Characterization of a Simian Hepatitis A Virus (HAV): Antigenic and Genetic Comparison with Human HAV

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Received 1 May 1989/Accepted 31 July 1989

PA21, a strain of hepatitis A virus (HAV) recovered from a naturally infected captive owl monkey, is indistinguishable from human HAV in polyclonal radioimmunoassays and cross-neutralization studies. However, cDNA-RNA hybridization has suggested a significant difference at the genomic level between PA21 and a reference human virus, HM175. Further characterization of this unique HAV was undertaken in an effort to determine the extent of genetic divergence from human HAV and its relation to the conserved antigenic structure of the virus. The close similarity between PA21 and HM175 antigens was confirmed with an extended panel of 18 neutralizing murine monoclonal antibodies: a reproducible difference in binding to the two viruses was detected with only one antibody (B5-B3). The nucleotide sequence of the P1 region of the PA21 genome had only 83.2% identity with HM175 virus, a difference approximately twice as great as that found between any two human strains. Most nucleotide changes were in third base positions, and the amino acid sequences of the capsid proteins were largely conserved. Amino acid replacements were clustered in the carboxy terminus of VP1 and the amino-terminal regions of VP2 and VP1. These data indicate that PA21 virus represents a unique genotype of HAV and suggest the existence of an ecologically isolated niche for HAV among feral owl monkeys.

Hepatitis A virus (HAV) is a uniquely pathogenic hepatotropic picornavirus associated with acute viral hepatitis in humans (17). Infection with HAV results in solid, lifelong immunity, due in part to the absence of antigenic variation among HAV isolates. Although direct comparisons are limited in number, human HAV strains are antigenically indistinguishable from each other in solid-phase immunoassays and neutralization tests employing either convalescent human polyclonal antibodies (18, 22) or murine monoclonal antibodies (20). At the genomic level, the nucleotide sequences of these viruses have been estimated by T₁ oligonucleotide mapping to differ by only 1 to 4% (38), while a comparison of cDNA sequences derived from several human HAV isolates indicates a 3 to 9% difference (5, 23, 25, 29, 32, 36; J. Ticehurst, personal communication). Thus, existing human isolates of HAV represent a relatively homogeneous collection of closely related viruses, having as a group significantly less genetic diversity than that found among isolates of single poliovirus serotypes (31).

Most previous studies have suggested that natural infection with HAV is confined to humans (8). However, a variety of higher primate species, including chimpanzees (*Pan troglodytes*) (13), marmosets (*Saguinas* sp.) (9), and owl monkeys (*Aotus trivirgatus*) (16), have been shown to be susceptible to infection. Serologic evidence of infection with HAV has been found among primates held in captivity (12), but such infection generally has been considered to be of human origin (8, 28). HAV appears capable of bidirectional transmission between captive primates and their human keepers, although the ultimate origin of virus causing hepatitis A in animal handlers has never been entirely clear.

In 1980, an HAV strain (PA21) was recovered from a feral owl monkey shortly after its capture and admission to a primate-holding facility in Panama (22). Serologic testing of other owl monkeys demonstrated widespread infection with HAV within the colony during the preceding years, with

most wild-caught animals seroconverting within months of capture. Virus recovered from infected monkeys was found to be antigenically similar to human HAV by quantitative in vitro neutralization assays (19), in cross protection studies, and in preliminary studies with monoclonal antibodies (20). Yet slot blot cDNA-RNA hybridization with probes derived from human HAV suggested that the PA21 virus was distinct from human HAV isolates (20). We now report the further characterization of PA21 virus, undertaken in an effort to determine the basis of its genetic diversity and how this might relate to its highly conserved antigenic structure.

PA21 virus was adapted to growth in continuous green monkey kidney cells (BS-C-1) as described previously (2). The physical characteristics of PA21 particles produced in BS-C-1 cells were compared with those of the prototype human HAV strain, HM175, propagated in the same cell line. Centrifugation of both PA21 and HM175 virus harvests through linear rate-zonal sucrose gradients demonstrated similar sedimentation profiles, with two major peaks of antigen activity having sedimentation characteristics corresponding to empty and full capsids (Fig. 1) (21, 34). The PA21 antigen was distributed almost equally between the two particle types, while approximately threefold more HM175 antigen was associated with complete virions than with empty capsids. This difference in the distribution of antigen was constant for multiple purifications of both viral strains. Fractions recovered from these gradients were blotted onto nitrocellulose and hybridized with the pHAV_{CH}119-1 probe (representing nucleotides 158 to 843 of the HM175 genome). The resulting hybridization signal was strongest with fractions containing complete virus particles, but weaker hybridization signals were found with fractions tailing into the empty-capsid region of the gradient (Fig. 1). In both virus-containing gradients, there was a suggestion of a discrete population of particles yielding positive hybridization signals in fractions immediately preceding the empty-capsid peak (fractions 18 and 19). Thus, with the exception of proportionately more empty capsids in PA21 virus har-

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FIG. 1. HAV antigen profile of HM175 (top) or PA21 (bottom) virus after centrifugation through 10 to 30% (wt/vol) sucrose gradients. Antigen (■) was detected by a radioimmunoassay, and the refractive index was measured with an Abbe-type refractometer. A sample of each fraction was blotted onto nitrocellulose and hybridized with a ^{32}P -labeled pHAV_{CH}119-1 cDNA probe. The relative optical densities of the autoradiograms were determined by a laser densitometer and plotted as blot intensity (+).

vests, the sedimentation characteristics of PA21 and HM175 viruses were identical, including the possibility of a subpopulation of slowly sedimenting RNA-containing particles.

To more fully characterize the antigenic relatedness of the PA21 and HM175 viruses, we measured the binding of each of an expanded panel of 18 neutralizing murine monoclonal antibodies to virus that had been captured on a solid-phase support with polyclonal human antiserum (the sources of 13 of these antibodies have been described previously [30]; 14-1-C29, H29-C26, H10-C29, H80-C25, and H7-C27 were the generous gift of R. H. Decker, Abbott Laboratories, North Chicago, Ill.). Previously described direct and indirect radioimmunoassay methods were employed for this analysis, which utilized quantities of sucrose-CsCl gradient (7)-purified viruses that were roughly standardized according to viral RNA content (30). In replicate experiments employing an indirect immunoassay, reduced binding of monoclonal antibodies to the simian virus strain was suggested only with antibodies 10.09 and B5-B3 (82 and 78%, respectively; Fig. 2). Direct immunoassays, which compared the binding of radiolabeled monoclonal antibodies to HM175 and PA21, demonstrated markedly reduced binding (20 to 30%) of B5-B3 antibody to PA21 and only questionably reduced binding of 10.09 and 2D2 (Fig. 2). Thus, these studies confirmed the reduced affinity of the B5-B3 antibody for PA21 virus but failed to demonstrate conclusive differences in the binding of the other 17 monoclonal antibodies. As these antibodies are directed against immunodominant neutralization epitopes of HAV (30), the results indicate a high degree of conservation of the critical antigenic determinants of these two HAV strains.

To compare the genomic RNAs of the viruses, standardized quantities of RNA extracted from CsCl-sucrose gradient (7)-purified PA21 and HM175 viruses were electrophoresed through a formaldehyde-agarose gel, transferred to nitrocellulose paper, and hybridized with probe pHAV_{CH}119-1 (Fig. 3, lanes A and B). The size distribution of virion RNA from both virus strains was similar: most RNA was full-length, migrating with a 7.5-kilobase standard. However, in addition to a smear of shorter-length RNAs, discrete bands of subge-

nomic-size RNA were evident in both PA21 and HM175 RNA preparations. When the blot was stripped and then rehybridized with a probe derived from the P1 genomic region of PA21 virus (pHAV_{CH}D64, nucleotides 896 to 3661; see below) (Fig. 3, lanes C and D), full-length HM175 RNA failed to produce a significant signal. The failure of the PA21 probe to hybridize with full-length HM175 virus RNA confirmed the existence of significant genetic divergence between these viruses and indicated that previous hybridization results (20) were not related to deletions in the PA21 genome. However, the smaller PA21 RNA species evident after probing with the 5' HM175 cDNA probe (pHAV_{CH}119-1) were not seen after probing with the P1 region PA21 probe, suggesting that these subgenomic-length RNAs may represent virion RNA with deletions in the P1 region, as suggested by Nüesch et al. (26). When the blot was again stripped and rehybridized with a probe derived from the 3' region of the HM175 genome (pHAV_{CH}12-1, which extends from nucleotides 6058 to 7463), there was a much-reduced hybridization signal with PA21 RNA (Fig. 3, compare lanes E and F). However, subgenomic-length RNA was again identified in the HM175 RNA lane. These findings are thus consistent with the presence of minor populations of particles containing genomes with substantial deletions or truncations in both PA21 and HM175 virus stocks.

An examination of RNA associated with more slowly sedimenting particles (fractions 18 and 19 of the gradients shown in Fig. 1) revealed RNA species similar to those demonstrated in Fig. 3. No RNA was detected in the empty-capsid fractions, while the more slowly sedimenting RNA-containing particles were associated with full-length and much lesser amounts of subgenomic-length RNA (results not shown).

The apparent genetic divergence of the P1 regions of PA21 and human HAV genomes in the face of high-level conservation of the immunodominant neutralization epitopes prompted the determination of the nucleotide sequence of the simian virus. cDNA was synthesized from PA21 virus RNA by the method of Gubler and Hoffman (14) and cloned into the *Eco*RI site of pTZ18R after linker addition. Single-stranded DNA was prepared from pTZ18R clones with

FIG. 2. Binding of murine monoclonal antibodies to immobilized PA21 viral antigen expressed as the mean percentage of the amount of antibody bound to PA21 compared with HM175 antigens in replicate assays. The results of all 18 monoclonal antibodies in an indirect assay (▨) and results of tests with four antibodies in a direct immunoassay (■) are shown. Error bars reflect 1 standard deviation.

FIG. 3. Northern (RNA) blot analysis of RNA extracted from HM175 (lanes A, C, and E) and PA21 (lanes B, D, and F) virions. The blot was sequentially hybridized with three cDNA probes: lanes A and B, 5' HM175 probe; lanes C and D, P1 PA21 probe; lanes E and F, 3' HM175 probe. The marker was a 7.5-kilobase poly(A) tailed RNA.

helper bacteriophage M13K07 and sequenced by the dideoxy-chain termination method of Sanger et al. (33). Overlapping cDNA clones spanning part of the 5' noncoding region, the complete P1 region, and the 5' portion of the P2 region of PA21 virus were sequenced completely, and the derived sequences were compared with known sequences of human HAV strains. Additional sequence was obtained from the 3' noncoding region and the 3' terminus of the P3 genomic region of PA21 virus. Altogether the 5' noncoding, P2, P3, and 3' noncoding regions were found to have 89.2, 79.6, 84.4, and 93.7% nucleotide identity, respectively, with respect to the human HM175 virus (Table 1) (5). The G+C content of PA21 RNA was found to be 39.5%, only slightly higher than that of other HAV strains (5). The typical HAV bias against the CG nucleotide pair was present (frequency of use, 0.4%) (5).

The P1 region of PA21 genomic RNA was found to comprise 2,373 nucleotides and to encode 791 amino acids. The nucleotide identity between PA21 and sequenced human HAV strains was approximately 83% in the capsid-encoding region (Table 1). The region encoding VP1 (Fig. 4) demonstrated the greatest divergence (78.4 to 81.3% identity with six human HAV strains), while the VP3-encoding region, as in comparisons of other picornaviruses, was the most conserved (84.7 to 86.0% identity). The majority of the nucleo-

tide changes occurred in the third codon position (343 of 398, in comparison with HM175), while differences present in the first or second positions often did not lead to a change in the amino acid. Thus, the amino acid sequence of the capsid proteins is largely conserved between the simian and human viruses.

Overall, the PA21 nucleotide sequence indicates that its capsid differs at only 23 (HM175 and MBB) to 31 (HAS15) amino acid residues (of 791) compared with the capsids of human HAV strains (Table 2). Most of these changes (16 of 23) are conservative in nature. Although the nucleotide changes are distributed randomly across the P1 region (except for the region encoding the carboxy terminus of VP1, at the 3' end of the P1 region), amino acid replacements are clustered near the amino termini of VP2 and VP1 and the carboxy terminus of VP1 (Fig. 5). Of the 31 carboxy-terminal amino acids of VP1, 8 are different from those of HM175 virus. When the amino acid sequence was aligned with a proposed tertiary structure of HAV capsid proteins (27), all of the nonconservative amino acid changes were found to be in surface loops and not in alpha helices or the beta pleated-sheet backbone structure. Posttranslational cleavage sites proposed by either Cohen et al. (5) or Diamond et al. (11) are unchanged.

It is possible that the genetic divergence evident between PA21 and the human HAV strains could reflect multiple passages of the PA21 strain through an alternate (owl monkey) host within the primate colony in Panama (38), even if PA21 was originally derived from humans. However, an analysis of sequences derived from several HM175 variants argues against this. The human HM175 strain has been sequenced in its entirety three times: as a wild-type variant passed three times in marmosets (wt HM175) (37), as a cell culture-adapted variant passed a total of six times in marmosets and then 16 times in cell culture (p16 HM175) (15), and as another cell culture-adapted and attenuated variant passed 35 times in cell culture after independent isolation directly from human feces (p35 HM175) (4). The sequences of these three HM175 variants, which are separated by three to six passages in marmosets, differ only by two to three nucleotides in the P1 region, making it difficult to contend that passage of an originally human strain of HAV in owl monkeys held in the primate colony could account for the large number of nucleotide changes found in PA21 virus.

TABLE 1. Percent nucleotide identity between PA21 and human HAV isolates

Genomic region	% Nucleotide identity to:					
	HM175	LA	MBB	CR326	HAS15	MS1
5' noncoding (bases 456 to 734)	89.2	88.9	89.6	88.6	89.0	
P1						
VP4 ^a	92.8	94.2	89.9	92.8	92.8	
VP2	83.0	82.7	83.0	82.4	83.5	
VP3	84.8	85.5	86.0	85.2	84.7	
VP1	81.3	80.0	80.3	80.6	78.4 ^b	80.6
Total (bases 735 to 3107)	83.2	82.9	83.1	82.9	82.1 ^b	
P2 2A (bases 3108 to 3661)	79.6	80.1	81.0		80.7	
P3 3D _{pol} (bases 6879 to 7415)	84.4	82.1	84.2		82.0	
3' noncoding (bases 7416 to 7493)	93.7	85.0	85.3		93.2	

^a Cleavage points between the PA21 capsid proteins were those proposed by Cohen et al. (5) for HM175 virus.

^b Includes 18-base deletion in VP1.

FIG. 4. Nucleotide and predicted amino acid sequence of the PA21 capsid protein VP1. Numbering and cleavage sites are based on those of Cohen et al. (5). The differences present in the HM175 sequence are displayed above (nucleotides) or below (amino acids) the PA21 sequence.

Although it has generally been accepted that captive primates become infected with HAV by transmission of virus from humans, there are several studies that suggest that HAV infection may occur naturally among some simian species in the wild (3, 6, 35). The identification of PA21 virus in captive owl monkeys in Panama prompted a serologic analysis of newly captured owl monkeys: 2 of 145 animals were found to have antibody to HAV (22). Similarly, Burke and Heisey determined that 18 of 54 cynomolgus monkeys freshly captured at a remote jungle location had anti-HAV antibodies (3). The prevalence of antibody among these animals was correlated with weight and thus age, indicating infection in the wild. Others have also found evidence of infection with HAV in recently captured primates (6, 35).

Although the possibility that PA21 was acquired from humans either recently or in the distant past cannot be ruled out with complete certainty, the substantial differences between PA21 and multiple human strains of HAV strongly suggest that this strain is native to the owl monkey. At the nucleotide level, PA21 is almost twice as different from the human strains as they are from each other. To further assess these differences, the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package DISTANCES program (10) was used to assign quantitative measures of the distances between the HAV strains. The HAV strains can be placed into two major groups: LA, HAS15, CR326, HM175, and MBB (the human isolates) and PA21 (Fig. 6). The human strains can be further divided into two groups. Those that are the most closely related genetically (LA, HAS15, and CR326) are also the most closely related geographically, their origins being California, Arizona, and Costa Rica, respectively. The other pair, MBB and HM175, originate from geographically distant areas

(North Africa and Australia, respectively) but have identical amino acid sequences. The most striking finding in this analysis, however, is the distance of PA21 from all other strains, even though it was recovered relatively close geographically to members of the LA-HAS15-CR326 group.

A similar analysis was carried out at the 3' end of the viral genome, adding the recently reported sequence of a strain isolated from an old-world cynomolgus monkey (1). The nucleotide sequences of the 3' 1,251 bases of the RNA of this

TABLE 2. Percent amino acid identities between capsid proteins of HAV strains

Virus	Protein	% Amino acid identity to protein of:					
		HM175	LA	MBB	CR326	HAS15	MS1
PA-21	VP4	100.0	95.7	100.0	95.7	95.7	94.3
	VP2	97.7	98.2	97.7	96.4	98.2	
	VP3	98.4	98.0	98.4	98.0	98.0	
	VP1	95.3	95.3	95.3	95.0	93.3 ^a	
	Total P1	97.1	97.0	97.1	96.3	96.2 ^a	
HM175	VP4		95.7	100.0	95.7		98.3
	VP2		99.1	100.0	97.3	99.1	
	VP3		99.6	100.0	99.6	99.6	
	VP1		99.3	100.0	99.0	97.0 ^a	
	Total P1		99.2	100.0	98.6	98.3 ^a	
LA	Total P1			99.2	99.1	99.0 ^a	
MBB	Total P1				98.6	98.3 ^a	
CR326	Total P1					98.2 ^a	

^a Includes six-amino-acid deletion.

FIG. 5. Amino acid differences between individual HAV strains and the consensus human virus sequence in the capsid region. The consensus sequence at each position was determined by the amino acid present in the majority of the human HAV strains. Each sequence is represented by a horizontal line, with regions encoding capsid proteins marked below this line. Positions where the encoded amino acid differs from the consensus sequence are shown by marks above the line.

strain (CYNO55) and PA21 virus are equivalently distant from HM175 (results not shown). The relationship between the human strains and PA21 in the 3' region is similar to that found with the P1 region (Fig. 6), but the CYNO55 strain is as different from PA21 as each simian virus is from the human strains. This suggests that there may be several genetically distinct types of HAV, each unique to a different primate species, a concept that is new to the field of hepatitis A.

Rico-Hesse et al. (31) have defined "genotypes" of poliovirus as groups of poliovirus isolates that differ at the nucleotide level by less than 15%. While arbitrary, this criterion has allowed useful distinctions to be made between geographically diverse isolates of poliovirus. According to this definition, all human HAV strains comprise a single HAV genotype, while the primate PA21 and CYNO55 isolates represent unique genotypes of HAV. It is notable that there are multiple genotypes among individual serotypes of poliovirus currently in circulation worldwide. A similar degree of genetic diversity is not seen among geographically diverse isolates of human HAV, although the reason for this distinction is not apparent at present. On the basis of limited genomic sequencing of the VP1-2A region, the poliovirus serotypes have an overall divergence of 29%, while within poliovirus serotype 1 the maximum divergence is 22.6% (31). These differences are slightly greater than that found between the PA21 and human HAV isolates (Table 1).

Differences in the nucleotide sequences of PA21 and HM175 viruses are randomly distributed throughout the capsid region, with the exception of a cluster of changes near the 3' end of the VP1-encoding region. The majority of these changes (85%) occur in the third base positions of codons, allowing for continued conservation of the amino acid sequence (96.7% average amino acid relatedness to the known human HAV sequences). In the derived amino acid sequence, changes are clustered into three areas: the amino termini of VP2 and VP1 and the putative carboxy terminus of VP1. Although most of these changes are conservative in nature, the frequency of substitutions within these regions (particularly at the carboxy terminus of VP1) argues that the primary structure of these domains is less critical for viral function or stability than other regions of the capsid proteins. Excluding the replacements evident at the termini of VP1 and VP2, there are few other differences between PA21

FIG. 6. Relationship among HAV strains according to the capsid protein nucleotide sequences. The distance along the horizontal lines connecting any pair or group of strains to the first common node denotes the percent nucleotide identity shared by those strains.

and the consensus human virus capsid protein sequence (Fig. 5). Four amino acid replacements are scattered throughout VP3. Two of these are nonconservative, a proline to alanine at 3-040 (residue 40 of capsid protein VP3) and a glycine to histidine at 3-147. Although VP3 is highly conserved among human HAV strains and has been shown to play an important role in the antigenic structure of the virus (30), it is impossible at this time to determine the importance of these changes or their relevance to the slight differences seen in the antigenic structures of PA21 and human HAVs.

The only difference apparent in a comparison of the neutralization epitopes of the PA21 and HM175 viruses was a slightly reduced binding of the B5-B3 monoclonal antibody to PA21 (Fig. 2). This suggests that at least one of the amino acid changes found in PA21 is within or near the B5-B3-binding site. Mutations found by Ping et al. (30) in neutralization-resistant HAV variants include amino acid substitutions at residues 3-070, 1-102, and 1-276. Mutations at 3-070 and 1-102 have each been shown to confer weak resistance to neutralization with B5-B3, while a mutation at 1-276 has not yet been shown to result by itself in antigenic changes in the virus. These residues do not correspond to any specific amino acid replacements found in PA21 virus. However, multiple changes do surround residue 1-276 in PA21, including a nonconservative serine-to-aspartic acid change at 1-277 and an alanine-to-leucine change at 1-281. Except for the isoleucine-to-valine change at 2-063 (24), these are the only changes in the PA21 capsid which align with recognized antigenic sites in other picornaviruses (27), providing at least indirect evidence for the possible involvement of this region of VP1 in the conformationally defined antigenic site of HAV.

This work was supported in part by Public Health Service grants AI07151, AI07001, and AI22279 from the National Institutes of Health.

We appreciate the advice and assistance of Shannon Kenny.

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