CNS) of 11 chimpanzees (3). Yet explantation and cocultivation of these same tissues yielded 56 strains of other viruses. CMV has also been recovered from kidney cultures established from healthy Vervet monkeys (10) and African green monkey (1).

MZM-CMV appears to be of chimpanzee origin. Although species specificity generally exists for CMV, cross-reactivity has been demonstrated between human and simian cell lines (1; L. J. Charamella, R. B. Reynolds, L. T. Ch'ien, and C. A. Alford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V373, p. 256). Such cross-reactivity has also been shown for the Towne strain of human CMV and for MZM-CMV. Each of these two viruses regularly produced CPE in

both human and simian cell cultures. As indicated in Fig. 5B, MZM virus shares restriction fragment patterns with both chimpanzee strains of CMV used in this study.

It is of interest that the 4-month-old chimpanzee (4X78) that received the MZM-13 virus at birth developed subclinical neurological lesions. The pathological changes seen were consistent with chronic viral encephalitis and a more acute inflammatory radiculitis. These features are similar to those seen in both postinfectious encephalomyelitis and experimental allergic encephalomyelitis and in experimental allergic neuritis. Although serological evidence exists suggesting that demyelinating disease in the peripheral nervous system of humans may be linked to CMV (2, 14), ours is the only experimental evidence, to our knowledge, in which CMV has been associated with demyelination in the CNS. The relationship between MZM-CMV and demyelinating disease and the possible activation of MZM-CMV by multiple sclerosis brain cell inoculation remain to be determined.

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