Infection of a chimpanzee with hepatitis C virus grown in cell culture

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Culture supernatant harvested from Daudi cells, a lymphoplastoid cell line, after 58 days of infection with the H77 strain of hepatitis C virus (HCV), was inoculated into a chimpanzee. HCV RNA, as detected by RT–PCR, first appeared in the serum and liver 5 and 6 weeks, respectively, after inoculation. Peripheral blood mononuclear cells (PBMC) collected on week 7 were also positive for HCV RNA. The major sequences of hypervariable region 1 (HVR1) of the viral genome recovered from the inoculated chimpanzee were the ones which were the majority in the original H77 inoculum and not those which were in the majority in the culture supernatant. Only the sequence recovered from PBMC was the same as the major one found in the cell culture.

We previously reported that HPBMa10-2 cells, a human T cell line, and Daudi cells were capable of supporting productive infection of hepatitis C virus (HCV) (Shimizu et al., 1993; Shimizu & Yoshikura, 1994, 1995). Sequence analysis of the HCV genomes recovered from these cultures revealed that only specific subsets of the virus could replicate well in the lymphocyte cell lines (Nakajima et al., 1996). As the in vitro infectivity may not necessarily be correlated with the in vivo infectivity, we inoculated culture-grown HCV into a susceptible chimpanzee (Pan troglodytes), and compared the sequences recovered from the chimpanzee with those present in the culture fluid used as the inoculum.

One ml of a suspension containing 5 × 10⁶ Daudi cells was mixed with 10 μl of undiluted plasma H77 which contained 10⁸–⁹ 50% chimpanzee infectious doses of HCV/ml (Feinstone et al., 1981), and incubated at 37 °C for 2 h. After washing, cells were resuspended at a concentration of 10⁵/ml and cultured at 37 °C in a CO₂ incubator. To maintain a long-term culture of HCV-infected Daudi cells, an approximately equal number of uninfected cells was added periodically to the infected cells at subculture after removal of the supernatant. Cell suspensions were harvested at various times during the culture period, separated into supernatants and cell pellets, and tested for HCV RNA by RT–PCR. HCV RNA was detected intermittently in both cells and supernatants during the follow-up period. Cells and culture supernatants remained positive for HCV RNA for more than 2 years. Details of the infection of HCV in the Daudi cell culture have been described; detection of HCV core and envelope antigens by immunofluorescence and detection of virus-like particles by electron microscopy (EM) in the cells harvested on day 14 were reported elsewhere (Shimizu & Yoshikura, 1995; Shimizu et al., 1996).

To test the in vivo infectivity, 10 ml of supernatant harvested from the Daudi cell culture on day 58 was intravenously inoculated into chimpanzee 1496. It had an HCV genome titre of 10²/ml by endpoint dilution RT–PCR. Serum samples and liver biopsy specimens were collected before inoculation, weekly after inoculation for a period of 25 weeks, and then monthly until week 42. Serum, liver and peripheral blood mononuclear cell (PBMC) samples were tested for HCV RNA by RT–PCR. For detection of the E1/E2 region of the HCV genome, we used primer sets 5′GGGGAGTCTCCTGG-CGGCGATA 3′ (nucleotide positions 1399–1418) and 5′ CGTCCGACCTGGCGAACCT 3′ (nucleotide positions 1723–1704) as an external pair and 5′ GGAATTCCTCCAT-GTTGGGAACCTGGCCG 3′ (nucleotide positions 1429–1447) and 5′ GGAAGGCTTGCCAGTATTGATGTGCAA 3′ (nucleotide positions 1618–1599) as an internal pair (italicized nucleotides represent restriction enzyme sites which were attached to HCV sequences). For detection of the 5′ noncoding region, we used primer sets 5′ TGGGGCGAC-ACTCCACCAT 3′ (nucleotide positions 14–33) and 5′ GTGCGACGTCTACGAGACC 3′ (nucleotide positions 341–322) as an external pair and 5′ GGAATTCCTCCATGCGAGTACCACA 3′ (nucleo-
Fig. 1. (a) Outcome of HCV infection in chimpanzee 1496. Symbols at the top indicate the specimen was positive (+) or negative (−) for HCV RNA by RT–PCR. For detection of HCV RNA, total RNA was extracted from 100 µl of serum, an approximately 1mm³ portion of liver, and a pellet of approximately 10⁶ PBMC. RT–PCR was carried out with nested primers as described previously (Nakajima et al., 1996). The primers were synthesized based on the sequence of HCV strain H77 (Inchauspe et al., 1991) to detect the 5’ non-coding (nucleotide positions 63–301) and the E1/E2 (nucleotide positions 1429–1618) region of the viral genome. Day 0 represents the day of inoculation. For the liver and PBMC samples, detection of the minus-strand of the viral genome was carried out by the rTth method with the thermostable rTth reverse transcriptase RNA PCR kit (Perkin Elmer) which is reportedly strand-specific (Lanford et al., 1995). (b) HCV HVR1 sequences detected in plasma H77, the supernatant collected on day 58 from the Daudi cell culture (used as the inoculum) and the samples from the recipient chimpanzee. The RT–PCR products were subcloned and sequenced. Dashes indicate nucleotides identical to those of H1-1.

tide positions 301–288) as an internal pair. In the RT step, the external antisense primer was used to prime cDNA synthesis from positive-strand HCV RNA and the external sense primer was used to prime cDNA synthesis from negative-strand HCV RNA. Serum was also assayed for alanine aminotransferase (ALT) activity and for antibodies to HCV (anti-C25, anti-C33 and anti-NS5) by ELISA using the HCV antibody test system (Ortho Diagnostics). In addition, a liver biopsy specimen was tested for a cytoplasmic interferon-inducible antigen (p44) by immunofluorescence using monoclonal antibody M17 (Honda et al., 1990) and for the characteristic morphological changes by EM. The p44 antigen and the ultrastructural changes have been shown to be indirect measures of HCV replication (Shimizu et al., 1979, 1985; Shimizu, 1992). The chimpanzee was housed and maintained under conditions that met all relevant requirements for the care and use of primates in an approved facility.

Fig. 1(a) shows the outcome of HCV infection in chimpanzee 1496. HCV RNA became detectable in the serum 5 weeks after inoculation. In the liver, HCV minus-strand RNA was first detected 6 weeks after inoculation. The PBMC sample taken on week 7 was also positive for the minus-strand of HCV RNA. Fig. 1(b) compares the HVR1 nucleotide sequences of the viral genome in plasma H77 used for infecting Daudi cells, in the culture supernatant harvested on day 58 from the Daudi cell culture, and in the samples collected from inoculated chimpanzee 1496. In plasma H77, ten different HVR1 sequences were detected by cloning, and sequence
**In vivo infectivity of cell-cultured HCV**

H1-1 was the major variant. From the culture supernatant used as the inoculum, sequences H1-1, H1-2, H14, H2-1 and H4 were recovered, and H1-2 was the major variant.

In inoculated chimpanzee 1496, sequences H1-1, H4, and H59 were detected from the serum and liver. H1-1, which was the major species in the inoculum H77, was recovered as the majority, i.e., the major HCV subset recovered from the chimpanzee was different from that in the inoculum culture supernatant. Sequence H1-2, which was the majority in the inoculum culture supernatant, was recovered from the PBMC only. Sequence H1-2 was previously found to be present as a lymphotropic HCV *in vivo* (Shimizu et al., 1997).

Fig. 2(a) shows a positive immunofluorescence reaction with anti-p44 monoclonal antibody M17 in the liver tissue collected on week 8; the p44 antigen was detectable in the liver between weeks 8 and 16. Convoluted membrane structures (type II alteration) were found by EM in hepatocytes obtained on week 16 (arrows in Fig. 3). As shown in Fig. 1(a), ALT values remained in the normal range, except for an elevation on week 1 which is sometimes detected non-specifically after injection. Serum anti-HCV antibodies (anti-C25, anti-C33 and anti-NS5) were not detected by ELISA during the follow-up period of 42 weeks.

The incubation period before the appearance of the HCV genome in chimpanzee 1496 was 5–6 weeks. This was unexpectedly long; we previously found that *de novo* RNA synthesis of HCV occurred as early as 3 days after experimental inoculation of chimpanzees with serum-derived HCV (Shimizu et al., 1990). In addition, in an infectivity titration study of a HCV inoculum using chimpanzees, M. Sugitani and others (personal communication) observed that there was no direct correlation between the dilution of the inoculum and the time of appearance of HCV RNA in the infected chimpanzees. Therefore, the delay in appearance of markers of the virus

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**Fig. 2.** Immunofluorescence detection of the p44 antigen in liver from chimpanzee 1496 with mouse monoclonal antibody M17. (a) positive reaction (arrows) in liver obtained at week 8. (b) Negative reaction in liver obtained prior to inoculation. Magnification ×400.

**Fig. 3.** Electron micrograph of the cytoplasm of a hepatocyte obtained from chimpanzee 1496 at week 16. Arrows indicate the characteristic convoluted membrane structures. (Bar, 500 nm).
replication for cell culture-derived HCV in chimpanzee 1496 was probably not due to the small amount of HCV in the inoculum. Rather, it may have been due to the time-lapse required for overgrowth of HCVs which replicated better in vivo.

In conclusion, our results indicated that the HCV variant which grew preferentially in the cultured lymphocytes did not replicate favourably in vivo, and the replication advantage in vitro did not correlate with the in vivo infectivity. The discordance between in vivo and in vitro data was probably brought about by the different cell tropism among the HCV quasispecies in the H77 inoculum.

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