Immunity in Chimpanzees Chronically Infected with Hepatitis C Virus: Role of Minor Quasispecies in Reinfection

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Received 30 January 1997/Accepted 18 November 1997

We have previously reported that chimpanzees chronically infected with hepatitis C virus (HCV) could be reinfected, even with the original infecting strain. In this study we tested the hypothesis that this might reflect the presence of minor quasispecies to which there was little or no immunity. To evaluate this hypothesis, we sequenced multiple clones taken at intervals after primary infection and rechallenge from four chronically infected chimpanzees. The inoculum used in these studies (HCV-H, genotype 1a) revealed 17 separate variants among 46 clones sequenced. Following challenge, each of the four challenged animals showed marked alterations of their quasispecies distribution. The new variants, which appeared 1 to 6 weeks after challenge, were either identical to or closely resembled variants present in the challenge inoculum. These results, paralleled by an increase in viremia in some of the challenged animals, suggest that quasispecies in the challenge inoculum were responsible for signs of reinfection and that there was little immunity. However, the newly emerged quasispecies completely took over infection in only one animal. In the remaining three chimpanzees the prechallenge quasispecies were able to persist. The natural evolution of infection within chimpanzees resulted in variants able to compete with the inoculum variants. Whether through reexposure or the natural progression of infection, newly emerged quasispecies are likely to play a role in the pathogenesis of chronic HCV infection.

Hepatitis C virus (HCV) is estimated to chronically infect about 400 million people worldwide. More than half of these develop chronic active hepatitis, cirrhosis, or hepatocellular carcinoma. The HCV genome consists of a single-stranded RNA molecule approximately 10 kb long which contains a single open reading frame encoding approximately 3,000 amino acids (1, 5). There are at least six genotypes of HCV, and within a given patient the genomes are distributed among quasispecies which show sequence variation, particularly in the variable regions of the genome (4, 9). Hypervariable region 1 (HVR1) is a 27-amino-acid segment in the amino terminus of the second envelope protein which has been identified as the most variable region of the viral genome (11, 20). Sequential changes have been observed during the course of chronic HCV infections in chimpanzees and in humans (4, 11, 12). It has been postulated that these reflect immune system selection of neutralizing epitopes encoded by HVR1 (18, 19) and that persistent infection depends on the ability of the virus to continuously evade the effects of neutralizing antibody (7, 10, 15, 17, 20). Due to its variability, HVR1 has been used extensively as an indicator of viral evolution.

We have previously reported that chronically infected chimpanzees could seemingly be reinfected, even with the original infecting strain (13). In a recent report a similar phenomenon was observed in patients with posttransfusion hepatitis (6). We postulated that this might reflect the presence of minor quasispecies in the inoculum to which there was little or no immunity (13). Here we test this hypothesis by sequencing multiple clones of HVR1 derived at intervals after initial infection and after rechallenge.

MATERIALS AND METHODS

Chimpanzees. The chimpanzees were housed in the New York Blood Center’s primate laboratory, Vihl II, at the Liberian Institute for Biomedical Research in Robertsfield, Liberia. The animals were housed in minimum groups of two in spacious outdoor enclosures. As shown in Table 1, the chimpanzees in this study were initially infected with HCV-H (genotype 1a), and they subsequently developed chronic infection. At varying periods (1.3 to 4.2 years) after infection, they were rechallenged with the same inoculum. Serum samples were taken at weekly or biweekly intervals throughout the study. These samples were flash frozen and maintained continuously at −70°C.

Extraction of viral RNA. The HCV RNA kit (Qiagen, Chatsworth, Calif.) was used to extract viral RNA from 120 μl of serum taken 6 weeks after the primary infection, 1 week before rechallenge, and 1, 3, and 6 weeks after rechallenge.

RT and PCR. Ten microliters of each RNA extract was added to 10 μl of reverse-transcription (RT) master mixture to form 20 μl reaction mixtures containing viral RNA, 20 U of RNAsin (Promega, Madison, Wis.), 1X Thermo DNA polymerase reaction buffer (Promega), 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco, Grand Island, N.Y.), 0.5 mM dNTPs, and 50 μg of primer X(E2) 18J (20), and 4 mM MgCl2 (Promega). The RT reaction mixtures were incubated at 43°C for 1 h and at 95°C for 5 min and then cooled to 4°C.

Initially Tag polymerase (Perkin-Elmer, Foster City, Calif.) was used for PCR. Several clones for chimpanzee 88 and most of the inoculum clones were obtained by following the nested PCR procedures described by Weiner et al. (20). However, the procedure was changed for the remainder of the chimpanzee serum samples to utilize the higher-fidelity Pfu DNA polymerase (Stratagene). Thirty microliters of PCR master mixture was added to each tube, with final concentrations according to the Stratagene guidelines for cloned Pfu DNA polymerase. After a 95°C hot start for 45 s, 25 PCR cycles (95°C for 45 s, 55°C for 45 s, 72°C for 2 min) were performed in a Perkin-Elmer Cetus GeneAmp 9600 PCR thermal cycler, followed by a final extension at 72°C for 10 min. Ten microliters of the first PCR product were then added to 40 μl of a second, nested PCR master mixture, and the reactions were amplified for 25 cycles as outlined above. The four nested sense and antisense primers, producing first-round PCR products 244 bp long and nested products 176 bp long, have been described by Weiner et al. (20).

Extensive precautions were employed to avoid PCR contamination. A dedicated room and laminar flow hood were used for preparing RNA extractions, for cDNA synthesis, and for first-round PCR reactions. The second round was performed in a second room with a different flow hood. Aerosol-resistant pipette tips were used routinely, and at least four negative controls were used in each PCR. The Perkin-Elmer Cetus GeneAmp 9600 PCR thermal cycler was routinely cleaned according to the manufacturer’s protocol, and all experimental surfaces were decontaminated with a 10% Clorox solution before and after each use.
TABLE 1. Characteristics of chimpanzees used in this study

<table>
<thead>
<tr>
<th>Chimpanzee</th>
<th>Approx age when infected (yr)</th>
<th>Sex</th>
<th>Initial infection strain</th>
<th>Mo to challenge</th>
<th>Challenge strain</th>
<th>Challenge dose (log_{10} CID_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4.5</td>
<td>M</td>
<td>HCV-H</td>
<td>HCV-H</td>
<td>NT*</td>
<td>c</td>
</tr>
<tr>
<td>69</td>
<td>3.5</td>
<td>F</td>
<td>HCV-H</td>
<td>HCV-H</td>
<td>2.0</td>
<td>10</td>
</tr>
<tr>
<td>88</td>
<td>5.4</td>
<td>M</td>
<td>HCV-H</td>
<td>HCV-H</td>
<td>4.5</td>
<td>10</td>
</tr>
<tr>
<td>238</td>
<td>4.2</td>
<td>F</td>
<td>HCV-H</td>
<td>HCV-H</td>
<td>6.2</td>
<td>10</td>
</tr>
</tbody>
</table>

* F, female; M, male.

† CID_{50}, log_{10} 50% chimpanzee infectious doses.

‡ NT, not titrated.

Cloning PCR products. When Taq DNA polymerase was used to generate the PCR products the protocol from the TA cloning kit (Invitrogen, San Diego, Calif.) was used to clone the products. However, Pfu DNA polymerase does not generate terminal overhangs; therefore, the 176-bp PCR products were selectively precipitated according to the protocol outlined in the pCR-Script Amp SK cloning kit (Stratagene). The kit was then utilized to ligate the PCR products into the pCR-Script vector and to transform the vector into the XL1-Blue competent cells according to the manufacturer’s protocol. Colonies containing plasmids with inserts were selected and grown overnight. DNA from the resultant bacterial suspensions was isolated with the Qiaprep spin plasmid kit (Qiagen).

Sequencing the inserts. Sanger’s chain-terminating method was used to sequence the inserts. Cycle sequencing was performed with AmpliTaq DNA polymerase FS (Perkin-Elmer) and fluorescent terminators on an automated sequencer (model 373A); Applied Biosystems). The 176-bp inserts were sequenced with T7 forward primers. pUC reverse primers were also used to sequence inserts with the dye terminator. Cycle sequencing was performed with AmpliTaq DNA polymerase.

Quantifying viral RNA. A fluorescent PCR detection system (Amplisensor Quantifying viral RNA) and a modified Biotronics procedure was used to quantify the HCV RNA content in chimpanzee serum samples. A modified Biotronics procedure was used to quantify the HCV RNA content.

RESULTS

All amino acid sequences were deduced from the nucleic acid sequences of individual 176-bp clones of HCV cDNA. Approximately two-thirds of the nucleotide changes resulted in amino acid changes (data not shown). All sequences were compared to the HCV-H strain amino acid sequence deduced from the published nucleotide sequence (5).

HCV-H inoculum. A series of clones were obtained for the HCV-H inoculum. The sequencing data from these clones revealed 15 amino acid changes and 17 variants (Fig. 1).

The most dominant quasispecies (HQ2) accounted for 41.3% of the clones. Quasispecies HQ2 consisted of 19 clones possessing serine, histidine, arginine, and serine substitutions at positions 391, 394, 401, and 404, respectively. The quasispecies homologous to the published sequence (HQ1) accounted for 26.1% of the clones (5). In addition to the most dominant and the prototypic quasispecies, there were 15 other variants. The variant designated HQ3 was a clone that had glutamic acid and proline substitutions at positions 371 and 399, respectively. The remaining variants were not given sequence designations because they did not reappear in the chimpanzees after challenge.

Chimpanzee 10. The evidence suggesting reinfection in chimpanzee 10 was the sharp rise in alanine aminotransferase (ALT) values occurring 1 week after challenge inoculation. In addition, the characteristic hepatocellular ultrastructural changes that typify HCV infection had disappeared prior to challenge; however, 5 weeks after challenge the changes reappeared. This animal also had a >10-fold increase in anti-C100 antibody titer after challenge (Fig. 2).

Six weeks after infection chimpanzee 10 showed a mix of variants that bore no resemblance to the variants found in the inoculum. A variant resembling one of those present 6 weeks after infection became the dominant quasispecies a week before challenge. The new quasispecies present in chimpanzee 10 was designated HQ4, and the amino acid changes that characterized HQ4 occurred at positions 384, 386, 388, 391, 394, 396, 397, 398, 400, 403, 405, and 414 (Fig. 3). Variant HQ4 remained the dominant quasispecies 1, 3, and 6 weeks after challenge. Fourteen of the 31 clones sequenced after challenge were not variant HQ4. Three weeks after challenge a clone representing a new quasispecies appeared. The new quasispecies was very similar to the HQ3 quasispecies present in the challenge inoculum, except for an additional serine and valine substitution at positions 383 and 414, respectively.

Chimpanzee 69. Evidence for reinfection in chimpanzee 69 came from a marked increase in the virus load occurring 6 weeks after challenge (Fig. 2). As shown below, this correlated with the emergence of a quasispecies present in the challenge inoculum. The major quasispecies (HQ5) present in chimpanzee 69 (Fig. 4) 6 weeks after the original infection closely resembles quasispecies HQ4 previously identified in chimpanzee 10 (Fig. 3). HQ5 contains all of the amino acid substitu-

† The sequence designation of recurring clones.
‡ The number of clones obtained having the indicated amino acid sequence for a particular serum sample.

FIG. 1. Sequence analysis of HVR1 of 46 clones from the inoculum. A multiple amino acid sequence alignment of the published amino acid sequence of HCV-H HVR1 (7) and those of clones representing HVR1 of HCV quasispecies present in the homologous challenge inoculum (5) is shown. Amino acids homologous to the published sequence are indicated with periods. All amino acids were derived from nucleotide sequences.
tions of HQ4 except that a histidine is substituted at position 384, an additional arginine is substituted at position 408, and an amino acid substitution is omitted at position 388. One week before challenge, three clones were sequenced. HQ5 and two additional variants were present. One week after challenge the major quasispecies in chimpanzee 69 had completely changed. A new quasispecies (HQ6) was characterized by serine, histidine, isoleucine, and threonine substitutions at po-

FIG. 2. Evidence supporting reinfection in the study animals. The time of rechallenge is shown by the open arrowhead in each chart. C100 is the first-generation anti-C100 enzyme-linked immunosorbent assay (Ortho, Raritan, N.J.) (solid bar). The asterisk indicates a >10-fold titer increase. EM denotes positive ultrastructural changes characteristic of HCV infection in chimpanzees. Open squares, negative; closed squares, positive. CAP denotes reactivity in an enzyme-linked immunosorbent assay with microtiter wells coated with recombinant capsid antigen produced in *Escherichia coli* (solid bar). The solid lines with open circles denote HCV RNA molecules per milliliter. The dashed lines show ALT levels.

FIG. 3. Sequence analysis of HVR1 of 47 clones from chimpanzee 10. A multiple amino acid sequence alignment of the published amino acid sequence of HCV-H HVR1 (7) and those of clones representing HVR1 of HCV quasispecies present in chimpanzee 10 is shown. Amino acids homologous to the published sequence are indicated with periods. All amino acids were derived from nucleotide sequences.
sitions 391, 394, 396, and 400, respectively. There were also variations on HQ6, including a glycine substitution at position 420 in one clone and an aspartic acid substitution at position 415, which took the place of the threonine substitution, in another clone. The major quasispecies present 1 week after infection was the prototypic HQ1 quasispecies (11 of 22 clones). Five weeks later (6 weeks after challenge) variant HQ6 was no longer present, and the major quasispecies was still the HQ1 variant.

Chimpanzee 88. Evidence for reinfection in chimpanzee 88 included the reappearance of characteristic hepatocellular ultrastructural changes 11 weeks following challenge and 10-fold increases in the titers of antibodies to capsid (CAP) and C100 proteins, 3 and 12 weeks, respectively, after challenge. In addition, the viral load rose steadily between 1 and 6 weeks after challenge (Fig. 2).

Six weeks postinfection two separate quasispecies were present in the clones of chimpanzee 88 (Fig. 5). The first quasispecies, designated HQ7, had seven amino acid changes at positions 391, 394, 396, 399, 400, 403, and 405. The second quasispecies had four amino acid substitutions in locations similar to those in HQ7; however, three of the substitutions resulted in different amino acids. After another 5.5 months, 7 months postinfection, the major quasispecies HQ3 was present in 9 of 11 clones and the other two clones had 1 to 2 amino acid changes that differentiated them from quasispecies HQ3. The next serum sample was taken 1 week before challenge and 11.5 months after the last serum sample. By that time the major quasispecies had completely reverted to HQ7, and the next two serum samples from 1 and 3 weeks postchallenge revealed only quasispecies HQ7 in all of the clones. However, 6 weeks after challenge a new quasispecies emerged. This was the prototypic HQ1; however, the HQ7 quasispecies was present in 3 of the 12 clones sequenced, and a week later the variant was present in 5 of 5 clones sequenced.

* The date of serum samples, in weeks after infection (±1, ±28) or weeks before or after challenge (C-1, C+1, etc.), from which the clones were obtained.
† The sequence designation of recurring clones.
‡ The number of clones obtained having the indicated amino acid sequence for a particular serum sample.

FIG. 4. Sequence analysis of HVR1 of 43 clones from chimpanzee 69. A multiple amino acid sequence alignment of the published amino acid sequence of HCV-H HVR1 (7) and those of clones representing HVR1 of HCV quasispecies present in chimpanzee 69 is shown. Amino acids homologous to the published sequence are indicated with a period. All amino acids were derived from nucleotide sequences.

FIG. 5. Sequence analysis of HVR1 of 46 clones from chimpanzee 88. A multiple amino acid sequence alignment of the published amino acid sequence of HCV-H HVR1 (7) and those of clones representing HVR1 of HCV quasispecies present in chimpanzee 88 is shown. Amino acids homologous to the published sequence are indicated with periods. All amino acids were derived from nucleotide sequences.
**Chimpanzee 238.** Evidence for reinfection in chimpanzee 238 was the finding of a moderate rise in ALST levels, peaking at 73 U/liter 2 weeks after challenge. In addition anti-C100 rose from an undetectable level 2 weeks after challenge to a peak optical density of 1.14 8 weeks after challenge. The HCV viral load rose from 3.34 log 10 HCV RNA U/ml 1 week before challenge to 5.64 log 10 HCV RNA U/ml 1 week after challenge (Fig. 2).

Six weeks after infection the major quasispecies in chimpanzee 238 was HQ2 (Fig. 3). Four years later, and 1 week prior to challenge, the extent to which they were affected varied. In chimpanzee 69, the naturally evolved quasispecies were able to either coexist with or outcompete quasispecies introduced by homologous challenge. In chimpanzee 238, which was chronically infected with HQ1 at the time of challenge, revealed the emergence of a second inoculum quasispecies, HQ2, 1 week after challenge. The postchallenge appearance of clones homologous to those found in the challenge inoculum, and the coinciding signs of reinfection, suggest that there is limited immunity to viral variants which occur within a quasispecies population.

While each of the chimpanzees quasispecies were affected by the challenge, the extent to which they were affected varied. In addition to a lack of immunity to minor quasispecies within an inoculum, our data also suggests that there are viral-host interactions that contribute to the evolution of a quasispecies specific for the host. In the case of chimpanzees 10, 88, and 238, the naturally evolved quasispecies were able to either coexist with or outcompete quasispecies introduced by homologous challenge. In chimpanzee 69 the homologous inoculum variant dominated the infection 1 week following challenge and persisted throughout the course of the study. While the quasispecies present in the challenge inoculum quickly took over the infection of chimpanzee 69, the natural evolution of infection in chimpanzees 10, 88, and 238 resulted in variants that were able to compete with the inoculum variants.

Farci et al. conducted similar experiments with a limited number of animals and reported that when PCR-positive chimpanzees were rechallenged with a heterologous-strain inoculum the nucleotide sequence of the virus following rechallenge was not that of the rechallenge inoculum but was homologous with the sequence present before rechallenge (2). They concluded that the replication of the challenge virus was either inhibited or masked. The findings of our current study suggest that their second conclusion was probably more accurate and that they did not observe clones of the challenge inoculum because of the limited number of clones sequenced.

**DISCUSSION**

In all of the chimpanzees we examined, we found that the quasispecies variants present 6 weeks following original infection bore little resemblance to the variants in the inoculum. We recognize that, due to the limited number (n = 46) of inoculum clones sequenced, not all variants present in the inoculum were identified; however, the new variants also could have resulted from mutations during replication. The differences in the quasispecies present in each of the chimpanzees following initial infection, and the uniqueness of the changes in composition of the quasispecies at the time points prior to challenge, suggest that what determines the variants present and the course the infection will follow are factors not limited to the virus. It is more likely a combination of host and viral factors that determines which variant is most fit to persist within a host.

While all of the chimpanzees demonstrated unique viral patterns in the course of their initial infections, the quasispecies distributions of their infections were similarly affected following a homologous challenge. At time points ranging from 1 to 6 weeks postchallenge new viral variants emerged in all of the chimpanzees. These sequences of variants were either very similar (chimpanzee 10) or homologous to variants present in the challenge inoculum, and they were not observed previously in the chimpanzees. In chimpanzee 10 the major quasispecies before challenge was HQ4, and this quasispecies persisted until 3 weeks postchallenge, when a variant that resembled the inoculum-derived HQ3 emerged. Similar patterns appeared 1 to 6 weeks after chimpanzees 69 and 88 were challenged. The major clones in the initial infections were not similar to those present in the inoculum, but after challenge, the prototypic HQ1 sequence appeared in the clones of both animals. Even chimpanzee 238, which was chronically infected with HQ1 at the time of challenge, revealed the emergence of a second inoculum quasispecies, HQ2, 1 week after challenge. The postchallenge appearance of clones homologous to those found in the challenge inoculum, and the coinciding signs of reinfection, suggest that there is limited immunity to viral variants which occur within a quasispecies population.

**FIG. 6.** Sequence analysis of HVR1 of 38 clones from chimpanzee 238. A multiple amino acid sequence alignment of the published amino acid sequence of HCV-H HVR1 (7) and those of clones representing HVR1 of HCV quasispecies present in chimpanzee 238 is shown. Amino acids homologous to the published sequence are indicated with periods. All amino acids were derived from nucleotide sequences.
Our findings following homologous challenge of chronically infected chimpanzees support our hypothesis that there are quasispecies present in the challenge inoculum to which there is little or no immunity. It is still not clear whether neutralizing antibodies reactive with epitopes in HVR1 play a major role in host resistance; however, Farci et al., Shimizu and coworkers, and Weiner et al. reported that antibodies to peptide epitopes of HVR1 are neutralizing in cell culture systems (3, 16, 20). It is likely that cytotoxic lymphocyte epitopes also play a role in host immunity (8, 14). In addition to a lack of immunity, our findings indicate that host-specific viral fitness is also a factor that could be influencing viral persistence. The exact mechanisms of viral selection are unclear; more than likely they include both immune interactions and interactions with host cells. While the mechanisms are unclear, both the lack of immunity and the possibility of host-specific viral fitness have implications for the development of an HCV vaccine or HCV infection treatment procedures.

ACKNOWLEDGMENTS

We are grateful to Amy Weiner for help in the establishment of HVR1 sequencing in our laboratory and for comments on the manuscript. Simon Wain-Hobson also reviewed the manuscript and provided invaluable comments. Pat McCormack and Annie Mae Moffat provided dedicated technical assistance.

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