SHORT COMMUNICATION

A Hyperimmune Serum against a Synthetic Peptide Corresponding to the Hypervariable Region 1 of Hepatitis C Virus Can Prevent Viral Infection in Cell Cultures

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To investigate whether a principal neutralization epitope exists in hypervariable region 1 (HVR1) within the putative envelope of hepatitis C virus (HCV), we generated a hyperimmune rabbit serum against a synthetic peptide corresponding to HVR1 of HCV isolate H77. The reactivity of the serum in the enzyme-linked immunosorbent assay was correlated with the 13 amino acids (position 398 – 410) in HVR1. The serum prevented infection with H77 virus in cell cultures but did not prevent infection with H90 virus, a genetically divergent isolate from the same patient. The study demonstrated that neutralization of HCV was mediated, in part, by isolate-specific antibody recognizing HVR1.

Hepatitis virus C (HCV) infection is a worldwide health problem. It persists in over 80% of infected cases and may result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The development of an effective vaccine would be the most efficient method to prevent these diseases. However, HCV is known to undergo genetic mutation during infection even within one individual (1-3). Hypervariable region 1 (HVR1), the N-terminal 27 amino acids within the putative envelope protein E2, comprises the most variable region of the HCV genome (4, 5). The variation is assumed to be caused by random mutation and selection of mutants capable of escaping from neutralizing antibodies produced in the patients. We previously established in vitro systems for detection of HCV infectivity using the human lymphocyte cell lines, HPBM-a10.2 (6, 7) and Daudi cells (8), and demonstrated that anti-HCV antibody can be detected by blocking the adsorption of HCV virions to the HPBM-a10-2 cells (9) and by the formation of antigen-antibody complexes (10). The antibody was isolate-specific and the specificity changed over time (9). Recently, Zibert et al. demonstrated, employing the adsorption-blocking method, that the majority of neutralizing antibodies in the patient sera were directed against the HVR1 of HCV (11). The study presented here examines whether immune serum containing antibodies to a synthetic peptide with the sequence of a specific HVR1 neutralizes a strain of HCV that contains the same HVR 1 sequence.

A peptide with 21 amino acids (peptide A in Fig. 1A) was synthesized based on the published HVR1 sequence of strain H77 (12), a pedigreed isolate of HCV, and coupled with tetanus toxin at the N-terminus. Rabbit 87 received two immunizations at 1-month intervals intradermically, using 500 μg of the conjugated peptide in complete Freund’s adjuvant, followed by a booster 6 months later. Serum samples were obtained prior to (coded as 87P) and 6 months after injection (coded as 87I). Serum 87I was reactive in enzyme-linked immunoabsorbant assay (ELISA) against the homologous peptide and the recombinant E1/E2 protein complex expressed by a vaccinia vector. In order to locate amino acids which affect the reactivity of serum 87I, 4 additional peptides (B, C, D, and E in Fig. 1A) were synthesized and tested by ELISA. Microtiter plates were coated overnight at 4°C with each peptide at a concentration of 5 μg/well and then blocked for 2 days with phosphate-buffered saline containing 0.2% bovine serum albumin and 1% Tween-20 (Sigma). The plates were incubated with serial dilutions of serum 87P or serum 87I overnight at 4°C, washed, and incubated with peroxidase-labeled anti-rabbit IgG (MBL, Japan) for 2 hr at 37°C. Enzyme reactions with substrates were carried out according to the standard procedure. The optical density (OD492) was measured in a MTP-32 Reader (Corona, Japan). Preimmune serum 87P was not reactive with any of the peptides. As shown...
H90 has been shown to be positive for anti-HCV C22 and C100 antibodies by ELISA (9). Both have been shown to be infectious in chimpanzees; plasma H77 contained $10^{6.5}$ 50% chimpanzee infectious doses of HCV per milliliter (13) and 1 ml of undiluted plasma H90 was infectious in a chimpanzee (unpublished data).

To determine the adsorption efficiency of the H77 and H90 viruses to the cells, plasmas H77 and H90 were serially diluted in 10-fold increments and 100 ml of each dilution was inoculated into 1 ml of a suspension of $5 \times 10^5$ HPBMa10.2 cells. After incubation for 2 hr at 37°C, the cells were washed twice and tested for cell-adsorbed HCV RNA by reverse transcription (RT)/PCR using nested primers which detect the E1/E2 region of the HCV genome. Plasmas H77 and H90 had genome titers of $10^6/100$ and $10^5/100$ ml, respectively, by end-point dilution RT/PCR assay, but the adsorption titer was $10^3/100$ ml for H77 and $10^1/100$ ml for H90 (Fig. 2A). We also checked the HVR1 sequences of HCV adsorbed by the cells, since a high level of genetic diversity has been reported for H77 (14, 15). The HCV genomes adsorbed by the cells at the dilutions of $10^{-3}$ for H77 and $10^{-1}$ for H90 were RT/PCR-amplified and the products were cloned into pUC118 (Takara, Japan) and 10–12 subclones were sequenced by ABI 373A sequencer with the Taq dye terminator cycle sequencing kit (Perkin-Elmer). For H77, 5 of 12 clones had the HVR 1 amino acid sequence which was the same as peptide A used for immunization except in Fig. 1B, immune serum 87 I was reactive with homologous peptide A and chimeric peptide D (amino-terminal H90 and carboxy-terminal H77) but not with peptide B (derived from the sequence of H90) or chimeric peptide C (amino-terminal H77 and carboxy-terminal H90). The substitution of three amino acids (positions 395–397), which were TTA in H77 and SVL in H90, appeared not to affect the reactivity of the peptide with serum 87I, suggesting that the 13 amino acids in positions 398–410 determined the reactivity. This was confirmed by the positive reaction of serum 87I with peptide E, consisting primarily of these amino acids (Fig. 1B).

In the neutralization test, isolate H77, representing the inocula. The detection of HCV RNA by RT/PCR was performed using nested primers that amplify the E1/E2 region (nucleotide positions 1437–1709) of the HCV genome. (B) Genetic variation in the HVR1 (between residues 390 and 410) of the cell-adsorbed HCV after exposure to dilutions of $10^{-3}$ for H77 and $10^{-1}$ for H90. Number of clones with individual sequences among 12 sequences for H77 and among 10 sequences for H90 are indicated on the right. Dashes indicate identity with the published H77 sequence.
for one amino acid at position 391 and 7 of 12 clones retained the sequence common to peptides A, D, and E, which reacted with the 87I serum. For H90, 5 of the 10 clones had the same sequence as peptide B (Fig. 2B).

To determine whether serum 87I blocks the binding of HCV to the cells, we examined it in HPBMa10-2 cells as we reported previously (9). Daudi cells were not employed for the adsorption inhibition assay since it was found that virus–antibody complexes could be adsorbed by Daudi cells, a lineage of B cells, probably through the Fc receptor. A 100-μl sample of each target virus (at a 10^{-3} dilution for H77 and a 10^{-1} dilution for H90, which corresponded to one adsorption titer per milliliter; see above) was incubated overnight at 4°C with an equal volume of a 1:10 dilution of serum 87P or serum 87I previously heated at 56°C for 30 min, inoculated into 1 ml of a suspension containing 5 × 10^6 HPBMa10.2 cells, and incubated for 2 hr at 37°C. After washing, the cell–adsorbed HCV RNA was assayed by RT/PCR. As shown in Fig. 3A, serum 87I inhibited the binding of H77 to the cells (Exp. 1 and Exp. 2 in Fig. 3A). However, in one experiment among three trials (Exp. 3 in Fig. 3A), cell–adsorbed HCV RNA was detected following incubation of H77 with serum 87I. This was probably due to the sequence heterogeneity of the inoculum used (see above). Serum 87I did not inhibit the binding of H90 to the cells in any of the three trials.

To further investigate whether the adsorption inhibition by serum 87I mentioned above was correlated with the inhibition of viral replication, we performed neutralization experiments in HPBMa10.2 cells and Daudi cells, whose culture was continued for 12 days after adsorption to monitor the presence of the intracellular HCV genome. Treatment of the target viruses with serum 87P or serum 87I and inoculation into the cells were carried out in the same manner as described above for the binding-inhibition assay. Here the concentration of the target viruses was twofold higher than that for the adsorption inhibition. The presence of HCV RNA in the cells was examined by RT/PCR at intervals and the products were subjected to direct sequencing. Figure 3B shows the results. Serum 87I inhibited detection of the H77 virus later than Day 0 in HPBMa10-2 cells and later than Day 3 in Daudi cells, while serum 87P failed to do so in both cultures. When neutralization was attempted with the H90 virus, both serum 87P and serum 87I failed to prevent infection in both cultures. Thus, neutralization by serum 87I appeared to be isolate-specific. The cell cultures were sometimes intermittently positive during the course of infection, but such intermittent positivity was characteristic of HCV-carrier cultures (7).

We also examined the sequences of HCV strains recovered from the neutralization experiments. When HPBMa10.2 cells were incubated with H77 treated with preimmune serum 87P, the amino acid sequence recovered from cells harvested on Days 0 and 9 was the same as the sequence representing a major population in the H77 inoculum (15). Similar results were obtained with Day 0 and Day 12 harvests of Daudi cells. At the nucleotide level, however, the sequence recovered on Day 0 and the sequence recovered on Day 9 from HPBMa10-2 cells or on Day 12 from Daudi cells differed by one nucleotide at position 1517: C for Day 0 and T for Days 9 and 12 (asterisks in Fig. 3C indicate A (alanine) encoded by GCT instead of GCC). The sequence with T at position 1517 was a minority at adsorption, but this
sequence was the one that persisted as a selected variant during long-term culture of H77 in HPBMa10-2 and Daudi cells and may have had a replication advantage in lymphocyte cells (15). When HCV, the H77 virus, was treated with hyperimmune serum 87I and inoculated into cells, the HCV genome was not detected after Day 3. Although at least some of the HCV virions were successful in adsorbing to the cells, the sequences recovered on Day 0 from HPBMa10-2 cells or Daudi cells were different from the sequence of the antigen peptide (peptide A in Fig. 1A) and different from the sequences detected in the cells inoculated with H77 incubated with the preimmune serum. The variants probably escaped from the antibody. However, since they could no longer be detected after Day 0 from the HPBMa10-2 culture or after Day 3 from Daudi culture, it is possible that the variants might also have been affected by the polyclonal antibody, leading to an aborted infection during later stages of the infection. For the H90 virus, which was not neutralized by serum 87I, the same sequence was recovered on Day 0 from the samples treated with serum 87P as from those treated with serum 87I. The sequence recovered from the cells on Day 12 was, however, different from the sequence detected on Day 0. A similar selection for lymphotropic virus may have occurred in this inoculum as well.

When Daudi cells were used as the host cells, the adsorption inhibition by 87I serum was not clear-cut, though in the replication inhibition experiment essentially the same data as with HPBMa10-2 cells were obtained. This was found to be due to the binding of HCV-antibody complexes, probably through the Fc receptor as the Daudi cells were of B-cell origin. Therefore, for the neutralization test, HPBMa10-2 cells are superior to Daudi cells.

In conclusion, this study demonstrated that a hyperimmune serum against HVR1 prevented HCV infection in cell culture, suggesting that the HVR1 is one of the critical domains for neutralization. ELISA data suggested that the epitope determining the isolate-specific neutralization was within amino acid positions 398–410.

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