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Identification, Using Sera from Exposed Animals, of Putative Viral Antigens in Livers of Primates with Callitrichid Hepatitis

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Callitrichid hepatitis (CH) is an acute, frequently fatal viral hepatitis which affects members of the primate family *Callitrichidae* (R. J. Montali, E. C. Ramsay, C. B. Stephensen, M. Worley, J. A. Davis, and K. V. Holmes, J. Infect. Dis. 160:759–765, 1989; E. C. Ramsay, R. J. Montali, M. Worley, C. B. Stephensen, and K. V. Holmes, J. Zoo Wildlife Med. 20:178–183, 1989). Outbreaks of the disease occur in zoos and animal parks. In this study, CH-specific antigens were identified in the livers of infected animals by using immune sera from primates with CH and CH-exposed asymptomatic animals. Three CH-specific antigens with apparent molecular masses of 34, 54, and 65 kDa were identified. A polyclonal antiserum was raised against the 54-kDa antigen. These antigens were not found in the livers of uninfected animals and may be viral proteins. Our results suggest that at least five of the six outbreaks of CH considered here were caused by the same virus or by an antigenically related virus.

Callitrichid hepatitis (CH) affects members of the primate family *Callitrichidae*, which includes tamarins and marmosets. It is an acute hepatitis, characterized by hepatocellular necrosis with inflammation and acidophilic body formation throughout the liver. CH occurs in zoos and animal parks. It appears to have a high attack rate and a fatality rate of approximately 75%. CH is not caused by a known primate hepatitis virus. We previously transmitted CH to 3 common marmosets (*Callithrix jacchus*) via a 0.22-μm liver filtrate from an infected zoo tamarin. The filterable nature of this agent strongly suggests a viral etiology for CH. Enveloped virions, 85 to 105 nm in diameter, were observed in the livers of the inoculated marmosets (6, 7).

Given the serious nature of this disease and the close phylogenetic relationship of callitrichids to humans, it is of considerable interest to characterize the agent of CH. With this goal in mind, we have sought to identify and characterize antigens found in the livers of infected animals which react with sera from CH patients and CH-exposed animals. We reasoned that sera from animals with CH, particularly from convalescent animals, should contain antibodies against the etiologic agent of CH. It also seemed likely, given the acute, often fatal, course of the disease, that rapid viral replication during the early phase of infection might lead to accumulation of sufficient viral antigen in the liver to be detectable with such sera. Our earlier report (6) supported this hypothesis, and we have expanded upon this preliminary observation in the work described here.

Livers and sera were available from five animals naturally infected with CH (liver, 3; serum, 4), eight asymptomatic zoo animals naturally exposed to CH (liver, 1; serum, 7), five asymptomatic zoo animals never exposed to CH (liver, 2; serum, 3), three animals experimentally infected with CH, and three uninoculated experimental controls. Table 1 indicates the source of these specimens and gives some characteristics of the animals from which they came.

Liver tissue was mechanically disrupted in cold TEN buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl [pH 7.4]) and centrifuged at 1,000 × *g* for 10 min at 4°C. This supernatant was centrifuged at 27,000 × *g* for 15 min at 4°C, and the pellet was washed twice and resuspended in TEN. Protein concentration was determined by the method of Bradford (2). Dot-blots were performed as previously described (6). Liver samples were applied to nitrocellulose paper by using a dot-blot apparatus (5 μg per dot) (Minifold; Schleicher & Schuell, Keene, N.H.). Blots were cut into strips and incubated in skim milk blocking buffer (12% [wt/vol] nonfat dry milk dissolved in 50 mM Tris–150 mM NaCl–1 mM EDTA–0.05% Tween [pH 7.4]) for at least 1 h. All incubations were done at 24°C. Strips were washed once in B3 buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.05% Tween, 0.1% bovine serum albumin [pH 7.4]), incubated with antisera diluted in B3 plus 2% bovine serum albumin for 1 h, washed five times for 5 min per wash in B3, incubated with 1×10^5 to 2×10^5 cpm ^{125}I-staphylococcal protein A (low specific activity; DuPont-NEN, Wilmington, Del.), washed five times for 5 min per wash in B3, blotted dry, and exposed to X-ray film (XAR-5; Kodak, Rochester, N.Y.) at −70°C with intensifying screens.

In our earlier report (6), we showed that sera from one CH patient (LPZ) and one asymptomatic exposed animal (OKCZ2, the sole survivor of a family group) reacted with CH-specific antigens found in the livers of the 3 marmosets experimentally infected with CH, as well as antigens found in the liver of CH patient OKCZ1, the source of the inoculum for the experimentally infected animals. We have now expanded these observations by testing three additional serum samples from patients with CH, five additional serum samples from asymptomatic, exposed animals, and six serum samples from experimentally infected and uninfected control marmosets. Sera from a total of four zoo callitrichids with CH (OKCZ1, MW2, LPZ, and MW3) were available for testing. They all reacted with the liver samples from infected

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TABLE 1. Characteristics of CH patients and controls

Study identification	Zoo identification	Source of animal (yr of outbreak)[a]	Species[b]	Comments[c]
Zoo callitrichids with naturally acquired CH				
OKCZ1 (OKC1)	947307	Oklahoma City Zoo, Oklahoma City, Okla. (1986)	EMT	Euthanized after 3 days of symptoms; serum collected day before euthanasia, liver at necropsy; liver used to inoculate experimental marmosets (see below)
MW1	Ira	Marineworld, Vallejo, Calif. (1988)	ST	No symptoms before death; liver collected at necropsy
MW2	Miracle	Marineworld, Vallejo, Calif. (1988)	ST	MW2 had high serum AST and bilirubin during CH outbreak; serum drawn 79 days after death of cagemate (MW1) from CH; MW2 survived
MW3	Tammy	Marineworld, Vallejo, Calif. (1988)	ST	MW3 had high serum AST and bilirubin during CH outbreak; serum drawn 33 days after death of cagemate (MW1) from CH; MW3 survived
LPZ	7975	Lincoln Park Zoo, Chicago, Ill. (1987)	EMT	Euthanized after ≥8 days of symptoms; serum and liver taken at euthanasia
Asymptomatic zoo callitrichids exposed to CH				
OKCZ2 (OKC2)	717337	Oklahoma City Zoo, Oklahoma City, Okla. (1984)	GLT	Serum drawn 3 yr after death of all cagemates from CH
HDZ	4704	Henry Doorly Zoo, Omaha, Nebr. (1980)	GLT	Serum drawn 7 yr after outbreak; no cagemates with CH
MW4	Spider	Marineworld, Vallejo, Calif. (1988)	ST	Infant which died at time of outbreak with no histologic evidence of CH; liver taken at necropsy
BZ1	Espiritu	Brookfield Zoo, Chicago, Ill. (1986-1987)	GM	Serum drawn 18 mo after outbreak; no cagemates with CH
BZ2	Gertrude	Brookfield Zoo, Chicago, Ill. (1986-1987)	GM	Serum drawn 18 mo after outbreak; no cagemates with CH
BZ3	Mini	Brookfield Zoo, Chicago, Ill. (1986-1987)	GM	Serum drawn 18 mo after outbreak; no cagemates with CH
BZ4	Kris	Brookfield Zoo, Chicago, Ill. (1986-1987)	GM	Serum drawn 18 mo after outbreak; no cagemates with CH
BZ5	James	Brookfield Zoo, Chicago, Ill. (1986-1987)	GLT	Serum drawn 18 mo after outbreak; no cagemates with CH
Asymptomatic zoo callitrichids not exposed to CH[d]				
NZP1	8441	National Zoological Park, Washington, D.C.	GLT	Liver from infant trauma death
NZP2	77300	National Zoological Park, Washington, D.C.	GLT	Liver from infant trauma death
NZP3	106161	National Zoological Park, Washington, D.C.	GLT	Serum
NZP4	102217	National Zoological Park, Washington, D.C.	GLT	Serum

Continued on following page

TABLE 1—*Continued*

Study identification	Zoo identification	Source of animal (yr of outbreak)[a]	Species[b]	Comments[c]
Asymptomatic zoo callitrichids not exposed to CH[d]				
NZP5	104954	National Zoological Park, Washington, D.C.	GLT	Serum
Callitrichids experimentally infected with CH				
EXPTL1 (EXP1)		—[e]	CM	Symptomatic 4 days p.i.[e]; euthanized 8 days p.i.; liver taken at necropsy; serum drawn before inoculation and at euthanasia
EXPTL2 (EXP2)		—[e]	CM	Asymptomatic; died 7 days p.i.; liver taken at necropsy; serum drawn before inoculation
EXPTL3 (EXP3)		—[e]	CM	Symptomatic 7 days p.i.; euthanized 9 days p.i.; liver taken at necropsy; serum drawn before inoculation and at euthanasia
Uninoculated experimental controls				
EXPTL4 (EXP4)		—[e]	CM	Liver and serum
EXPTL5 (EXP5)		—[e]	CM	Liver and serum
EXPTL6 (EXP6)		—[e]	CM	Liver and serum

[a] See reference 7 for further description of CH outbreaks.
[b] EMT, Emperor tamarin (*Saguinus imperator*); ST, saddleback tamarin (*Saguinus fusciollis*); GM, Goeldi's monkey (*Callimico goeldii*); GLT, golden lion tamarin (*Leontopithecus rosalia*); CM, common marmoset (*C. jacchus*).
[c] AST, Aspartate aminotransferase; p.i., postinoculation.
[d] No CH outbreaks have occurred at the National Zoological Park, where these animals were born and reared.
[e] —, See reference 6.

animals but not from uninfected controls (Fig. 1 and 2A). Three (OKCZ2, BZ1, and BZ5) of the seven asymptomatic exposed animals also reacted in this CH-specific manner (Fig. 1 and 2B). In contrast, none of the unexposed animals (including the zoo tamarins [Fig. 1, NZP3-5], the uninoculated experimental marmosets [data not shown], and the experimentally infected marmosets bled before inoculation [data not shown] showed evidence of a CH-specific serum antibody response.

Acute-phase sera, available from two of the three inoculated marmosets, were negative for CH-specific antibody (data not shown). This was not unexpected since these sera were collected just 8 and 9 days after inoculation, insufficient time for a significant immunoglobulin G (IgG) response to develop. CH-specific IgM antibody might have been present, but ^{125}I-staphylococcal protein A does not detect IgM. Collection of serum samples during the acute phase of disease also could explain the low antibody titer in the OKCZ1 serum, evident from the weak signal given by this serum in Fig. 1. OKCZ1 was symptomatic for just 2 days before blood was drawn. LPZ, on the other hand, had a higher CH-specific serum antibody titer (again, judging from Fig. 1) and had a longer clinical course (at least 8 days). The longer clinical course of LPZ combined with a prodrome of uncertain duration apparently allowed sufficient time for the development of a vigorous IgG response in this animal. The other sera with CH-specific antibody titers were drawn during or after convalescence from symptomatic (MW2 and MW3) or, presumably, asymptomatic (OKCZ2, BZ1, and BZ5) infection. These sera gave strong dot-blot signals, suggesting that they have high CH-specific IgG titers, as expected for convalescent sera.

In addition to showing that sera from patients with CH and from asymptomatic exposed animals contain CH-specific antibody, Fig. 1 shows that the liver from a patient naturally infected with CH, previously unreported (MW1), contained CH-specific antigen, although at an apparently lower concentration than that in the liver from EXPTL3. On the other hand, Fig. 2A shows that the liver from the CH patient LPZ did not contain sufficient antigen to be detected by this method. Knowing that the livers of all three patients experimentally infected with CH contained abundant CH-specific antigen (6) and that the OKCZ1 and MW1 livers contained less antigen (Fig. 1), the following course of events can be postulated: rapid virus replication may occur during the prodrome and early symptomatic phase of this disease, followed by immune system-mediated clearance. This scheme is consistent with our findings that CH-specific antigen was most abundant in the livers of the patients experimentally infected with CH (which received large inocula, had short disease courses, and no IgG response); less abundant in the livers of the two patients naturally infected with CH, which had short clinical courses and low serum IgG titers (OKCZ1 and MW1); and least abundant in the liver of LPZ, which had the longest clinical course and highest IgG titer. In the case of a viral hepatitis, serum IgG (and IgM) may prevent infection of additional hepatocytes while cytotoxic T cells and virus-induced cell death eliminate cells already infected, thus clearing viral antigen from the liver.

The data presented thus far are consistent with the proposition that the CH-specific antigens are viral proteins: sera from 100% of patients naturally infected with CH (four of four), from 43% of asymptomatic animals exposed to CH

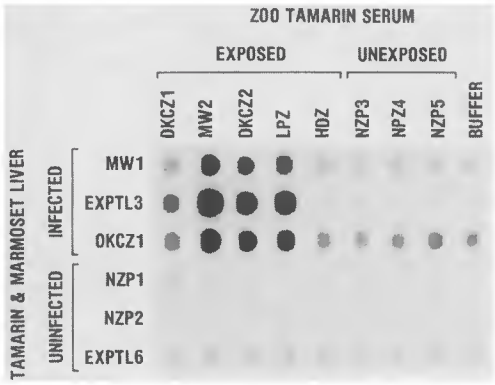

FIG. 1. Dot blot of liver extract from patients naturally (MW1, OKCZ1) and experimentally (EXPTL3) infected with CH and from uninfected control animals (NZP1, NZP2, and EXPTL6). The blot was probed with sera from patients with CH (OKCZ1, MW2, and LPZ) and from exposed (OKCZ2, HDZ) and unexposed (NZP3, NZP4, and NZP5) animals and with dilution buffer alone. See Table 1 for details of animals. All sera were preabsorbed with uninfected marmoset liver (prepared as described in the text) and were diluted 1:100.

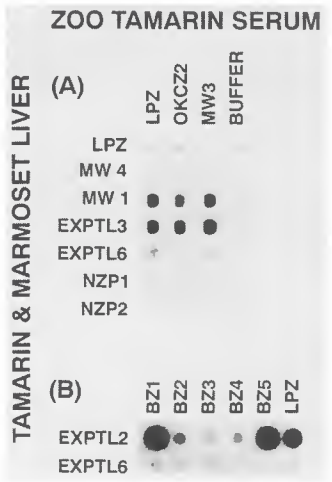

FIG. 2. Dot blot of liver extract (10 [A] or 5 [B] μg per dot) from marmosets and tamarins, including patients naturally (LPZ and MW1) and experimentally (EXPTL2 and 3) infected with CH, an animal which died during an outbreak of CH but did not show histopathologic evidence of CH (MW4), and unexposed animals (NZP1, NZP2, and EXPTL6). The blots were probed with sera from CH patients (LPZ and MW3), from exposed animals (OKCZ2 and BZ1-5), and dilution buffer alone. See Table 1 for details of animals. The LPZ serum was preabsorbed with uninfected marmoset liver (prepared as described in the text). MW3 was diluted 1:50; all other sera were diluted 1:100.

(three of seven), and from 0% of unexposed animals (zero of nine, including three zoo golden lion tamarins, three uninoculated experimental marmosets, and the three animals from which preinoculation sera were taken) showed CH-specific antibody titers. CH-specific antigen was found only in the livers of infected animals, and it was apparently cleared during the symptomatic phase of CH. Presuming that the CH-specific antigens are viral, our observations suggest that the two Oklahoma City Zoo outbreaks (in 1984 and 1986), the two separate outbreaks in Chicago-area zoos (Brookfield and Lincoln Park), and the Marineworld outbreak were all caused by the same virus or by serologically related viruses. Findings from two additional animals exposed to CH do not directly support this conclusion, but neither do they detract from it: while the Henry Doorly Zoo outbreak was typical of CH (7), the single animal from that zoo tested here was seronegative (Fig. 1, HDZ), suggesting that it was not infected. This is plausible since neither HDZ nor its sole cagemate was symptomatic at the time of the outbreak. Figure 1A also shows that the liver of MW4, a 2-month-old infant which died during the CH outbreak at Marineworld, did not contain detectable CH antigen. This is consistent with the histopathologic findings from this animal, which did not support a diagnosis of CH. MW4 was apparently not infected and probably died from neglect as a result of the infection of both parents.

To characterize the CH-specific antigens in more detail, we performed Western immunoblot analysis. Liver samples, prepared as described above, were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels by the method of Laemmli (4) by using a Mini-Protean II gel apparatus (Bio-Rad; Rockville Centre, N.Y.). Samples were boiled for 5 min in Laemmli sample treatment mix, including 5% 2-mercaptoethanol. Electroblotting to nitrocellulose paper (Schleicher & Schuell) was performed in a Mini-Transblot cell (Bio-Rad) by standard methods (9). The transfer buffer consisted of 25 mM Tris, 192 mM glycine (pH 8.3), and 20% methanol. After transfer, electroblots were incubated with serum and ^{125}I-staphylococcal protein A and exposed to film exactly as described for the dot blots.

If the CH-specific antigens are viral, Western blots could reveal the same viral antigens in liver extracts from different patients with CH. The LPZ serum identified a 54-kDa antigen in the livers of all 3 patients with CH (MW1, OKC1, and EXP2) that was not found in the control animals (EXP5, NZP1, and NZP2) (Fig. 3A). As expected from the dot blot results, the concentration of antigen appeared highest in the EXP2 lane. In this lane, two minor bands were also seen, at 32 and 34 kDa. The MW2 serum also reacted with the 54-kDa antigen in the EXP2 liver (Fig. 3B), but this band was not detected in the livers of the two patients naturally infected with CH (MW1 and OKC1), probably because of the lower concentrations of antigen in these livers and because of low titers of 54-kDa-antigen-specific antibody in the MW2 serum. The MW2 serum appeared to have a higher titer of antibody specific for an antigen which forms a broad band at 34 kDa (Fig. 3B, EXP2 lane). This antigen may be distinct from the 54-kDa antigen since the MW2 serum produces only a faint band at 54 kDa in the EXP2 lane. This 34-kDa antigen was also found in the livers of the patients naturally infected with CH (MW1 and OKC1) (Fig. 3B). The MW2 serum also identified a third 65-kDa antigen in the EXP2 liver which was not seen in the livers of the patients naturally infected with CH, again, possibly because of lower concentrations of CH-specific antigens in those livers.

The minor 32- and 34-kDa antigens identified by the LPZ serum in the liver of EXP2 (Fig. 3A) appear to be antigenically related to the 54-kDa antigen. This was shown by the following experiment. The liver from EXP2, prepared as described above, was subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel was fractionated and homogenized, and the fraction containing the 54-kDa antigen was identified by dot blot with the LPZ serum and with reference to molecular weight standards. Gel fractions containing this antigen were used to immunize a rabbit (R306). The resulting antiserum reacted not only with

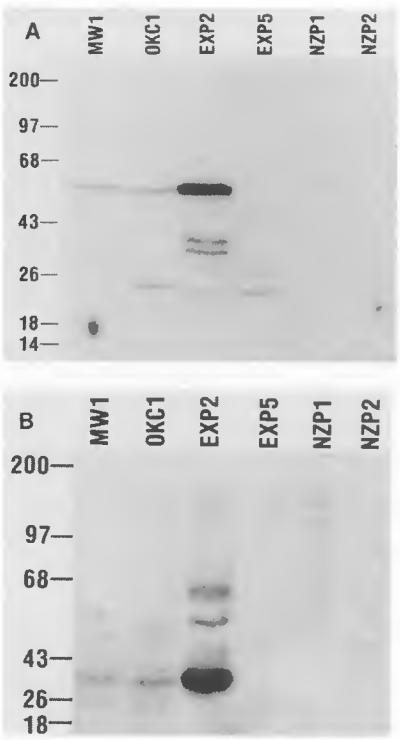

FIG. 3. Western blots of liver samples (50 μg per lane) from two naturally infected tamarins (MW1 and OKC1), an experimentally infected marmoset (EXP2), two uninfected zoo tamarins (NZP1 and NZP2), and an uninfected experimental marmoset (EXP5) using serum from CH patient LPZ (preabsorbed with excess guinea pig liver powder [GIBCO, Grand Island, N.Y.] and diluted 1:50) (A) or CH patient MW2 (1:100) (B).

the 54-kDa antigen in the EXP2 liver, but also with the minor 34- and 32-kDa antigens (Fig. 4).

These data indicate that there may be three distinct CH-specific antigens of 65, 54, and 34 kDa. A side-by-side Western blot analysis (Fig. 5) using sera from several seropositive animals and R306 showed that the 54-kDa antigens identified by LPZ, BZ1, BZ5, and R306 all had the same

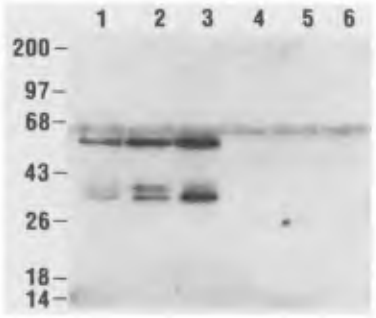

FIG. 4. Western blot of liver samples (50 μg per lane) from three marmosets experimentally infected with CH (lanes 1 through 3) and from three uninfected controls (lanes 4 through 6). Serum was from rabbit R306, immunized with the 54-kDa CH antigen which was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel fractionation, as described in the text. The serum was absorbed with excess guinea pig liver powder (GIBCO) and with excess marmoset liver antigen prepared from an uninoculated control marmoset, as described in the text, and was diluted 1:25.

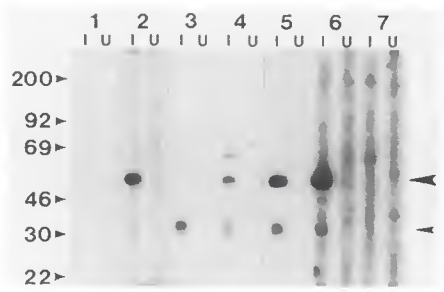

FIG. 5. Western blot of liver samples (40 μg per strip) from a patient experimentally inoculated with CH (EXPTL2; lanes I) and an uninoculated control (EXPTL6; lanes U), probed with the following: dilution buffer alone (lane 1), sera from CH patients LPZ (lane 2; absorbed with acetone powder of a normal marmoset liver and diluted 1:50) and MW2 (lane 3; diluted 1:100 and used previously to probe other blots), sera from CH-exposed animals BZ5 (lane 4; diluted 1:50) and BZ1 (lane 5; diluted 1:100), and anti-54 kDa antigen serum R306 and R306 preimmune serum (lanes 6 and 7, respectively; both absorbed as LPZ and diluted 1:25). Liver samples were run on a preparative sodium dodecyl sulfate-polyacrylamide electrophoresis gel and blotted to nitrocellulose paper, which was cut into strips and incubated with sera; the strips were realigned in order to compare mobility of the antigens identified with the various sera. Film was exposed at −70°C for 15 h (lanes 2 and 5 through 7) or 48 h (lanes 1, 3, and 4). Antigens banding at 54 and 34 kDa are indicated by the large and small arrow, respectively.

electrophoretic mobilities, reinforcing our conclusion that these sera react with the same antigen. The 32- to 34-kDa doublet seen by the R306 serum did not resolve on this gel. Thus, the 34-kDa band identified by the BZ1 serum on this same blot could be due to either this 54-kDa antigen-related doublet or the possibly distinct 34-kDa antigen identified by the MW2 serum (also seen in Fig. 5).

It is unlikely that these CH-specific antigens are normal liver proteins. Autoantibodies reacting with liver proteins have been observed in patients with human A, B and non-A, non-B hepatitis, including autoantibodies against the 57-kDa intermediate filament vimentin (3) and another 45-kDa liver protein (1). These autoantibodies, however, react with proteins in the livers of normal individuals as well as of hepatitis patients. The CH-specific antigens described here clearly are not found in normal livers. These CH-specific antigens also seem to be distinct from the infection-specific cytoplasmic antigen found in livers of chimpanzees with non-A, non-B and delta hepatitides (8). These infections have a mild, chronic course, quite dissimilar from the acute, often fatal course of CH. In addition, the molecular mass of this cytoplasmic antigen is 44 kDa (6), different from the molecular masses of the CH-specific antigens.

The more likely possibility is that these CH-specific antigens are proteins encoded by the virus which causes CH. This is strongly suggested by (i) their occurrence in livers of infected animals, (ii) their apparent clearance as infection progresses, (iii) their absence from livers of uninfected animals, (iv) the development of serum antibody against these antigens during infection, and (v) the absence of CH-specific serum antibody in animals never exposed to CH. Further analysis of these putative viral antigens may aid in identifying the CH virus. Use of the sera and antigens identified here should be helpful in seroepizootiologic studies and in adapting the virus to cell culture.

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sera and tissue samples from natural outbreaks of CH by the veterinary staffs of Marineworld, Vallejo, Calif.; of the Lincoln Park Zoo, Chicago, Ill.; of the Brookfield Zoo, Chicago, Ill.; and of the Henry Doorly Zoo, Omaha, Nebr.

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