

Duration of Viremia in Hepatitis A Virus Infection

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The duration of viremia and time course for development of IgM antibodies were determined prospectively in natural and experimental hepatitis A virus (HAV) infection. Serial serum samples from HAV-infected men ($n = 13$) and experimentally infected chimpanzees ($n = 5$) were examined by nested reverse-transcriptase polymerase chain reaction analysis to detect HAV RNA and by ELISA to detect IgM antibodies to HAV. Among infected humans, HAV RNA was detected an average of 17 days before the alanine aminotransferase peak, and viremia persisted for an average of 79 days after the liver enzyme peak. The average duration of viremia was 95 days (range, 36–391 days). Results were similar in chimpanzees. In addition, HAV RNA was detected in serum of humans and chimpanzees several days before IgM antibodies to HAV were detected. These results indicate that adults with HAV infection are viremic for as long as 30 days before the onset of symptoms and that the duration of viremia may be longer than previously described.

Infection with hepatitis A virus (HAV) is the leading cause of clinically apparent viral hepatitis in the United States [1]. HAV infection is generally self-limiting and is primarily transmitted via the fecal-oral route through contact with an infected person [2]. Although a rare occurrence, transmission through the parenteral route is possible, with cases reported after blood transfusions in neonates and adults [3–5]. Recently, cases of hepatitis A have been linked to receipt of factor VIII concentrates in Europe [6–9] and South Africa [10] and to receipt of factor VIII and IX concentrates in the United States [11].

We used gene amplification by the polymerase chain reaction (PCR) to determine the frequency and duration of hepatitis A viremia in persons with disease and in experimentally infected chimpanzees. In addition, the genetic variation that occurred during the course of infection was determined for selected regions of the HAV genome.

Materials and Methods

Human subjects and serum samples. A retrospective analysis was conducted on archived serum specimens obtained from par-

ticipants in a clinical trial to determine the efficacy of hepatitis B vaccine [12]. As part of the study, serum was collected from each subject at ~1-month intervals and were tested for alanine aminotransferase (ALT) levels and serologic markers of hepatitis B virus (HBV) infection. Subjects with an elevated ALT level were followed by repeat testing until the ALT returned to normal. Subjects with elevated ALT levels and no serologic markers of HBV infection were subsequently tested for acute HAV infection. Archived serum specimens and associated data have been maintained at the Centers for Disease Control and Prevention with no link to participant identity.

Subjects for the present study were chosen from individuals who converted from negative to positive for IgM antibody to HAV (IgM anti-HAV), had an available serum sample drawn <1 month before their peak ALT, and had frequent follow-up serum samples. Because of the retrospective nature of the study, however, subjects often did not have an archived serum specimen that corresponded to each recorded ALT value. Serum specimens were stored at -20°C or -70°C until analyzed.

Experimental HAV infection in chimpanzees. Serum samples were retrospectively analyzed from 2 chimpanzees inoculated intravenously with 10^6 chimpanzee infectious doses (CIDs) of an HAV inoculum designated HLD-2 [13], 2 chimpanzees inoculated intravenously with 1 CID of HLD-2, and 1 chimpanzee orally inoculated with 100 CIDs of HAV inoculum designated SD-11 [14, 15]. After inoculation, serial serum samples were obtained at intervals of 3–4 days and were stored at -70°C until analyzed. Again, because of the retrospective nature of the study, sufficient sample quantity was not available for each time point in every animal.

Anti-HAV IgM detection. Qualitative detection of IgM antibody to HAV was performed by enzyme immunoassay (HAVAB-M EIA; Abbott Laboratories, Abbott Park, IL), using the overnight incubation method.

Hepatitis C virus (HCV) antibody detection. Qualitative detection of total antibody to HCV was performed by ELISA (OR-THO HCV, version 3.0; Ortho Diagnostic Systems, Raritan, NJ).

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This study used archived unlinked serum samples from participants in the original clinical trial. For the original study, informed consent was obtained from each test subject, and human experimentation guidelines of the US Department of Health and Human Services and those of the Centers for Disease Control and Prevention were followed in the conduct of clinical research and animal experimentation.

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Location and Primers	Nucleotides	
	No.	Sequence
VP3-VP1 junction (N terminal)		
External (Human)	2172	5'GCTCCTCTTTATCATGCTATGGAT3'
	2415	5'CAGGAAATGTCTCAGGTACTTTCT3'
Internal (Human)	2195	5'ATGTTACTACACAAGTTGGAGAT3'
	2380	5'GATCCTCAATTGTTGTAGCT3'
External (Chimpanzee)	2132	5'GTTATTGTTTATCTTTAGCAAT3'
	2451	5'GATCTGATGATGCTCTGGATCT3'
Internal (Chimpanzee)	2172	5'GCTCCTCTTTATCATGCTATGGAT3'
	2415	5'CAGGAAATGTCTCAGGTACTTTCT3'
VP1-P2A junction (C terminal)		
External	2870	5'GACAGATTCTACATTGGATTGGT3'
	3381	5'CCATTTCAGAGTCCACACACT3'
Internal	2897	5'CTATTGAGATTGCAAAATTACAAT3'
	3288	5'AACTTCATTATTCATGCTCTCT3'
5' UTR		
External	236	5'CTCTCCCTTGCCCTAGGCTCT3'
	514	5'CCAGTCTCCGCGTGAATGGT3'
Internal	244	5'CTTGCCCTAGGCTCTGGCCG3'
	459	5'CAATATCCGCGCTGTACCTAT3'

Figure 1. Oligonucleotide primers used for the amplification of hepatitis A virus (HAV) RNA from clinical specimens. External primers were used for first-round amplification, and internal primers were used for second-round nested amplification. Nucleotides are numbered according to the wild-type HAV HM 175 sequence [18].

Specimens that initially tested positive for anti-HCV by ELISA were confirmed by strip immunoblot assay (RIBA HCV 3.0; Chiron, Emeryville, CA).

HAV RNA detection. RNA was extracted from serum by using TriPure reagent (Boehringer Mannheim, Indianapolis, IN) in the presence of a glycogen carrier, as described elsewhere [16]. HAV RNA was detected by nested reverse-transcriptase PCR analysis, using primers that identified 3 distinct areas of the genome: the VP3/VP1 region, the VP1/P2 junction, and the 5' untranslated region (UTR). Amplification of the capsid regions among infected chimpanzees was performed, using previously described primers and methods [16], whereas different primers were used for amplification of the VP3/VP1 capsid region among infected humans [17]. All primers used are listed in figure 1.

In specimens negative for HAV RNA when tested with capsid-region primers, HAV RNA was reextracted and reamplified, using primers for the 5' UTR, with minor modifications to the technique described above. The modifications were as follows: 2 mM di-thiothreitol (Promega, Madison, WI) was added to the cDNA reaction, and 5% dimethylsulfoxide was added to the first-round PCR amplification reaction. In all instances, PCR products were identified by electrophoretic separation on 2% agarose gels, followed by ethidium bromide staining for visualization.

Nucleic acid sequencing. One hundred microliters of PCR product were purified and sequenced as described elsewhere [16]. Sequences were compared, using Sequence Navigator software (Applied Biosystems, Foster City, CA), and further analysis was preformed with Wisconsin package 9.1 (Genetics Computer Group, Madison, WI) [19].

Serum HAV concentration. The concentration of HAV was estimated as both infectious virus plaque-forming units (pfu) per milliliter and particles per milliliter. Cell culture-derived HAV of known concentrations (5×10^7 pfu/mL) was diluted in serum, and concentrations ≥ 0.05 pfu/mL were detected by the amplification methods described above (data not shown). On the basis of a

particle:pfu ratio of $\sim 79:1$ [20], the limit of detection for this assay was ~ 4 HAV particles/mL. Study samples with an electrophoretic product visible by ethidium bromide staining after first-round PCR amplification were considered to have an HAV concentration >5 pfu/mL (~ 400 HAV particles/mL). These samples were subsequently diluted in normal human serum, and HAV RNA was extracted and amplified by nested PCR to determine the end point positive dilution. The HAV concentration was estimated by comparing the signal from the end point dilution with the signal obtained from cell culture-derived HAV of known concentration serially diluted in normal human serum [21].

Results

HAV-Infected Humans

HAV RNA in serum. Thirteen persons met the selection criteria and had an average of 8 (range, 3–18) serum samples collected before and after their peak ALT. These samples were collected from 94 days before to 677 days after the ALT peak. HAV RNA was detected in serum specimens from all 13 study subjects. In 1 subject, however, HAV RNA could be detected only by amplification of the 5' UTR.

Two study subjects had an ALT pattern consistent with relapsing hepatitis A. Each had an initial rise and fall in ALT, followed 1–2 months later by a second rise and fall in ALT. HAV RNA was present in serum during the initial rise in ALT, became undetectable once ALT levels dropped, and reappeared during the second ALT peak. In one individual, HAV RNA was detected throughout the initial episode of hep-

Table 1. Comparison of number of days before and after peak alanine aminotransferase (ALT) for polymerase chain reaction and anti-hepatitis A virus IgM results.

Subject type and no.	Days from peak ALT		
	First positive		Last positive RNA
	RNA	IgM	
Human			
GO1-3664	−21	+25	+115
GO1-4839	−33	+15	+65
GO1-4680	0	−13	+69
GO1-5731	−29	+11	+18
GO1-1771	−17	−7	+53
GO1-5826	0	0	+36
GO1-2646	−12	0	+53
GO1-6834	−29	0	+34
GO1-5665	−13	0	+383
GO2-28177	−20	0	+62
GO3-912	0	0	+62
GO3-1120	−9	−9	+36
GO3-2289	−20	+9	+44
Chimpanzee (dose)			
CH-1357 (10 ⁶ CID)	−22	−11	+69
CH-1537 (10 ⁶ CID)	−17	−4	+53
CH-1487 (10 ² oral CID)	−18	−3	+26
CH-1562 (10 ⁰ CID)	−14	+4	+11
CH-1568 (10 ⁰ CID)	−14	0	+11

NOTE. CID, chimpanzee infectious dose.

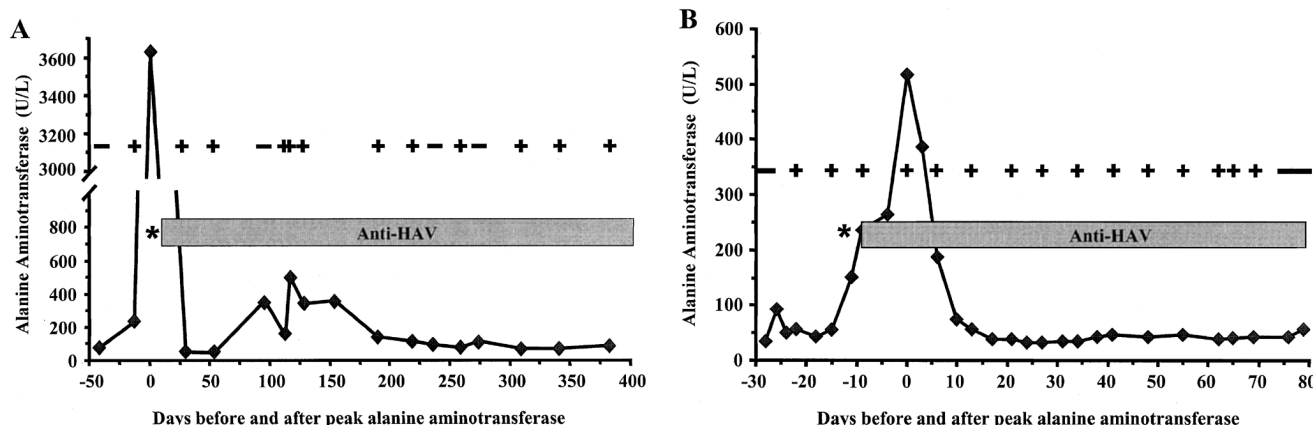


Figure 2. Relationship of detection of hepatitis A virus (HAV) RNA to levels of antibody and liver enzyme (alanine aminotransferase, U/L) in serum. +, Positive for HAV RNA by polymerase chain reaction (PCR) analysis; −, negative for HAV RNA by PCR analysis. *First detection of IgM antibody to HAV (anti-HAV) in human (GO1-5665; *A*) and in experimentally infected chimpanzee (CH-1357; *B*).

atitis and the relapse and was intermittently detected for an additional 150 days after the period of relapse (figure 2*A*).

The longest period that HAV RNA was detected before the peak ALT was 33 days; however, HAV RNA was not detected in 7 study subjects from whom specimens were collected 38–94 days before their ALT peak. HAV RNA was detected in 70% (95% confidence interval [CI] 35%–92%) of study subjects ($n = 10$) with specimens collected 15–37 days before peak the ALT and in 80% (95% CI, 30%–99%) of subjects ($n = 5$) with specimens collected within 14 days before the ALT peak. All study individuals ($n = 11$) tested were HAV RNA positive at the time of the ALT peak, and viremia continued in all study subjects ($n = 13$) for 18–69 days after peak ALT. In samples collected ≥ 70 days after peak ALT, the number of viremic patients declined rapidly, with only 2 (40%; 95% CI, 7%–83%) remaining HAV RNA positive (figure 3). These 2 individuals had HAV RNA detected 115 and 383 days after peak ALT. For all study subjects, the mean duration that HAV RNA was detected in serum was 95 (range, 36–391) days. The first and last days of HAV RNA positivity related to the peak ALT for each study subject are listed in table 1.

Hepatitis A viremia was not always associated with an elevated ALT. In subjects who were HAV RNA positive ≥ 20 days before peak ALT, 67% (95% CI, 24%–94%) had normal ALT levels (< 53 U/L). ALT levels in all (95% CI, 72%–99%) study subjects became elevated by 17 days before the peak ALT, and all were HAV RNA positive at the time of the first documented ALT elevation. ALT values returned to normal in 42% (95% CI, 17%–71%) of study subjects 20–69 days after the ALT peak. For the 2 patients with viremia that persisted > 70 days after the ALT peak, one had consistently normal ALT levels, and the other initially had normal ALT levels, but they again became elevated as part of a clinical picture consistent with relapsing hepatitis A. In addition, 3 subjects had ALT levels that

did not return to normal. One was HAV RNA positive at the time he was lost to follow-up, 34 days after peak ALT. The other 2 subjects were HAV RNA positive 65 and 383 days after peak ALT and had persistently elevated ALT levels for 302 and 677 days, respectively, before they were lost to follow-up. The individual with persistently elevated ALT levels for > 1 year (677 days) was also anti-HCV positive and had the longest documented viremia (396 days). All other study subjects were anti-HCV negative.

Serum samples collected > 20 days before the ALT peak or > 29 days after the ALT peak were found to be positive for HAV RNA only after nested PCR and were considered to contain < 5 pfu/mL HAV (~ 400 HAV particles/mL) (see Materials and Methods). By contrast, all samples obtained in the 20-day period before the ALT peak were HAV RNA positive after first-round PCR amplification, as were 25% (95% CI, 5%–64%) of samples obtained ≤ 29 days after the ALT peak. The estimated HAV concentrations by end point positive dilution during this 49-day period were 10^4 – 10^5 pfu/mL ($\sim 790,000$ – $7,900,000$ HAV particles/mL) before the ALT peak, 10^2 – 10^4 pfu/mL (~ 7900 – $790,000$ HAV particles/mL) at the ALT peak, and 10 – 100 pfu/mL (~ 790 – 7900 HAV particles/mL) after the peak. The estimated HAV concentration 30 days after the peak ALT was 1–10 pfu/mL (~ 79 – 790 HAV particles/mL; table 2).

IgM anti-HAV. IgM anti-HAV was first detected in available serum samples ($n = 3$) 7–13 days before the ALT peak, whereas specimens ($n = 9$) collected 17–47 days before the ALT peak were IgM anti-HAV negative. Among 10 study subjects with serum samples available for testing 0–13 days before the ALT peak, 9 were IgM anti-HAV positive. The one IgM anti-HAV–negative person did not become IgM anti-HAV positive until > 7 days after the ALT peak. Three study subjects had sufficient serum to test only from those specimens collected > 7 days after peak ALT, and all were positive (table 1).

Table 2. Hepatitis A virus (HAV) concentration at times before and after peak alanine aminotransferase (ALT).

Subject type and no.	Days from peak ALT	ALT (U/L)	HAV (pfu/mL)
Human			
GO1-1771	-17	121	10 ⁴
	-7	1790	10 ⁵
	0	3080	10 ²
	+53	55	10 ¹
GO2-28177	-20	76	10 ⁴
	0	230	10 ²
	+29	47	10 ²
	+62	39	10 ²
GO3-1120	-9	670	10 ⁵
	0	1040	10 ³
	+36	66	10 ¹
	0	5200	10 ⁴
GO3-912	+12	400	10 ²
	+42	390	10 ¹
Chimpanzee (dose)			
CH-1357 (10 ⁶ CID)	-22	57	10 ³
	-9	235	10 ²
	0	517	10 ⁴
	+13	57	10 ²
CH-1487 (10 ² oral CID)	+69	42	10 ¹
	-18	67	10 ⁰
	-8	66	10 ²
	-3	262	10 ⁴
CH-1568 (10 ⁰ CID)	0	982	10 ³
	+4	530	10 ³
	+23	58	10 ²
	+26	68	10 ⁰
CH-1568 (10 ⁰ CID)	-14	47	10 ⁰
	-7	43	10 ³
	+4	122	10 ²
	+11	52	10 ¹

NOTE. The upper limit of normal ALT levels in human subjects is 53 U/L; the upper limit of normal ALT levels of CH 1357 is 50 U/L; the upper limit of normal ALT levels of CH 1487 is 91 U/L; and the upper limit of normal ALT levels of CH 1568 is 63 U/L. pfu, Plaque-forming units; CID, chimpanzee infectious dose.

Experimentally Infected Chimpanzees

HAV RNA in serum. The 2 chimpanzees that received the highest HAV intravenous concentration (10⁶ CIDs) were HAV RNA positive at the initial serum collection 5 and 6 days, respectively, after inoculation. In these animals, liver enzymes peaked at 22 and 28 days, respectively, after inoculation, and HAV RNA became undetectable in serum specimens collected 53 and 63 days after peak ALT. The duration of viremia in these animals was 70 and 91 days, respectively (figure 2B).

In the chimpanzee that received 100 CIDs of HAV orally, HAV RNA was first present in serum 21 days after inoculation, was present for an additional 18 days until the ALT peaked, and persisted for 26 days after the ALT peak. Altogether, HAV RNA was detected in the serum for 44 days.

In the 2 chimpanzees that received 1 CID of HAV intravenously, HAV RNA was first detected in serum 14 days after inoculation, and this persisted for an additional 14 days until the ALT peak. In addition, both animals remained HAV RNA

positive for 11 days after the ALT peak, for a total viremic period of 25 days (table 1).

As observed in infected humans, HAV RNA was detected in serum before ALT elevations. Chimpanzees that received 10⁶ CIDs were viremic 4–6 days before elevation of ALT levels, and HAV RNA was detected 32–53 days after ALT levels returned to normal. The chimpanzee that received 100 CIDs orally was viremic 10 days before ALT levels became elevated and remained viremic 9 days after hepatitis resolved. Similarly, the chimpanzees that received 1 CID intravenously had HAV RNA present for 7 days before elevation in ALT and remained viremic for 3 days after liver enzymes returned to normal.

Serum HAV concentration in chimpanzees that received the largest infectious inoculum (10⁶ CIDs) rose from 10² pfu/mL (~7900 HAV particles/mL) 17 days after inoculation to 10⁴ pfu/mL (~790,000 HAV particles/mL) at the time of the ALT peak and subsequently decreased to 1–10 pfu/mL (~79–790 HAV particles/mL) during the next 35 days (table 2). The chimpanzee that was inoculated orally had low initial serum HAV concentrations (10–100 pfu/mL) that rose to 10⁴ pfu/mL 3 days before peak ALT. After the ALT peak, virus concentrations dropped to 10–100 pfu/mL, and eventually virus was no longer detected. The chimpanzees that received the lowest infectious inoculum had the lowest serum HAV concentrations at all time points; the maximum serum concentration was 10³ pfu/mL 7 days before peak ALT. There were not, however, sufficient samples to determine virus concentration immediately before and after the peak ALT.

IgM anti-HAV. Antibody was not detected until 3–11 days before the ALT peak in all experimentally infected animals. IgM anti-HAV was detected 11 days before ALT peak in 1 chimpanzee that received 10⁶ HAV CIDs. However, the second

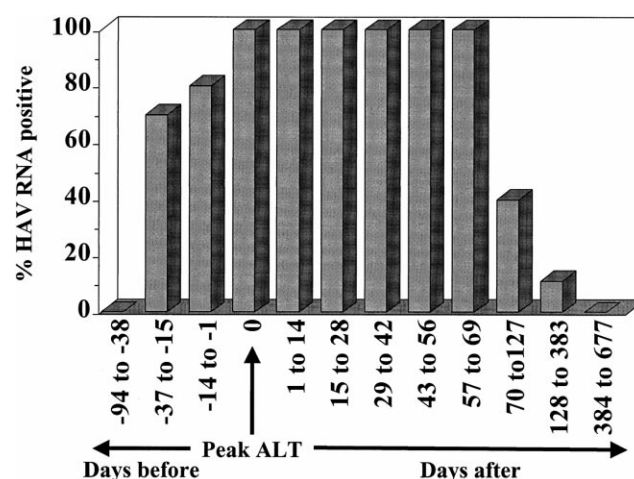


Figure 3. Percentage of human study subjects positive for hepatitis A virus (HAV) RNA before and after (in days) peak alanine aminotransferase (ALT) levels during HAV infection.

chimpanzee that received 10^6 HAV CIDs and the chimpanzee that received 100 oral HAV CIDs were not IgM anti-HAV positive until 3 and 4 days before peak ALT, respectively. Of the 2 chimpanzees that received 1 HAV CID, 1 animal became positive at the time of the ALT peak, and the other did not become positive until 4 days after the peak ALT (table 1).

Nucleotide sequence variation during infection. The nucleotide sequence of the 2 capsid-coding regions was determined for the earliest and latest HAV RNA-positive specimens available for each infected person and chimpanzee. In addition, the starting inocula were sequenced for the experimentally infected chimpanzees. In a pairwise analysis, all amplified isolates were genotype 1a. Among the 497 nucleotides examined for each isolate obtained from the infected humans, there was no variation between an individual's first and last specimens, a period ranging from 36 to 391 days.

In the experimentally infected chimpanzees, there was no sequence change in the ~530 nucleotides examined in the inoculum and the first HAV RNA-positive serum specimen for both HLD-2 and SD-11. In addition, no changes were observed between the first and last positive serum specimens for all 5 chimpanzees over a period that ranged from 25 to 91 days.

Discussion

Retrospective studies of human HAV infection have in general not been able to determine the duration of viremia, because of the lack of samples obtained during the preclinical stage of infection. The current study took advantage of serum specimens collected during a previous clinical trial, which allowed the prospective evaluation of serum HAV RNA during the entire course of human infection. Our results confirm earlier findings showing that serum infectivity was present for ~3–4 weeks before the onset of jaundice in human volunteers experimentally infected with HAV [22, 23] and that viremia develops 1–2 weeks after inoculation in experimentally infected nonhuman primates [24–26]. In addition, our study showed that high concentrations of virus were present during the period that precedes the onset of liver enzyme elevations and that IgM antibody to HAV was generally not present until shortly before the onset of hepatitis.

The results of the present study also indicate that viremia may be present for a much longer period during the convalescent phase of hepatitis A than was previously appreciated. In earlier human studies, serum infectivity was present ≤ 3 days after the onset of jaundice [22, 23], and HAV RNA was detected for an average of 18 days after the onset of clinical symptoms [27, 28]. We found that HAV RNA was present for an average of 95 days during human HAV infection and that the viremia persisted longer after the onset of symptoms (average, 79 days); however, HAV concentration was lowest during the convalescent period. In addition, IgM anti-HAV was present during this time, and previous studies have shown the presence of IgG anti-

HAV [29]. In nonhuman primates, previous studies found viremic periods of ~20 days [24–26]. Again, we found the viremic period to be longer (range, 25–91 days), and, in general, viremia was present for a longer period after the onset of symptoms.

Blood collected before the onset of symptoms appears to be at highest potential risk of transmitting HAV infection because of the high concentrations of virus and essentially no antibody. The risk for parental transmission of HAV after the onset of symptoms, however, is less clear. The PCR method used in this study detected HAV RNA at the level of ~4 HAV particles/mL and appears to have been more sensitive than assays used in other studies [24–28]. This level of detection, however, may be below the number of viral particles required to establish an acute infection.

Among the experimentally infected chimpanzees, the onset and duration of viremia were directly related to virus concentration in the inoculum. Previous studies in experimentally infected chimpanzees have not shown a relationship between the virus concentration in the inoculum and the duration of incubation period, the duration of enzyme elevations, or the degree of inflammatory response [30, 31]. The route of inoculation, however, has been shown to shorten the incubation period [30, 32].

Serum HAV RNA was generally detected before detection of IgM anti-HAV. In the experimentally infected chimpanzees, HAV RNA was detected an average of 14.4 days before IgM antibody, and there was no association between the inoculum size and the time between detection of HAV RNA and detection of anti-HAV IgM antibody. The human data were similar to the chimpanzee data in that, except for one subject, HAV RNA was detected before anti-HAV IgM detection. Because of the timing of the serial samples, the interval between detection of HAV RNA and anti-HAV IgM could not be estimated precisely.

The sequence data from humans indicate that no change occurred during the course of the infection, that relapsing infection was related to the initial infecting virus, and that no change occurred during periods of resolution that lasted for as long as 1 year. The chimpanzee sequence data similarly show no change between the inoculating virus, the virus isolated at the beginning of the infection, and the virus isolated at the resolution of the infection. This suggests that the HAV genome remains stable throughout an infection and during passage from person to person.

As demonstrated by case reports of hepatitis A outbreaks related to blood products [3–11], there appears to be an interval during the incubation period when parenteral transmission is most likely to occur. Although PCR positivity does not equal infectivity, our data suggest that the period during which blood can potentially transmit HAV infection may be longer than was previously appreciated.

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