

## Isolation of an Arenavirus from a Marmoset with Callitrichid Hepatitis and Its Serologic Association with Disease

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Callitrichid hepatitis (CH) is an acute, often fatal viral infection of New World primates from the family Callitrichidae. The etiologic agent of CH is unknown. We report here the isolation of an arenavirus from a common marmoset (*Callithrix jacchus*) with CH by using in vitro cultures of marmoset hepatocytes and Vero-E6 cells. Enveloped virions 67 to 133 nm in diameter with ribosomelike internal structures were seen in infected cultures. Immunofluorescence and Western immunoblot analysis using CH-specific antisera (principally from animals exposed to CH during zoo outbreaks) revealed three antigens in cells infected with this CH-associated virus (CHV). These antigens had the same electrophoretic mobilities on sodium dodecyl sulfate-polyacrylamide gels as did the nucleocapsid, GP2, and GPC proteins of lymphocytic choriomeningitis virus (LCMV). Monoclonal antibodies specific for these arenavirus proteins also reacted with the three CHV antigens. Conversely, the CH-specific antisera reacted with the nucleocapsid, GP2, and GPC proteins of LCMV. CHV thus appears to be a close antigenic relative of LCMV. The serologic association of CHV with several CH outbreaks implicate it as the etiologic agent of this disease.

Callitrichid hepatitis (CH) occurs in discrete epizootics in zoo collections of tamarins and marmosets (14). Twelve outbreaks have occurred in the United States since 1980, killing 57 animals. Included among the deaths were callitrichid species that are considered endangered in the wild, such as the golden lion tamarin (*Leontopithecus rosalia*), which is being bred in U.S. zoos for release into its native habitat in Brazil. CH epizootics are thus a threat to this breeding program. Premortem signs of CH are nonspecific, including dyspnea, anorexia, weakness, and lethargy, and are frequently followed by prostration and death. Postmortem findings include jaundice, pleural and pericardial effusions (occasionally sanguinous), subcutaneous and intramuscular hemorrhage, and hepatosplenomegaly. Diffuse hepatocellular necrosis with the formation of acidophilic bodies and mild inflammatory infiltrate is a consistent finding (12, 14). The typical CH syndrome can be experimentally produced in marmosets by parenteral inoculation with a bacteria-free liver filtrate from an animal with CH (12). Sera from callitrichids with CH and from some asymptomatic animals exposed to the disease contain antibodies that react with three antigens found in the livers of animals that died of CH (17). These antigens appear to be viral proteins belonging to the suspected etiologic agent of CH, termed CH-associated virus (CHV).

In the studies described here, we set out to cultivate CHV in primary cultures of marmoset hepatocytes (10) because hepatocytes are one of the targets of CHV infection in vivo

and in vitro cultures of these cells are known to be permissive for the replication of other hepatotropic viruses (7, 8). Our initial goal was to demonstrate replication of CHV in cell culture by using CH-specific antisera from convalescent animals (17). Electron microscopy (EM) was then used to identify key morphologic characteristics of the CHV virion. Finally, since CHV appeared to be an ultrastructurally typical arenavirus, we tested (i) arenavirus-specific monoclonal antibodies (MAbs) for their ability to react with CH-specific antigens and (ii) CH-specific antisera for their ability to react with lymphocytic choriomeningitis virus (LCMV), the prototypic Old World arenavirus.

### MATERIALS AND METHODS

**Hepatocyte cultures.** A liver wedge biopsy was obtained from a 2.5-year-old male common marmoset (*Callithrix jacchus*). Hepatocytes were isolated by perfusion with collagenase as previously described (7, 8, 10) and were plated at a density of  $10^6$  cells per 60-mm dish (Primaria, Falcon). Dishes were pretreated with rat tail collagen. Hepatocytes were allowed to attach for 3 h in Williams medium E supplemented with 5% fetal bovine serum, at which time the cultures were washed and changed to a serum-free medium supplemented with growth factors and hormones (10). The cultivation and characterization of baboon and chimpanzee hepatocytes in serum-free medium have been described (7, 8, 10). Secretion of apolipoproteins A1 and E by the hepatocyte cultures was monitored by Western immunoblot as stringent markers of differentiation. Stable levels of secre-

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tion of these markers were observed over a 66-day period in culture (data not shown).

**Vero cell cultures.** Vero-E6 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, N.Y.) with 5% fetal bovine serum.

**Virus inoculation.** Liver (0.3 g) from experimentally inoculated common marmoset EXP2 (12) was thawed, placed in 6 ml of Dulbecco's modified Eagle's medium containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) buffer, homogenized, and spun at  $1,500 \times g$  for 10 min at 4°C, and the supernatant was frozen at -85°C until used. EXP2 was inoculated with liver filtrate from an emperor tamarin (*Saguinus imperator*) that died with CH at the Oklahoma City Zoo (14). EXP2 subsequently developed CH and died 7 days after inoculation (12). The homogenate was diluted five- or sixfold with cell culture medium and filtered through a sterile 0.22- or 0.45- $\mu$ m-pore-size filter, and 0.5 ml was used to inoculate cells cultured in 60-mm dishes. Vero cells were also inoculated with LCMV-Armstrong obtained from the American Type Culture Collection (Rockville, Md.) essentially as described for CHV.

**Immunofluorescence.** Hepatocytes were fixed in cold acetone, and Vero-E6 cells were fixed in a cold 1:1 mixture of acetone-methanol. Binding of callitrichid antisera (diluted in phosphate-buffered saline [PBS] containing 0.2% bovine serum albumin) and murine MAbs (diluted in PBS containing 0.5% normal goat serum) was detected by using fluorescein isothiocyanate-(FITC)-labeled protein A and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), respectively.

**Western immunoblots.** Cell monolayers were extracted by using Laemmli sample treatment buffer (9) with the following composition: 4% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 50 mM Tris, 100 mM NaCl, 10% glycerol, and 0.05 mg of bromophenol blue per ml. SDS-polyacrylamide gel electrophoresis (PAGE) and Western immunoblot analysis were performed as described previously (17), using 10% acrylamide gels and a Mini-Protein II gel apparatus (Bio-Rad, Rockville Centre, N.Y.).  $^{125}$ I-protein A was used as the reporter molecule. Two types of molecular mass markers were used: (i) prestained standards (rainbow markers; Amersham, Arlington Heights, Ill.) and (ii) unstained standards (high-range standards; Bethesda Research Laboratories, Gaithersburg, Md.) visualized by staining with India ink (6). Marmoset liver samples were prepared for Western blot analysis as described previously (17).

**EM.** Cell monolayers on 60-mm dishes were fixed in 1% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide in PBS, and dehydrated in graded ethanol solutions. The fixed monolayers were then released from the plastic surface by using propylene oxide, embedded in Polybed 812, and sectioned with a diamond knife. Sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope.

**Antisera.** CH-specific sera available for use in this study were drawn from callitrichids exposed to CH at the Oklahoma City Zoo, Oklahoma City, Okla. (OKCZ1; emperor tamarin), the Brookfield Zoo, Chicago, Ill. (BZ1; Goeldi's monkey; *Callimico goeldii*), the Lincoln Park Zoo, Chicago, Ill. (LPZ; emperor tamarin), and Marineworld, Vallejo, Calif. (MW2; saddleback tamarin; *Saguinus fuscicollis*). While BZ1 was asymptomatic, the other three tamarins had clinical and pathological evidence of CH (14). CH-specific serum R306 was raised in a rabbit against the 54-kDa putative viral antigen identified in the livers of marmosets with CH (17).

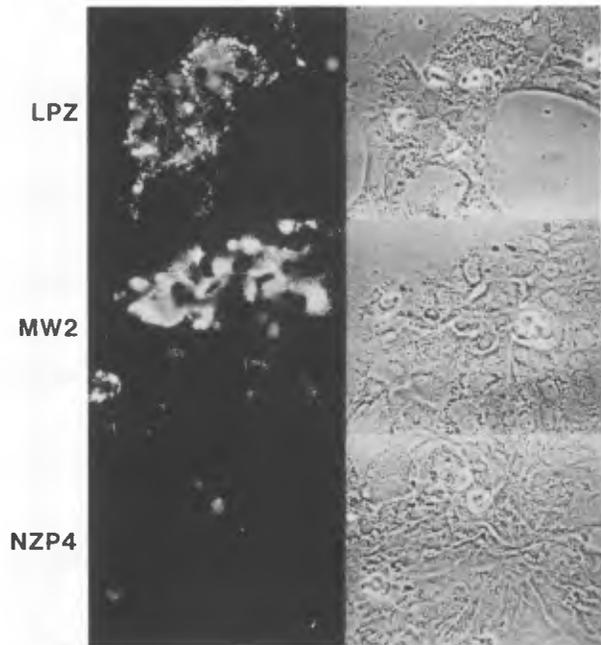


FIG. 1. Immunofluorescence analysis. CHV-inoculated marmoset hepatocyte cultures with CH-specific tamarin antisera LPZ and MW2 show punctate cytoplasmic fluorescence, while negative control serum NZP4 did not react (1:100 dilutions). Cultures were fixed in acetone 9 (LPZ) and 11 (MW2 and NZP4) days after inoculation with an infectious liver filtrate from a common marmoset (EXP2) with CH. Results were similar on days 3, 5, 7, and 28 after infection. The fluorescent (left) and phase-contrast (right) photographs are of the same field. CHV-specific sera BZ1 and OKCZ1 reacted similarly to LPZ and MW2 (data not shown).

Negative control sera were from two golden lion tamarins never exposed to CH (NZP3 and NZP4). The reaction of these sera with CH-specific antigens has been described in detail (17). A polyclonal anti-LCMV mouse ascitic fluid was purchased from Microbiological Associates (Rockville, Md.).

**MAbs.** MAb 3B-3.1, raised against Pichinde virus, recognizes a highly conserved epitope on the nucleocapsid protein of both New World and Old World arenaviruses (4), while MAb 1-1.3, raised against LCMV, recognizes a different epitope present only on the nucleocapsid protein of Old World arenaviruses (3). MAbs 33.6 and 9-7.9, both raised against LCMV, recognize two different epitopes within one antigenic site on the virion surface glycoprotein GP2. The epitope recognized by MAb 33.6 is found on both New World and Old World arenaviruses, while the epitope recognized by MAb 9-7.9 is restricted to Old World arenaviruses (20).

## RESULTS

**Evidence of CHV replication in cell culture.** Hepatocytes inoculated with liver filtrate from a marmoset with CH were examined by immunofluorescence using CH-specific sera from animals exposed to CH during four separate CH outbreaks (17). All four sera reacted with inoculated but not uninoculated cultures on all postinoculation days (Fig. 1). Typically, approximately 50% of cells in the inoculated cultures showed punctate cytoplasmic fluorescence, with

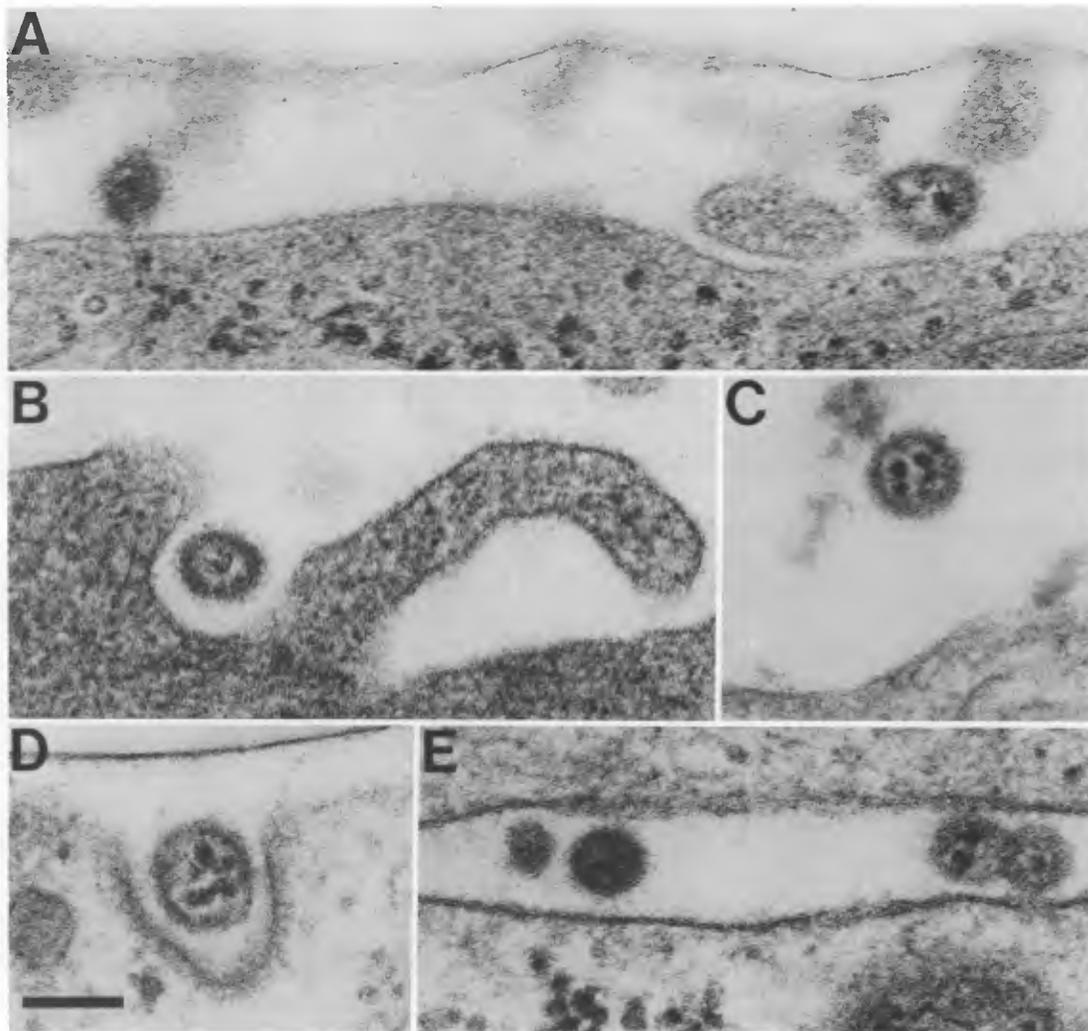


FIG. 2. Presence of typical arenavirus particles in CHV-inoculated marmoset hepatocyte and Vero-E6 cultures. Transmission EM analysis of marmoset hepatocytes (A and B) and Vero-E6 cells (C to E) 3 and 4 (E) days after inoculation with infectious liver filtrate from a common marmoset (EXP2) with CH revealed virions with morphologic characteristics of the family *Arenaviridae*. Final magnifications are  $\times 135,000$ . Bar represents 100 nm.

some cells containing large inclusions. Control sera from seronegative animals showed no specific fluorescence (Fig. 1). Hepatocytes inoculated with cell-free supernatants removed from liver filtrate-inoculated cells 11 to 13 days after infection showed the same pattern of fluorescence as the liver filtrate-inoculated cells, while sham-inoculated cultures showed no specific fluorescence (data not shown). No obvious cytopathic effects were seen in the inoculated hepatocyte cultures. These data indicate that CHV productively infects *in vitro* cultures of marmoset hepatocytes.

EM examination of hepatocyte cultures was performed to identify the ultrastructural characteristics of the CHV virion. Sections from parallel inoculated and uninoculated cultures fixed 3, 7, and 11 days after inoculation were examined under code to prevent bias. Enveloped virus particles 67 to 133 nm in diameter with an apparent glycoprotein fringe of approximately 10 to 15 nm and electron-dense internal structures which appeared to be ribosomes were seen extracellularly in the day 3 inoculated culture (Fig. 2). These virions resemble members of the family *Arenaviridae*. These

virus particles were not seen during extensive examination of the uninoculated cultures. Since arenaviruses can often be cultivated in Vero-E6 cells (11), cultures of these cells were inoculated with the infectious liver filtrate. No cytopathic effects were seen, but EM examination revealed typical arenaviruslike virions (Fig. 2). Inoculated Vero-E6 cells also showed a pattern of punctate cytoplasmic fluorescence when examined with CH-specific sera (data not shown). Uninoculated cultures showed no specific fluorescence. CHV thus appears to replicate in Vero-E6 cells as well as in hepatocytes.

**Antigenic relationship of CHV to arenaviruses.** To determine whether CHV was antigenically related to members of the family *Arenaviridae*, CHV-inoculated hepatocytes and Vero-E6 cells were examined by immunofluorescence using four arenavirus-specific MAb. MAbs 3B-3.1 and 1-1.3, which are specific for the nucleocapsid protein, reacted only with inoculated cells, showing a pattern of punctate cytoplasmic fluorescence in both hepatocytes (Fig. 3) and Vero-E6 cells (data not shown), similar to the pattern seen

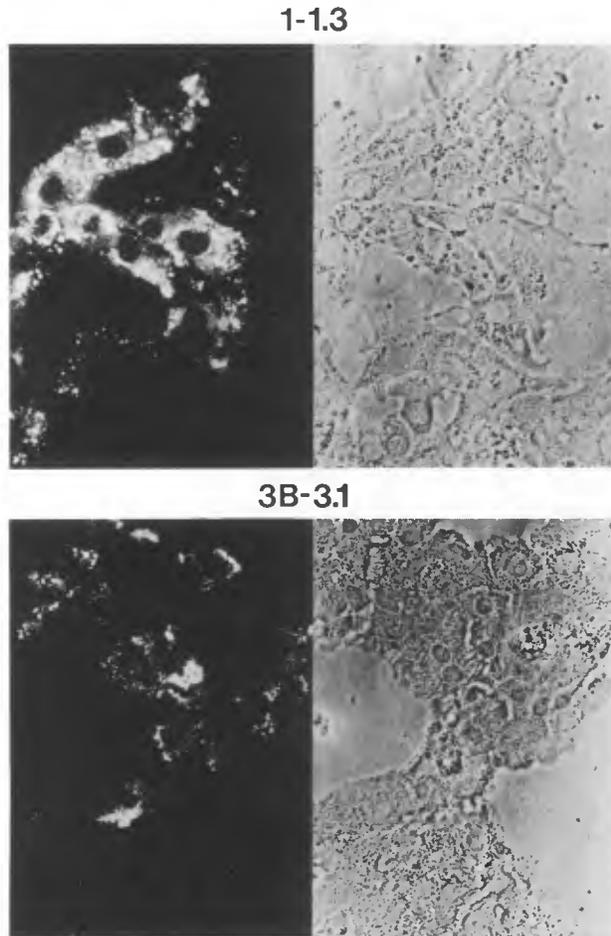


FIG. 3. Immunofluorescence analysis of inoculated marmoset hepatocyte cultures with arenavirus nucleocapsid protein-specific MAbs 1-1.3 and 3B-3.1, showing punctate cytoplasmic fluorescence. Cultures were fixed in acetone 3 (1-1.3) and 5 (3B-3.1) days after inoculation with infectious liver filtrate from a common marmoset (EXP2) with CH. The MAbs were diluted 1:20 (1:100 dilutions gave identical results). The fluorescent (left) and phase-contrast (right) photographs are of the same microscopic field.

with the CH-specific sera. MAbs 33.6 and 9-7.9, which are specific for the surface glycoprotein GP2, gave a pattern of diffuse cytoplasmic fluorescence when reacted with CHV-inoculated hepatocytes (Fig. 4) and Vero-E6 cells (data not shown). No specific fluorescence was seen when MAb 33.6 was incubated with uninoculated hepatocytes or Vero-E6 cells or when normal mouse ascitic fluid was incubated with CHV-infected hepatocytes (data not shown). Whereas MAbs 3B-3.1 and 33.6 recognize highly conserved epitopes found in both New and Old World arenaviruses, MAbs 1-1.3 and 9-7.9 recognize epitopes restricted to Old World arenaviruses. These data suggest that CHV belongs to the Old World family of arenaviruses. Polyclonal anti-LCMV ascitic fluid also reacted specifically with CHV-inoculated cells (data not shown), reinforcing this conclusion.

To determine the apparent molecular masses of the CHV antigens identified by the arenavirus-specific MAbs, extracts of CHV- and LCMV-infected cells were analyzed by Western blot. MAb 1-1.3 recognized the nucleocapsid protein of LCMV and an apparently homologous CHV protein of the

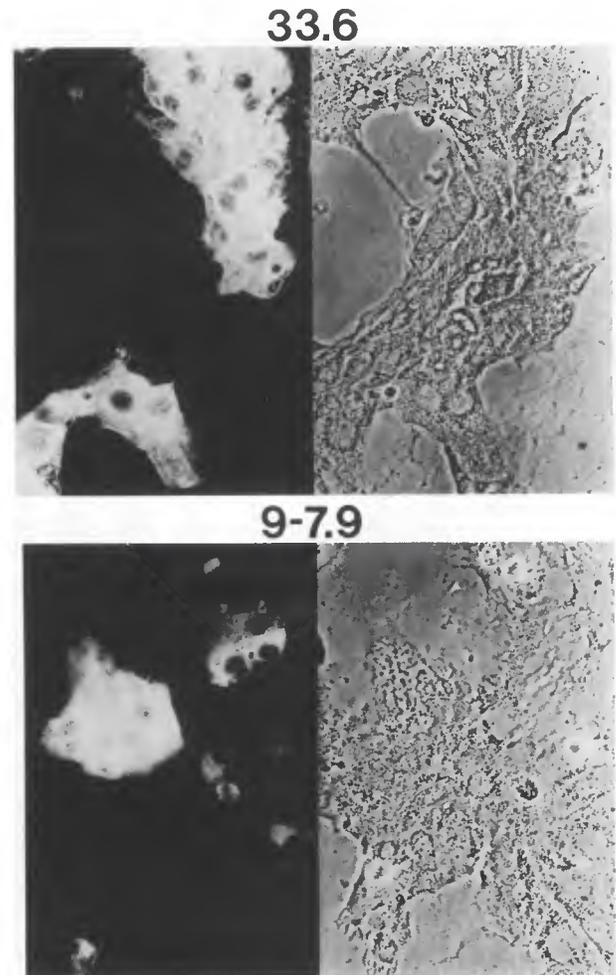


FIG. 4. Immunofluorescence analysis of inoculated marmoset hepatocyte cultures with arenavirus GP2-specific MAbs 33.6 and 9-7.9, showing smooth cytoplasmic fluorescence. Cultures were fixed in acetone 3 (33.6) and 5 (9-7.9) days after inoculation with infectious liver filtrate from a common marmoset with CH (EXP2). The MAbs were diluted 1:20 (1:100 dilutions gave identical results). The fluorescent (left) and phase-contrast (right) photographs are of the same microscopic field.

same electrophoretic mobility (Fig. 5). The molecular mass of the LCMV nucleocapsid protein is reported to be 63 kDa (2), although the mass relative to the standards used here is closer to 54 kDa, as we previously reported for CHV (17). This inconsistency is apparently due to the use of different molecular mass standards in these two studies. MAb 33.6 identifies the GP2 and GPC proteins of LCMV and also identifies two CHV antigens of the same electrophoretic mobilities (Fig. 5). These are presumably the homologous CHV glycoproteins. (MAb 33.6 also recognized an antigen of 47 to 51 kDa in LCMV-infected Vero cells [Fig. 5]. The identity of this antigen is uncertain.) When extracts of CHV-infected cells were run in adjacent lanes on the same gel and were subsequently probed with the arenavirus MAbs and the CH-specific sera from infected animals, the three CH-specific antigens identified by these sera were shown to comigrate with the nucleocapsid, GP2, and GPC proteins identified by the MAbs, suggesting that both the CH-specific

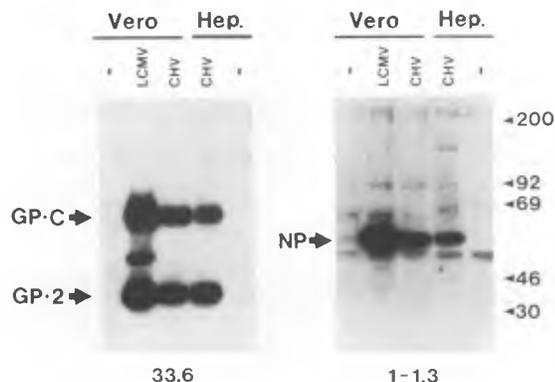


FIG. 5. Evidence that the GPC, GP2, and nucleocapsid proteins of LCMV have the same electrophoretic mobilities as the homologous CHV proteins. Cultures of marmoset hepatocytes (Hep.) and Vero-E6 cells (Vero) were inoculated with infectious liver filtrate from a common marmoset with CH (EXP2) and with LCMV-Armstrong (Vero-E6 cells only). Extracts (20  $\mu$ l per lane) made 3 days postinfection from parallel inoculated (LCMV and CHV) and uninoculated (-) cultures were analyzed by Western blot using MAbs specific for LCMV nucleocapsid (1-1.3) and GP2 and GPC proteins (33.6). The LCMV nucleocapsid protein (NP) and GPC and GP2 proteins are indicated. The MAbs were diluted 1:100, and a secondary rabbit anti-mouse immunoglobulin G (Sigma, St. Louis, Mo.) was used for amplification. Sizes on the right are indicated in kilodaltons.

sera and the MAbs identify the same viral proteins (data not shown).

Because arenavirus-specific antibodies were shown to react with CHV antigens (Fig. 3 to 5), CH-specific sera were tested by Western blot to determine whether reciprocal cross-reactions occurred against LCMV proteins. Sera from tamarins BZ1 and LPZ, and serum R306 raised against the 54-kDa CHV antigen, reacted with the nucleocapsid protein of LCMV in infected Vero-E6 cells and with the apparent nucleocapsid protein of CHV in infected hepatocytes and Vero-E6 cells (Fig. 6, closed arrow). Serum BZ1 also clearly identified the LCMV proteins GP2 (open arrow) and GPC (diamond) as well as antigens of similar mobility in the CHV-infected cells (Fig. 6; the CHV glycoproteins are less abundant in these extracts, making these bands much lighter than the corresponding LCMV bands). None of the CH-specific sera reacted with the arenavirus glycoprotein GP1. The significance of this is uncertain, since the presence of GP1 in these cultures was not confirmed by using anti-GP1 antibodies. The reaction of CH-specific sera with LCMV proteins further strengthens the close relationship of CHV to members of the Old World group of arenaviruses.

## DISCUSSION

CHV is an ultrastructurally typical arenavirus. It is enveloped, apparently buds from the cytoplasmic membrane (no intact virions were seen intracellularly), has a glycoprotein fringe, contains ribosomelike internal structures, and has a diameter ranging from 67 nm (condensed spherical particles) to 130 nm (pleiomorphic particles with electron-lucent areas within the virion cross section), as has been described for LCMV (5, 15).

CHV is antigenically related to the Old World arenaviruses. This was demonstrated clearly by the reaction of the arenavirus-specific MAbs with CHV and the reciprocal

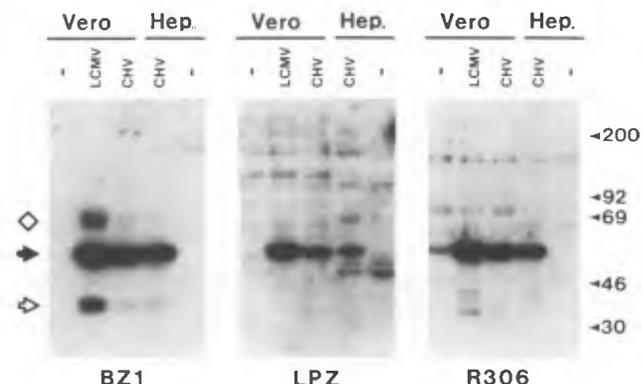


FIG. 6. Evidence that CH-specific antisera recognize the GPC (diamond), GP2 (open arrow), and nucleocapsid (closed arrow) proteins of LCMV. Cultures of marmoset hepatocytes (Hep.) and Vero-E6 cells (Vero) were inoculated with infectious liver filtrate from a common marmoset with CH (EXP2) and with LCMV-Armstrong (Vero-E6 cells only). Extracts (20  $\mu$ l per lane) made 3 days postinfection from parallel inoculated (LCMV and CHV) and uninoculated (-) cultures were analyzed by Western blot using CH-specific sera from callitrichids BZ1 (1:100 dilution) and LPZ (1:50 dilution) and rabbit serum R306 (1:25 dilution). Serum R306 was raised against the putative CHV nucleocapsid protein partially purified from the liver of an infected animal by preparative SDS-PAGE. This preparation also contained normal cellular proteins, some of which are recognized by this serum. Thus, a cellular antigen of similar molecular mass to the CHV nucleocapsid protein is seen faintly in the uninoculated Vero-E6 cell extract, even though R306 was preabsorbed against normal marmoset liver and acetone-methanol-fixed Vero-E6 cells. Sizes on the right are indicated in kilodaltons.

reaction of CH-specific sera with LCMV-Armstrong. The Old World arenaviruses are geographically limited to Africa, with the single known exception of LCMV, which can be found in Europe, Asia, Africa, and the Americas (11). Considering the geographic origin of this CHV isolate, Oklahoma City, CHV may be more closely related to LCMV than to other Old World arenaviruses. A close relationship between CHV and LCMV is also supported by the cross-reaction of several CH-specific sera with LCMV-Armstrong.

CHV is antigenically linked to several outbreaks of CH. Sera from animals exposed at the Oklahoma City Zoo, the Brookfield Zoo, the Lincoln Park Zoo and Marineworld (Vallejo, Calif.) reacted with CHV in infected hepatocytes and Vero-E6 cells by immunofluorescence and Western blot analysis. Similarly, the rabbit immune serum raised against the 54-kDa CHV antigen purified from an experimentally infected marmoset also reacted with CHV. These data strongly implicate CHV as the etiologic agent of CH.

The pathologic changes caused by CHV infection are strikingly similar to those seen in typical arenavirus infections. For example, Lassa fever and CH have several similarities, including an acute course, involvement of multiple organs (including liver and spleen), petechial hemorrhages (although not prominent in either CH or Lassa fever), nonsanguinous pleural and pericardial effusions, and, most strikingly, a pattern of microscopic liver changes that includes multifocal hepatocellular necrosis with formation of acidophilic bodies and minor inflammation. This type of liver lesion is characteristic of CH and is the most consistent microscopic finding of Lassa fever (12, 19). Experimental infection of cynomolgus monkeys with LCMV-WE also

produced an acute, often fatal disease with some similarities to CH, although hemorrhagic signs are much more pronounced than in CH (13). These similarities support the argument that CHV is the etiologic agent of CH.

Arenaviruses are typically maintained in rodent reservoirs by causing persistent infections during which infectious virus is excreted in the urine (11). This suggests that CHV may also persist in such a reservoir species. Rodents (including *Mus musculus*, a known host of LCMV) are common inhabitants of zoos and may be the principal reservoir of CHV. Another possible reservoir could be African rodent species (*Mastomys* and *Paromys*) that serve as reservoirs for the Lassa and Mopeia arenaviruses, respectively, in Africa. Such species are kept on exhibit in some zoos.

Zoo workers exposed to CHV-infected primates or rodents may be at risk of infection. Two zoo veterinarians who cared for callitrichids with CH were found to be seropositive for CHV, although neither recalled being ill at the time of exposure (unpublished observations). Human CHV infection could thus be asymptomatic, as is sometimes the case with LCMV (1). However, LCMV can also cause symptomatic disease in humans ranging from a flulike illness to meningitis to a fatal, hemorrhagic syndrome (16, 18), suggesting that CHV be treated as a serious health hazard for zoo personnel.

The data reported here strongly implicate CHV as the etiologic agent of CH, although marmoset inoculation studies with a clonally derived inoculum grown in cell culture are needed to definitively establish this etiologic relationship. The exact relationship of CHV to other arenaviruses should also be further explored. Sequencing of relevant portions of the genome and preparation of MAbs are now possible, since we have shown that the virus can be grown in cell culture. Preparation of CHV-specific reagents will also allow the examination of histologic specimens to determine whether affected animals from other CH outbreaks show antigenic evidence of infection with CHV. Finally, serologic studies will be facilitated by the availability of purified CHV antigen and will allow for surveillance of callitrichid populations (and reservoir species) for evidence of infection. Such information should enable zoos to prevent future CH outbreaks and avoid the losses that have plagued many U.S. zoos over the past decade.

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