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Transmission of Hepatitis A Virus Among Recently Captured Panamanian Owl Monkeys

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The presence of antibody to hepatitis A virus (anti-HAV) in 60% of procured owl monkeys (Aotus trivirgatus) held within the United States prompted a study of recently captured A trivigatus in Panama. Only 2 of 145 newly captured monkeys, but all of 35 A trivirgatus held within a colony for over 100 days, were found to have anti-HAV. Of 41 sero-negative, newly captured monkeys followed prospectively, 25 became infected with hepatitis A virus (HAV) as evidenced by seroconversion or demonstration of virus in the liver at death. Only one monkey that survived over 60 days within the colony was not infected. HAV was identified in the feces of most infected monkeys prior to the development of antibody and was antigenically indistinguishable from human HAV in cross-blocking radioimmunoassays. This colony-centered epizootic provides strong evidence that A trivirgatus is susceptible to HAV and should be investigated further as a potential model of human hepatitis A.

Key words: hepatitis A virus, anti-HAV, Aotus trivirgatus, owl monkey

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INTRODUCTION

Despite the fact that marmosets (Saguinas spp) and chimpanzees (Pan troglodytes) are the only subhuman primates to have thus far proven useful as models of hepatitus A virus (HAV) infection [Deinhardt, 1976], antibody to the virus (anti-HAV) has been frequently found in many other species of primates. Such species include the rhesus (Macaca mulatta), cynomolgus (Macaca fascicularis) and stump-tailed macaques (Macaca speciosa), baboons (Papio spp), owl monkeys (Aotus trivirgatus), and others [Eichberg and Kalter, 1980; Purcell and Dienstag, 1978]. However, attempts to infect directly many of these and other primate species with a known infectious inoculum of HAV, administered either intravenously or orally, have generally been unsuccessful [Purcell and Dienstag, 1978]. Although some macaques (rhesus, stump-tailed, and cynomolgus) have developed anti-HAV after challenge with virus, this has generally occurred without evidence of either hepatic dysfunction or shedding of virus in feces [Purcell and Dienstag, 1978; personal communication from D.S. Burke, Armed Forces Research Institute for Medical Sciences, Bangkok, Thailand]. Other than chimpanzees and marmosets, only the stump-tailed macaque has been shown, in a recent study, to shed infectious virus in feces following challenge with HAV [Mao et al, 1981]. Thus, given the apparent lack of susceptibility to the virus, the frequent presence of anti-HAV in many nonhuman primate species has remained somewhat of a mystery. It has been postulated, however, that the presence of anti-HAV in newly captured cynomolgus or Papio spp may reflect infection with HAV or a related virus prior to capture [Burke et al, 1981; Coursaget et al, 1981].

In a previous study, 4 of 18 caged A trivirgatus studied were found to be anti-HAV-positive [Purcell and Dienstag, 1978]. However, anti-HAV developed in only one A trivirgatus challenged intravenously and not in two challenged orally with human HAV. Furthermore, seroconversion in the one monkey was not associated with hepatic dysfunction, nor was fecal shedding of virus demonstrated. In contrast to these findings, we have observed a sustained outbreak of HAV infection among recently captured A trivirgatus* held at the Gorgas Memorial Laboratory (GML) in Panama. Newly captured monkeys were almost always sero-negative, but generally developed anti-HAV during their second month in the colony. Furthermore, HAV antigen, indistinguishable from human HAV, was detected by radioimmunoassay in both fecal and liver specimens from monkeys. These findings indicate that A trivirgatus may be useful for future studies of HAV infections and also provide an epizootiologic model that may explain the presence of anti-HAV in many other species of nonhuman primates.

MATERIALS AND METHODS

A trivirgatus Monkeys

A trivirgatus is a small, nocturnal, and arboreal primate that is widely distributed throughout Central and South America. Fully grown adults weigh 0.7 to 1.2 kg, and a number of different karyotypes have been described [Ma et al, 1978]. Several successful breeding colonies have supplied monkeys for the study of new antimalarial agents, and both wild-caught and colony-bred monkeys are available. A trivirgatus held at the

*In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Walter Reed Army Institute of Research (WRAIR) in Washington, DC were bled and studied for the presence of anti-HAV. Both colony-bred and procured (wild-caught) monkeys were included. Karyotypes were classified as described [Ma et al, 1978].

Newly captured A trivirgatus were bled at the Gorgas Memorial Laboratory (GML) in Panama City, Panama. These monkeys had been captured by local trappers in the forests that surround Panama City, most often within a radius of 100 km from the city. Upon arrival at GML, each monkey received attenuated small plaque herpes simplex and herpes tamarinus virus vaccines and was placed in a quarantine facility adjacent to the main colony. Monkeys were held in $1.5 \times 1 \times 1$ m wire mesh cages stacked two high, and those purchased as pairs were frequently caged together. Droppings were collected on solid aluminum pans beneath each cage floor that were removed daily, rinsed with water, and replaced. Between uses, cages were washed with water but were not sterilized. Monkeys were held in the quarantine facility for not less than 90 days and were then integrated into the main colony.

A trivirgatus admitted to the colony during the interval from September 25, 1980 to December 31, 1980 were studied prospectively. Feces were collected twice weekly and liver was taken at necropsy from monkeys dying during their first three months of captivity. Both liver and fecal specimens were stored at $-20°C$ until tested for HAV antigen. A portion of each liver was fixed in 10% neutral-buffered formalin, embedded in paraffin, and later stained with hemotoxylin and eosin for histopathologic studies.

Serologic Methods

Anti-HAV was determined by means of a commercial radioimmunoassay (Havab, Abbott Laboratories, N. Chicago, Ill). IgM antibody to HAV (IgM anti-HAV) was detected by radioimmunoassay as described [Lemon et al, 1980]. Antibody to hepatitis B virus core antigen (anti-HBc) was measured by commercial radioimmunoassay (Corab, Abbott Laboratories).

HAV Detection

HAV antigen was detected by a modification of the solid-phase radioimmunoassay method described by Purcell et al [1976]. The central 60 wells of a flexible microtiter "U" plate were coated for 4 hours at 30°C with 100 μl of human convalescent hepatitis A serum diluted 1:200 in 50 mM sodium carbonate buffer, pH 9.6. The plates were washed five times with phosphate-buffered saline, pH 7.4, (PBS) and each well filled with 1% bovine serum albumin (Calbiochem, La Jolla, Calif) in PBS. After one hour at 30°C, plates were washed with PBS containing 0.05% Tween-20 (PBS-Tween) (Fisher Scientific Co, Fairlawn, NJ) and 50 μl of a sample to be tested for HAV was placed into each well. The plates were incubated at 4°C for 18 hours, washed with PBS-Tween, and 40 μl of ^{125}I-labelled human anti-HAV [Lemon et al, 1980] was added to each well. After four hours at 4°C, plates were washed again with PBS-Tween, the wells cut apart, and bound ^{125}I determined by counting in a Rackgamma-II gamma counter (LKB-Wallac, Turku, Finland). Test specimens were compared with reference chimpanzee fecal specimens that either contained or did not contain HAV. A negative mean cpm value (N) was determined for each microtiter plate based on the results obtained from two wells loaded with HAV-negative chimpanzee material. The mean cpm value obtained from two wells containing a test sample (P) was divided by N to obtain a test specimen/negative mean value ratio (P/N). Sample generating

a P/N ratio of 2.1 or greater were considered positive. The specificity of each positive reaction was confirmed by blocking the reaction with hepatitis A convalescent sera; for this purpose, paired chimpanzee sera were obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md, and from previous experiments carried out at WRAIR. Twenty microliters of a 1:100 dilution of blocking sera was added to each microtiter well 15 minutes prior to the addition of the ^{125}I-labelled antibody. A positive radioimmunoassay reaction for HAV was considered confirmed if a greater than 50% reduction in cpm was noted in wells blocked with postinfection chimpanzee serum compared with wells blocked with homologous preinfection serum.

Fecal specimens were tested as 20% suspensions in PBS and were centrifuged for 30 minutes at 8,000g prior to testing. Previously frozen liver tissue was minced into fine pieces, subjected to 15 strokes with a Ten Broeck homogenizer as a 20% suspension in 0.05 M Tris-HCl pH containing 0.04 M KCl and 0.005 M sodium acetate, and clarified at 8,000g for 30 minutes prior to testing.

Electron Microscopy

Studies were carried out by Dr. Stephen Feinstone of the National Institute of Allergy and Infectious Diseases, Bethesda, Md, as previously described [Feinstone et al, 1979].

Aotus HAV Radioimmunoassay

To determine the degree of antigenic relatedness between virus recovered from A trivirgatus and human HAV, a parallel radioimmunoassay for HAV was developed using only Aotus-derived reagents. The assay was performed as described above, with the exception that A trivirgatus convalescent serum having an anti-HAV titer of greater than 1:6,400 was used as the coating antibody. A liver suspension (5%), prepared as above from an infected monkey, served as HAV antigen. A trivirgatus IgG was obtained by precipitation of 1 ml convalescent A trivirgatus serum (anti-HAV titer > 1:6,400) with 45% ammonium sulfate, followed by centrifugation at 8,000g for 30 minutes. The pellet was resuspended in 1 ml distilled water and dialyzed extensively against 0.0175 M potassium phosphate buffer, pH 7.2. The dialyzed material was then passed through a 10 cm × 1.5 cm DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) column and eluted with the same buffer. Fractions constituting the first protein peak were pooled, filter concentrated using an Amicon Xm100 filter (Amicon Corporation, Lexington, Mass) to approximately 1 mg protein/ml, and radiolabelled with ^{125}I as described [Lemon et al, 1980]. A human convalescent serum collected during an outbreak of hepatitis A in Alaska and a convalescent A trivirgatus serum obtained at the GML were diluted serially and the blocking activity of each serum was determined in simultaneously performed parallel radioimmunoassays using A trivirgatus or human reagents. Serum dilutions were made in PBS containing a fixed 1:100 dilution of anti-HAV-negative chimpanzee serum. For these experiments, every other well in the microtiter plates contained HAV antigen that was not immunologically blocked, and the degree of blocking activity obtained with a given serum dilution was based on a comparison with the cpm of unblocked adjacent wells. This procedure was followed in an effort to overcome regional differences in protein-binding across individual microtiter plates.

RESULTS

Serologic Surveys

An initial survey of A trivirgatus held at WRAIR demonstrated a high prevalence of anti-HAV (60%) among procured monkeys, but few sero-positive monkeys among those that were colony bred (3%) (Table I). Anti-HAV was equally present in both sexes and was found in almost all karyotypes studied. The procured monkeys were generally two to three years of age, and thus somewhat older than the colony-bred monkeys, which were less than two years of age. Nevertheless, these results suggest a high degree of exposure to an HAV-like agent among procured monkeys prior to their arrival at WRAIR and suggest that transmission of this agent has been uncommon within this colony.

To ascertain whether procured monkeys had been exposed in nature or had instead been infected or exposed to HAV antigen following capture, sera were tested from 50 A trivirgatus monkeys that had been held at the GML for various lengths of time following their capture in Panama. Thirty-eight of these sera (76%), which were collected in November 1979, contained anti-HAV antibody (Fig. 1). The highest antibody prevalence rates were found among those animals held in captivity longest. None of 10 animals held less than 30 days, but all of 35 animals held for longer than 100 days were positive. Furthermore, whereas immunoglobulin M (IgM) anti-HAV antibody was uniformly present in seropositive animals held for less than 300 days, it was not detected in any animal that had been held for over 600 days. None of these 50 monkeys had antibody to hepatitis B core antigen. These initial observations suggested that HAV was being transmitted to monkeys following their capture, probably within the colony at the GML.

To confirm the absence of HAV infection among wild A trivirgatus, 145 newly captured monkeys arriving at the GML between September 25, 1980 and May 31, 1981 were bled shortly after arrival and tested for the presence of anti-HAV. Only 2 of these 145 monkeys (1.4%) were sero-positive. Thus, exposure to HAV appears to have been a very rare event in these monkeys prior to their capture.

TABLE I. Serum Survey of A trivirgatus Held at WRAIR

Karyotype	Procured	Colony-bred
I	2/8 (25%)[a]	1/6 (17%)
II	21/27 (78%)	0/15 (0%)
III	24/36 (67%)	0/20 (0%)
IV	4/6 (67%)	0/6 (0%)
V	1/1 (100%)	–
VI	1/4 (25%)	0/2 (0%)
VII	–	–
VIII	0/1 (0%)	–
Hybrid	–	0/15 (0%)
Undetermined	2/8 (25%)	1/15 (7%)
Total	55/91 (60%)	2/79 (3%)

[a]Anti-HAV-positive/number tested (% positive).

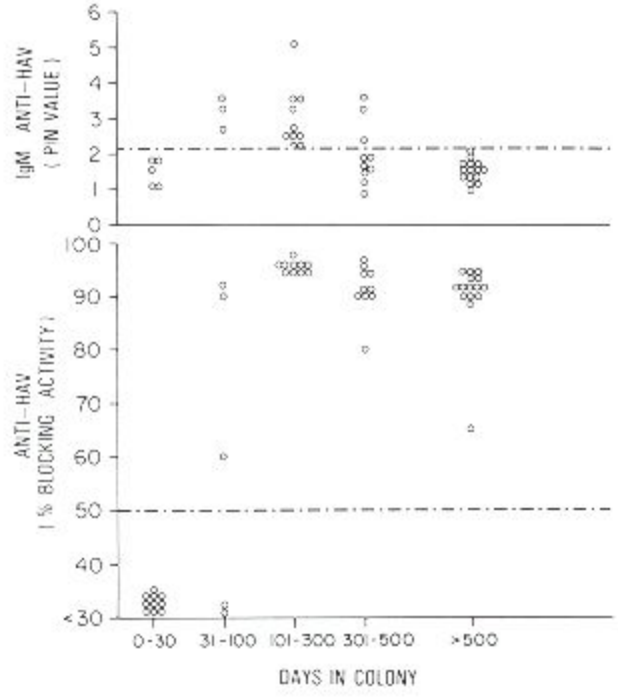

Fig. 1. Results of testing for HAV antibodies in a cross-sectional survey of the A trivirgatus colony at the GML. The length of time each monkey had been held in the colony is shown along the abscissa. Anti-HAV (lower panel) was tested by blocking radioimmunoassay (see Materials and Methods); a result greater than 50% was considered positive. IgM anti-HAV (upper panel) was measured by means of a solid-phase antibody-capture radioimmunoassay (see Materials and Methods) in which P/N ratio values greater than 2.1 were considered positive. These results demonstrate that monkeys held in the colony longer than 100 days uniformly possessed anti-HAV, whereas IgM anti-HAV was only found in monkeys held between 30 and 500 days.

Prospective Study of Newly Captured A trivirgatus

To more fully document the transmission of virus within the GML colony, all 41 monkeys arriving between September 25, and December 31, 1980 were followed prospectively with periodic collection of serum and fecal samples. Thirty-one of these animals (76%) had been held by trappers less than eight days prior to delivery to the GML, and only one had been held longer than 15 days. All lacked anti-HAV antibody upon arrival at the colony. Of the 41 monkeys in the original cohort, 11 died relatively soon after capture and were not bled later than 25 days after arrival at the colony. None of these monkeys showed serologic evidence of infection with HAV, and none of eight tested had HAV antigen detected in liver taken at necropsy. However, seroconversion to the virus was documented by radioimmunoassay in 21 of the remaining 30 monkeys. Antibody generally first appeared during the second month in the colony (Fig. 2).

To evaluate fecal shedding of virus by infected monkeys, fecal specimens (collected at 2- to 4- day intervals during the period between the last antibody-negative and first antibody-positive serum samples) were examined for the presence of HAV antigen by solid-phase radioimmunoassay. HAV antigen was detected in feces collected from 12 of 15 cages, containing a total of 23 monkeys (Table II). The quantity of antigen pres-

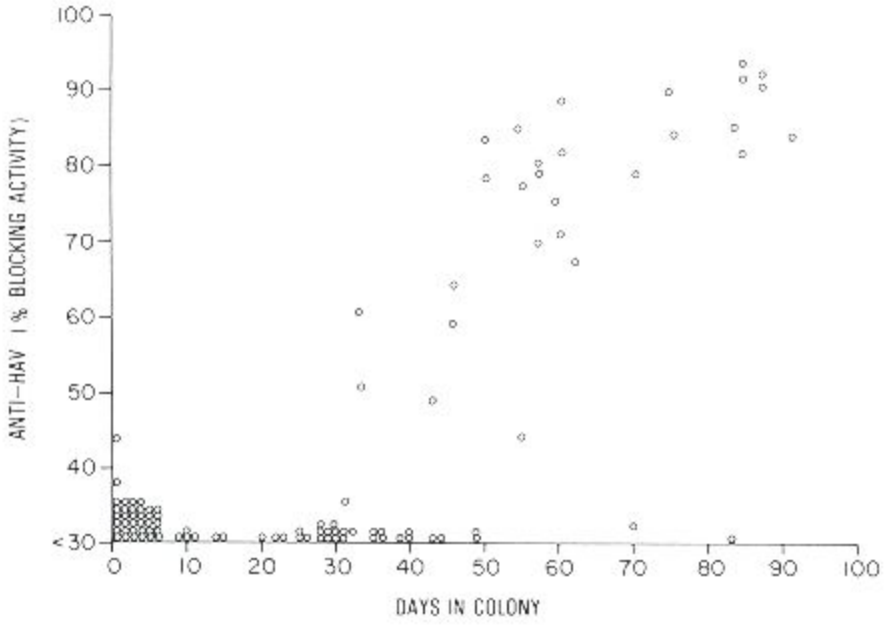

Fig. 2. Anti-HAV determined by solid-phase radioimmunoassay in 41 newly captured A trivirgatus held at the GML colony. Each symbol represents a single serum determination; greater than 50% blocking activity indicates the presence of anti-HAV. Only one monkey that survived over 60 days within the colony remained anti-HAV-negative.

TABLE II. Fecal Shedding of HAV by A trivirgatus*

			HAV Radioimmunoassay	
Cage #	Monkey(s)	Days in colony	P/N	(cpm/cpm blocked)[a]
254	10512	62	25.1	(427/85)
262	10538	34	9.1	(128/26)
263	10523	36	146.0	(2047/357)
264	10529	55	10.2	(326/40)
266	10542	32	55.6	(2434/598)
267	10515,16	57	1.8	(51/81)
269	10513	63	4.5	(77/32)
270	10539,40,41	49	1.3	(48/54)
271	10524,25	35	23.6	(532/78)
272	10526,27,28	32	12.5	(283/29)
275	10519,20	50	1.3	(38/29)
277	10537	36	159.0	(2704/299)
278	10532	42	26.6	(599/67)
279	10533	28	92.5	(2083/200)
280	10535,36	36	44.8	(1010/86)
Neg control	Chimp 273	—	1.0	(14–44[b]/not done)
Pos control	Chimp 273	—	94.4	(2077/175)

*Results shown are for the fecal specimen from each cage that yielded highest cpm in the HAV radioimmunoassay.

[a]Expressed as cpm when incubated with preinfection chimpanzee serum/cpm when blocked by postinfection chimpanzee serum (see Materials and Methods).

[b]Different microtiter plates.

ent, as estimated by the amount of ^{125}I-label bound, approximated that of feces from acutely infected chimpanzees. Two fecal specimens that gave positive results by radioimmunoassay were examined by immune electron microscopy. The presence of HAV in these specimens was confirmed by the finding of 27 nM particles that were aggregated in the presence of anti-HAV (data not shown).

Figure 3 depicts the radioimmunoassay results obtained with serial specimens from three cages. Viral antigen was present in feces over a period of four to 10 days, and there was no evidence of persistent viral shedding. The time interval between arrival at the colony and appearance of viral antigen in the feces ranged from 22 to 58 days. Cessation of fecal antigen shedding appeared to coincide or slightly preceed appearance of anti-HAV.

Of the nine remaining monkeys that survived over 25 days in the colony and that did not develop anti-HAV, eight died within 60 days of their arrival. Liver tissue, taken

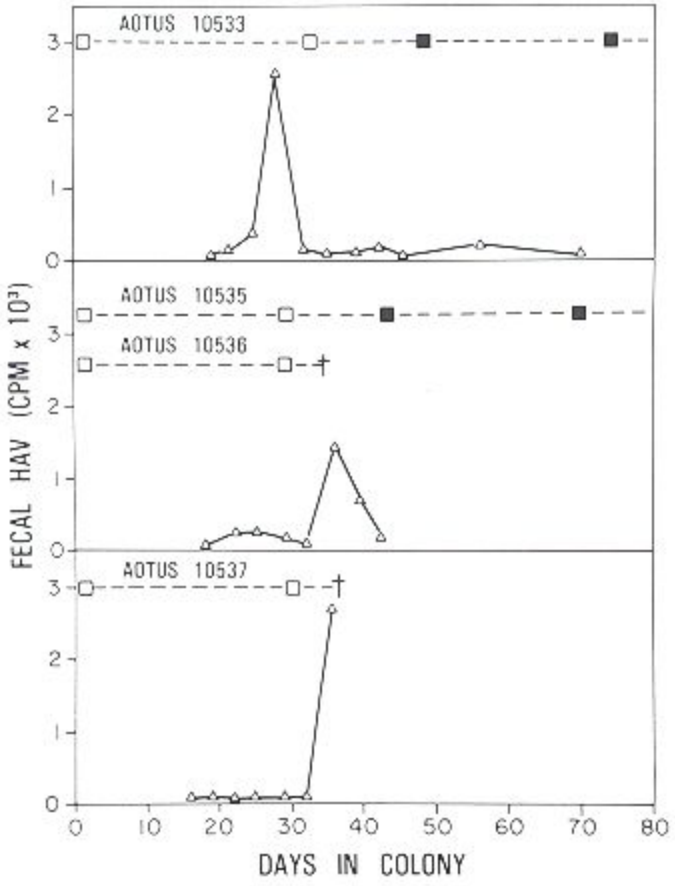

Fig. 3. Hepatitis A virus (HAV) infection among A trivirgatus monkeys held in three cages in a colony at the GML. Crosses represent monkey deaths. Boxes indicate results of testing for anti-HAV: open boxes indicate sera were antibody-negative, shaded boxes indicate antibody-positive. Triangles represent results obtained by testing fecal specimens for HAV antigen by solid-phase radioimmunoassay (see Materials and Methods). Peak antigen activity in each series of specimens was specifically blocked by postinfection but not preinfection reference chimpanzee sera.

at necropsy, was available from four of these eight monkeys and, in each case, contained HAV antigen detectable by radioimmunoassay (Table III). Antigen was also present in fecal specimens collected from three of these four monkeys just prior to their death; feces were not available from the fourth monkey. Three of the four liver specimens containing viral antigen were examined histologically, but none revealed changes suggestive of acute viral hepatitis in man (Table III). While minimal focal necrosis with neutrophilic infiltration was present in one liver specimen, similar lesions were also seen in other liver specimens that did not contain HAV antigen. Thus, although these four monkeys died during the acute phase of their infection, it appeared that death was not directly due to liver disease and perhaps was unrelated to HAV infection. Attempts were made to measure serum aspartate and alanine aminotransferase levels, but these studies were inconclusive. Many monkeys had elevated enzyme levels on admission to the colony, possibly reflecting either injury at the time of capture or other concurrent infections.

Comparison of Human and A trivirgatus HAV

To more fully examine the degree of antigenic relatedness between the HAV antigen recovered from the monkeys and human HAV (MS-1 strain), parallel radioimmunoassays were developed employing in one case antigen and antibody derived from human infections, and in the other, material obtained from A trivirgatus at the GML. Two sera, one collected from a convalescent human and the other from a convalescent monkey, were tested in each radioimmunoassay for blocking activity (Fig. 4). In replicate experiments, both monkey and human sera demonstrated a similar titer of blocking activity when tested against the A trivirgatus antigen. Although the monkey serum appeared to have a somewhat lower titer of blocking activity against MS-1 antigen when compared with the human serum, this difference was only one tube dilution. These results indicate that these two antigens were indistinguishable by this technique.

TABLE III. A trivirgatus With HAV Detectable in Liver at Necropsy

		HAV radioimmunoassay[a]		
Monkey #	Days in colony	P/N	(cpm/cpm blocked)[b]	Histologic findings in liver
10521	48	52.5	(814/139)	Marked hemosiderin accumulation. Minimal focal necrosis with neutrophilic infiltration.
10523	42	33.2	(514/154)	Marked hemosiderin accumulation. Minimal, focal, fatty metamorphosis (medium and large vacuoles). Moderate sinusoidal dilatation and congestion.
10529	58	35.0	(771/265)	Not available.
10537	37	246.0	(5419/875)	Moderate hemosiderin accumulation. Minimal intrahepatic, centrilobular cholestasis.

[a]Negative mean cpm = 16–22 (HAV-negative chimpanzee feces).
[b]See footnote to Table II.

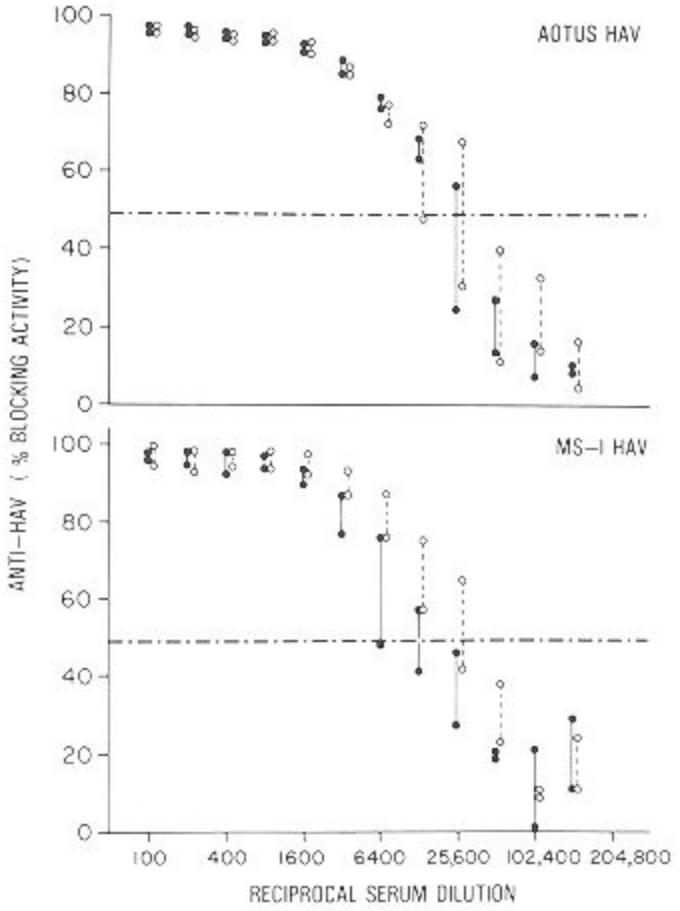

Fig. 4. Comparison of blocking activity of a convalescent human (○) and a convalescent A trivirgatus (●) serum in parallel radioimmunoassays employing either A trivirgatus-derived (upper panel) or human-derived (MS-1 strain) reagents (lower panel). The bars shown represent ranges of values obtained in separate experiments. The titer of blocking activity in each serum was similar when tested against each of the two antigens, indicating a high degree of antigenic relatedness between these two strains of HAV.

DISCUSSION

These data provide strong evidence that transmission of HAV (or a closely related virus) occurred within the A trivirgatus colony at the GML. Since initiating serological testing, all but two of 145 newly captured monkeys have arrived at the colony lacking antibody to HAV. In the cohort reported here, exposure appeared to occur during the first month in the colony. Twenty-five of 41 monkeys followed prospectively became infected with HAV following their arrival at the colony. Of the 16 monkeys without evidence of HAV infection, 15 died during their first two months in the colony and therefore may not have survived long enough to become infected. Only one monkey survived over 60 days and was not infected with the virus. Of special interest

was the demonstration of HAV in the feces of these monkeys. To our knowledge, this is the first identification of HAV in fecal material from nonhuman primates that had not been experimentally infected.

While possible, it would seem very unlikely that exposure had occured during the interval between capture and arrival at the colony, inasmuch as monkeys were procured from a variety of trappers and geographic areas. Cynomolgus monkeys and other primate species have also been shown to develop anti-HAV antibodies following their capture, suggesting that colony-centered transmission of HAV may occur among other species [Purcell and Dienstag, 1978; Burke et al, 1981]. In addition, the fact that most of the sero-positive A trivirgatus held at WRAIR (Table I) had not been obtained from the GML indicates that transmission of HAV among the A trivirgatus at the GML was probably not a unique event.

The course of HAV infection observed in A trivirgatus appears remarkably similar to that of HAV in man [Dienstag et al, 1975; Decker et al, 1979]. HAV antigen was present in liver tissue at the peak of infection, when antigen was also present in feces. Antigen appeared to be shed in feces for about one week, and the first detectable serologic response, consisting at least in part of IgM anti-HAV, coincided with cessation of fecal antigen shedding. While it is not possible to determine accurately the incubation period from our data, it appears to be similar to that of human hepatitis A.

Although the mechanism of transmission within the colony has yet to be established, the presence of HAV antigen in fecal specimens suggests that fecal-oral transmission was likely. The relatively large number of monkeys routinely purchased by the GML appears to have maintained a continuous supply of susceptible monkeys into the colony and probably explains how transmission had continued in the apparent absence of persistent viral shedding. Whether transmission was initiated in the colony by the addition of an infected monkey or from an infected human source is unknown. What is apparent, however, is that transmission within the colony under existing husbandry techniques was continuous over a period of at least two years and that virtually all susceptible monkeys eventually became infected.

The question remains as to whether the virus detected was human HAV or a closely related virus of A trivirgatus. The virus reacted well in conventional HAV assays, and antigen detected was specifically blocked in the radioimmunoassay by reference anti-HAV sera. Furthermore, cross-blocking experiments in parallel radioimmunoassays employing either human or A trivirgatus antigens did not reveal any significant differences between these strains. A significant biologic difference between the virus found in the GML colony and human HAV is, however, suggested by the fact that monkeys held at the GML colony appeared to be uniformly susceptible to infection with the GML strain of virus. In contrast, neither of two A trivirgatus inoculated orally with infectious human HAV developed anti-HAV in a previous study [Purcell and Dienstag, 1978]. Although this apparent difference in susceptibility might possibly be due to differences in inoculum size, it seems likely that the virus found at the GML has become adapted to A trivirgatus by multiple passage. Adaptation of human HAV to a nonhuman primate species, as evidenced by a shortening of the incubation period, an increase in the amount of viral antigen found in liver, and an enhanced ability to infect members of that species, has been observed following repeated passage of HAV in Saguinas mystax [Provost et al, 1977]. Whether or not the virus described here was ultimately of human origin, our observations suggest that A trivirgatus may be a useful animal for further investigation of the pathogenesis of human hepatitis A.

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