Val[675] and Leu[639] substitute for Thr[197] and Arg[165], suggesting that hydrophobic interactions may substitute for this ion pair in smMLCK. The CaM-dependent protein kinases are conspicuous in their lack of a conserved Arg[165] compared to all other protein kinases. The region containing the F helix–loop–G helix is reasonably conserved. smMLCK contains two substitutions with acidic residues at positions Asp[721] and Glu[723] (Gln[243] and Ile[245] in cAPK). The loop between the G and H helices is extended in smMLCK by inserts totaling four residues. In this acidic region of smMLCK, 7 out of 15 residues are either Glu or Asp. These residues form part of a perimeter of acidic residues together with those derived from the loop between the D and E helices (see Fig. 6). A deletion of five residues occurs in smMLCK in the loop between the H and I helix. This loop contains the last conserved core residue, Arg[280] (smMLCK, Arg[762]) that forms an ion pair with Glu[208] (smMLCK Glu[686]) and is located under the E and F helices (Figs. 2 and 3). Similar deletions are found in almost all other protein kinases except members of the protein kinase C subfamily. The I helix in smMLCK may be deleted, but we argue that the loop region is more likely to be shortened than the I helix deleted. It seems likely that Trp[773] (cAPK Trp[297]) in the sequence HPW is the most carboxyl-terminal conserved structural feature between the cAPK and the catalytic core of the MLCKs. Although the mammalian cAPK has the sequence HKW preceding this residue, the yeast cAPK isoenzymes, TPK1, 2 and 3 all have the HPW equivalent to MLCK (*8*). The hydrophobic environment of the Trp[773] in smMLCK is contributed by residues from the E and F helices and appears to be conserved.

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Lack of Protective Immunity Against Reinfection with Hepatitis C Virus

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Some individuals infected with hepatitis C virus (HCV) experience multiple episodes of acute hepatitis. It is unclear whether these episodes are due to reinfection with HCV or to reactivation of the original virus infection. Markers of viral replication and host immunity were studied in five chimpanzees sequentially inoculated over a period of 3 years with different HCV strains of proven infectivity. Each rechallenge of a convalescent chimpanzee with the same or a different HCV strain resulted in the reappearance of viremia, which was due to infection with the subsequent challenge virus. The evidence indicates that HCV infection does not elicit protective immunity against reinfection with homologous or heterologous strains, which raises concerns for the development of effective vaccines against HCV.

HCV, the principal cause of non-A, non-B (NANB) hepatitis, accounts for more than 90% of posttransfusion hepatitis (PTH) in the United States (*1*) and results in chronic infection in over 50% of cases (*2*). Although NANB hepatitis was first recognized in the 1970s (*3*), HCV has only recently been identified and partially characterized (*4*). Before the discovery of HCV, the observation of multiple, distinct episodes of acute NANB hepatitis in the same individual suggested the existence of more than one NANB hepatitis agent (*5*). A similar clinical pattern was documented in cross-challenge studies in chimpanzees (*6*, *7*) although most of these studies did not show a second episode of acute NANB hepatitis (*8*, *9*). Interpretation of these data was difficult and was further complicated by the fact that recurrent episodes of hepatitis were also observed in chimpanzees that were not rechallenged (*10*) and in chimpanzees rechallenged with homologous inocula (*11*), which suggests that there might be explanations other than the existence of multiple NANB agents to account for these observations. With the discovery of HCV and the availability of serologic assays that detect antibodies to this virus (*12*), it has become evident that HCV is the major cause of NANB hepatitis and that if other NANB agents exist they account for only a minority of such cases (*13*).

Thus, the recurrence of NANB hepatitis could be explained by viral factors, such as the existence of multiple HCV serotypes or the emergence of mutant viruses that escape neutralization by the host's immune system. Alternatively, the inability of the host to mount a protective immune response could lead to reactivation or reinfection with the same virus. These questions can now be explored with sensitive and specific techniques such as the polymerase chain reaction (PCR) (*14*). To investigate whether primary HCV infection elicits protective immunity against reinfection with homologous or heterologous strains of virus, we have re-evaluated a series of cross-challenge experiments in chimpanzees, previously conducted at the National Institutes of Health.

The patterns of HCV viremia and humoral immune response were analyzed in five chimpanzees (*15*) sequentially inoculated with different HCV strains derived from five unrelated prospectively studied individuals (G, F, H, K, and R) with posttransfusion NANB hepatitis (*7*, *16*). Three chimpanzees were challenged twice and two were challenged four times. The animals were followed for a mean period of 32 months (range, 12 to 51 months). One chimpanzee (number 963) was rechallenged with the homologous inoculum and four (numbers 189, 196, 502, and 793) with heterologous inocula; the interval between the challenges ranged from 6 to 19 months (mean was 11.2 months). The challenge inocula were prepared from serum or plasma (Table 1) used for other successful chal-

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lenge studies in chimpanzees (*7, 9, 17*). We used a nested PCR assay (*18*) to detect HCV RNA in serial serum samples obtained at weekly or biweekly intervals for the first 20 weeks and then every 4 weeks throughout the follow-up period after each virus challenge. To avoid false negative results caused by mismatch between primer and template, which could result from genetic heterogeneity among the different HCV strains (*19*), we tested all serial samples with a set of primers derived from the 5′ noncoding (NC) region (*18*), which represents the most conserved region of the HCV genome (*20*). Most of the sera were also tested with two sets of primers derived from the nonstructural (NS) regions NS3 and NS4 (*21, 22*). All sera were also analyzed for antibodies against structural and NS proteins of the HCV genome by enzyme-linked immunosorbent assay (ELISA) (Fig. 1).

After the first virus challenge, all five animals developed a classical NANB hepatitis (Table 1). Serum HCV RNA was first detected within 1 week after inoculation in four animals and within 5 weeks in the fifth animal (number 196) (Fig. 1). The viremia was transient, lasting for less than 18 weeks (range was 11 to 17 weeks) in four chimpanzees but was persistent in one animal (number 502). All chimpanzees seroconverted, as measured by first- or second-generation HCV antibody assays (Fig. 1) (*23*). The second-generation test was usually more sensitive for the early detection of antibodies to HCV (*24*). In contrast, none of the animals developed antibodies to the second envelope glycoprotein (E2, also called NS1) (*23*), and only two (793 and 502) became positive for antibodies to NS5 (anti-NS5) (*23*). At the time of the second challenge, all chimpanzees, with the exception of one with persistent HCV infection, were repeatedly negative for serum HCV RNA and antibodies to the C100-3 (part of the NS4 product) and NS5 (the putative viral polymerase) HCV proteins (Fig. 1). In contrast, antibodies to core and NS3 (*23*) were detected by the second-generation assay in four of the five animals (Fig. 1). In all four animals negative for serum HCV RNA, including the one reinoculated with the same strain, the second virus challenge resulted in the reappearance, within 2 weeks, of HCV RNA in the serum. This HCV viremia remained detectable intermittently throughout the observation period in one chimpanzee (793) but lasted for only 3 to 10 weeks in the other three animals (963, 189, and 196) (Fig. 1).

Two chimpanzees (189 and 196) were challenged a total of four times. In chimpanzee 189 (Fig. 1), the third challenge was followed by the reappearance within 3 weeks of serum HCV RNA that remained detectable until week 8, then became positive again at week 19 and was persistently positive at the time of the fourth challenge and thereafter during the remaining 15 months of follow-up. In chimpanzee 196 (Fig. 1), the third challenge induced a transient viremia (1 week), but the fourth challenge resulted in the reappearance of serum HCV RNA 3 weeks after inoculation and its persistence thereafter.

With one exception (chimpanzee 196, third challenge), the recurrence of viremia was always associated with the reappearance of antibodies against the NS proteins C100-3 or NS5 (Fig. 1), which suggests that viral replication had recurred. In animals in which the rechallenge induced a transient HCV viremia, antibody to C100 or anti-NS5 typically reappeared and then disappeared with the loss of HCV viremia (Figs. 1 and 2A). In contrast, these antibodies persisted in the animals that developed chronic HCV infection (Figs. 1 and 2B). Antibody responses to homologous or heterologous rechallenge occurred almost immediately after inoculation, as opposed to the long delay in response to primary HCV infection. This suggests an anamnestic antibody response, which may be characteristic of secondary infection. Antibodies detected by the second-generation assay were less influenced by the pattern of HCV viremia because they remained positive throughout the entire study. With one exception (chimpanzee 793) (Fig. 2B), we could not detect significant variations in the titer of these antibodies before and after rechallenge. Serial serum samples were also tested for antibodies to the E2/NS1 protein with an experimental assay (*23*), but these antibodies were never detected in any of the chimpanzees studied.

Analysis of serum alanine aminotransferase (ALT) concentrations during the cross-challenge studies in chimpanzees showed that the reappearance of viremia was usually associated with a mild elevation in the ALT concentrations (Table 1). Only

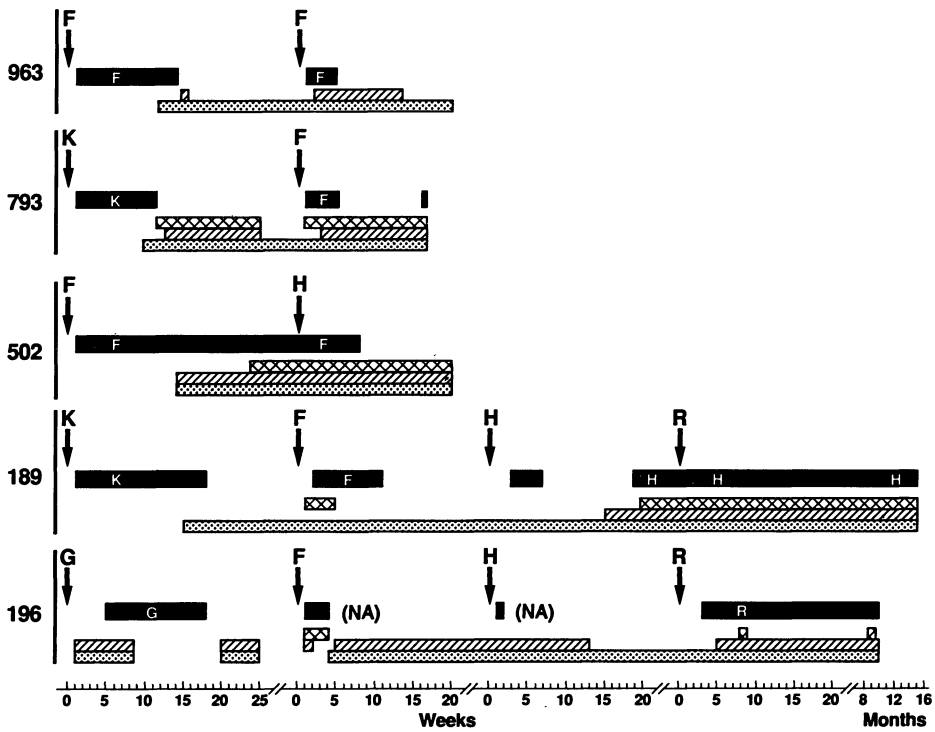

Fig. 1. Patterns of HCV viremia and serologic responses in multiple cross-challenge studies in chimpanzees; the numbers of animals are indicated to the left. HCV viremia was determined weekly or biweekly for the first 20 weeks after each virus challenge and then every 4 weeks throughout the observation period (*18*). Antibodies to HCV were tested weekly or biweekly (*23*). The arrows indicate the time of challenge with the HCV strains used for inoculation (Table 1). HCV RNA is indicated by the filled boxes, first-generation anti-HCV is indicated by the diagonal shading, second-generation anti-HCV is indicated by the stippled shading, and anti-NS5 is indicated by the crosshatching. In chimpanzee 196, antibodies to HCV detected by first- and second-generation assays appeared in the first sample tested, 1 week after inoculation, which reflects passive transfer of antibodies by the large (75-ml) inoculum (Table 1). Passively transferred antibodies became undetectable 8 weeks later, and seroconversion occurred 20 weeks after inoculation. None of the animals developed detectable concentrations of antibodies to E2/NS1 during the multiple cross-challenge studies. The letters in the PCR (HCV RNA) bar indicate the strain identified by sequencing after challenge (there was not sufficient serum available from chimpanzee 196 for PCR amplification of the E2/NS1 region after the second and third challenges); NA, not available.

one chimpanzee (196), after the fourth challenge, developed an acute hepatitis with ALT values similar to those observed during primary HCV infection. The interpretation of this second episode of acute hepatitis, however, was complicated by the fact that this animal was suffering from a wasting syndrome of unknown etiology at the same time. Coded liver biopsy specimens were analyzed in two chimpanzees (963 and 793) during primary acute resolving HCV infection and after the second challenge. Necroinflammatory changes were observed in the liver, coincident with biochemical evidence of hepatitis in both animals (Fig. 2). By the time of rechallenge, the liver biopsies and liver enzyme values had returned to normal. However, within 2 weeks of the second challenge, both animals again exhibited necroinflammatory changes that were suggestive of acute viral hepatitis. In a third animal (502) in which viremia was persistent after primary infection, serial liver biopsies also showed recurrent necroinflammatory changes after rechallenge (*25*). Thus, although rechallenge was usually associated with only mild elevations in ALT concentrations, distinctive histopathological signs suggestive of acute hepatitis were observed in the liver of rechallenged animals for which liver biopsies were available.

When we used the PCR technique to compare the ability of the three sets of primers to detect HCV sequences, we found that primers from the 5′ NC domain and NS4 identified HCV RNA in all sera used for inoculation, but the primers from NS3 reacted with only three of the five strains (strains F, H, and R) (Table 1). The same pattern of response was seen after inoculation of these HCV strains into chimpanzees; the primers from the NS3 region did not identify HCV sequences during primary HCV infection of chimpanzees 793, 189, and 196 (inoculated with strains K or G) (Table 1) but did detect HCV RNA after the second virus challenge (strain F). These data indicated that the variability in detection of HCV sequences was a result of sequence heterogeneity among specific HCV strains and suggested that the reappearance of viremia after rechallenge was not likely to be a result of the reactivation of the original virus strain.

To prove that the HCV sequences recovered after rechallenge corresponded to the HCV sequences used for inoculation, we extracted RNA from all sera used for inoculation and from selected samples recovered from the chimpanzees after each cross-challenge. The RNA was then reverse transcribed and amplified with a set of primers that covered the E2/NS1 domain of the HCV genome (*26*). The PCR products were purified and sequenced as described (*27*). We chose to sequence the E2/NS1 region of the HCV genome because reports have shown that the E2/NS1 gene of all HCV genomes examined has a hypervariable domain (*28*) that may be used to identify individual viral isolates. This domain (region V) consists of approximately 78 nucleotides and may be analogous to the third hypervariable region (V3) of the human immunodeficiency virus (*29*). The nucleotide sequences of the hypervariable region of the five HCV strains used for inoculation are shown in Fig. 3. Comparison of these sequences revealed an overall nucleotide identity ranging from 62.3 to 93.2% among the five HCV isolates (Table 2).

We then compared the nucleotide sequence of the hypervariable region of the HCV strain used for inoculation with those from 12 of the 14 challenges. At the time of inoculation, serum HCV RNA was repeatedly negative in 12 challenges but positive in 2 challenges (the second challenge in chimpanzee 502 and the fourth challenge in chimpanzee 189). Sequence comparisons of rechallenges inducing de novo reappearance of HCV viremia demonstrated that the reappearance of HCV RNA in serum after each cross-challenge was not a result of reactivation of the original strain but the result of reinfection with a different HCV strain. In the two chimpanzees positive for serum HCV RNA at the time of rechallenge (189 and 502), the nucleotide sequence of the virus recovered after rechallenge was not that of the HCV used for rechallenge but was identical to the sequence of the virus present before challenge. In chimpanzee 189, this observation

Table 1. Inocula and serum ALT values in a series of cross-challenge experiments in chimpanzees with HCV. The five animals were inoculated intravenously with different HCV strains obtained from five individuals with NANB PTH, documented in prospective studies (*7, 16*). All inocula used in this study were derived from standard viral stocks that had been used successfully to transmit NANB hepatitis to chimpanzees in independent studies (*7, 9, 17*). For two inocula obtained directly from the patients, strain H and strain F, the infectivity titer had been determined in titration studies in chimpanzees [$10^{6.5}$, 50% chimpanzee-infectious doses (CID) and ~10^{1} CID, respectively] (*9*). Numbers in parentheses after sources indicate volume in milliliters. ALT was measured in international units per liter; numbers in parentheses indicate the week after inoculation that the peak occurred. The normal ALT values in chimpanzees ranged between 6 and 38 U/liter. ND = not done.

Chimpanzee	First challenge			Second challenge			Third challenge			Fourth challenge		
	Source of HCV inoculum	Baseline ALT	Peak ALT	Source of HCV inoculum	Baseline ALT	Peak ALT	Source of HCV inoculum	Baseline ALT	Peak ALT	Source of HCV inoculum	Baseline ALT	Peak ALT
963	Chronic PTH, strain F, third chimpanzee passage (1)	30	220 (12)	Chronic PTH, strain F, third chimpanzee passage (1)	30	55 (2)	ND	ND	ND	ND	ND	ND
793	Acute PTH, strain K, first chimpanzee passage (1)	23	418 (12)	Chronic PTH, strain F, third chimpanzee passage (1)	25	52 (1)	ND	ND	ND	ND	ND	ND
502	Chronic PTH, strain F, third chimpanzee passage (1)	21	412 (13)	Acute PTH, strain H (1)	28	39 (6)	ND	ND	ND	ND	ND	ND
189	Acute PTH, strain K (3)	19	219 (15)	Chronic PTH, strain F (5)	19	25 (3)	Acute PTH, strain H (1)	19	43 (11)*	Acute PTH, strain R (1)	34	39 (1)
196	Chronic PTH, strain G (75)	17	62 (13)	Chronic PTH, strain F (5)	20	39 (2)	Acute PTH, strain H (1)	19	20 (1)	Acute PTH, strain R (1)	26	74 (10)

*ALT fluctuated between 43 and 59 U/liter during the duration of the study, in parallel with the persistence of HCV viremia.

Table 2. Percent identity in a 162-nucleotide domain that encompasses the hypervariable region of five HCV strains (F, G, H, K, and R) used for inoculation of chimpanzees and the P HCV strain (*21*).

Strain	P	F	G	H	K	R
P	–	88.9	67.2	92.0	65.4	83.3
F		–	67.2	93.2	67.3	82.1
G			–	66.7	79.0	66.0
H				–	64.8	85.2
K					–	62.3
R						–

was confirmed by sequencing data obtained during follow-up (*30*) and suggests that viral interference may inhibit the replication of heterologous HCV or mask its presence after rechallenge in these persistently infected chimpanzees. However, because of the small number of rechallenges analyzed in persistently infected animals and our inability to rule out a mixed infection, we must be cautious in the interpretation of these data.

Although HCV, an RNA virus, is subject to considerable nucleotide change in a relatively short evolutionary period (*31*), we did not observe a high mutation rate in the HCV viral strains recovered after one passage in chimpanzees. The nucleotide sequences of the HCV inocula and the virus recovered after challenge were identical in 10 of 12 cases. In the remaining two cases, the HCV recovered after challenge exhibited one nucleotide substitution [chimpanzee 793, second challenge with strain F: nucleotide (nt) 1543, G → A] and six nucleotide substitutions (chimpanzee 189, third challenge with strain H: nt 1522, G → A; nt 1527, A → G; nt 1536, C → A; nt 1540, T → C; nt 1548, C → T; and nt 1555, C → T), respectively, when compared to the sequence of the HCV used for inoculation. Sequencing of DNA was usually performed on PCR products obtained within 5 weeks after challenge. Only in chimpanzee 189 was DNA sequencing performed on a later sample (29 weeks after the third challenge). In consideration of the pattern of HCV viremia seen in this animal (serum HCV RNA became positive after challenge, then negative, and again positive), the six nucleotide substitutions may reflect selection of mutant virus as a result of immune pressure directed against the V region of the HCV genome. Three of the six nucleotide substitutions observed in chimpanzee 189 after rechallenge with strain H also appeared independently in the original individual from whom the inoculum was derived (individual H) in a study of the mutation rate of HCV over a period of 13 years (*32*).

We have demonstrated that, in chim-

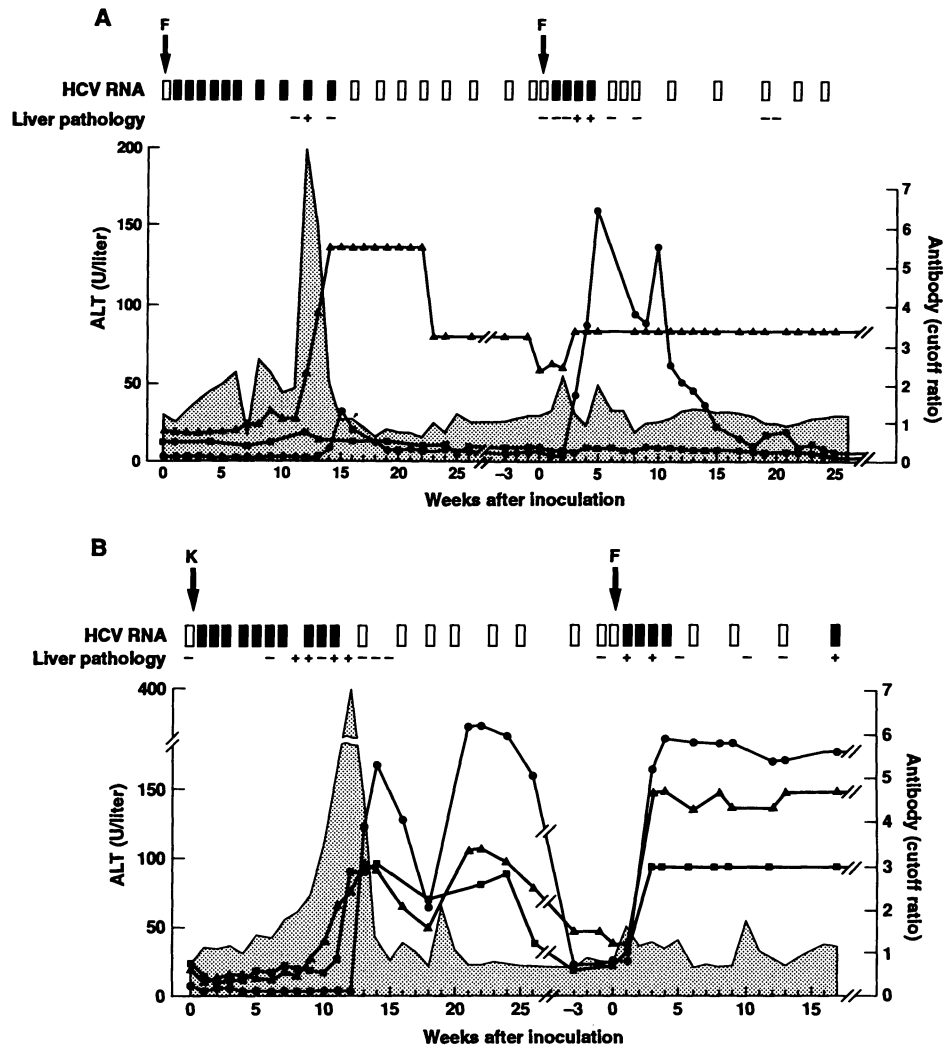

Fig. 2. Course of HCV infection in chimpanzee 963 rechallenged with the homologous HCV strain (**A**) and in chimpanzee 793 rechallenged with a heterologous HCV strain (**B**). The gray areas indicate the values of serum ALT. Normal ALT values in chimpanzees range between 6 and 38 U/liter. The arrows indicate the time of challenge and the strain used for inoculation. Open bars indicate negative assays for serum HCV RNA by PCR (*18*) and solid bars indicate positive assays. Liver pathology indicates necroinflammatory changes rated as negative or positive. First-generation anti-HCV is indicated by circles, second-generation anti-HCV is indicated by triangles, and squares indicate anti-NS5 (*23*). Cutoff ratio represents the ratio between the absorbance value for the test sample and that for the assay cutoff; values above 1 were considered positive.

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   1418                                                                1498
P  AGCGTATTTCTCCATGGTGGGGAACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCA
F  ..........................................A.....................................G.....
G  ...T..C.AT.......C.............T.....TT..A.T...T....A..C..C........T....G.A.C...GC
H  ..............................................................................
K  T..C..C.AT.....................T.....T...A.T...A....A..C...........T....G..TG..T..
R  ......C.....................................A.....C...A.......T..........G

   1499                                                                1579
P  CGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCGCACCAGGCGCCAAGCAGAACGTCCAGCT
F  ..............C.......G.G.C.CAG.....C...C.G.T..TT..A.....................A....A..
G  ...GT.A.....G.CG..A...T....C.CCCGC..CA...CGTC....T.T...GTC..GC.GTCT.....AA.......
H  ......................G...CAC.G....GC.....G.T.....TA.....................A....A..
K  ...GGTA.....G.CGCAA.C.TG.GACCAC..C..GG.AACGTC....T.....GTT..GC.GC.A.....TA.......
R  ....................CT.....C.C.G.G..GC.C.C........T.TAACAGG...T.........G.......A..
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Fig. 3. Alignment of the nucleotide sequences of the hypervariable region of the five HCV strains (F, G, H, K, and R) used for inoculation of chimpanzees in multiple cross-challenge studies. P, prototype. A region of 162 nucleotides of the HCV genome spanning map positions 1418 to 1579 was aligned. Map unit designations are taken from a published HCV sequence (*21*). Dots indicate nucleotide identity to the P sequence.

panzees convalescent from HCV infection, rechallenge with homologous or heterologous HCV strains consistently resulted in the reappearance of viremia. Sequencing data provided evidence for the first time that the recurrence of viremia was not a result of reactivation of the original strain but a result of reinfection with a different (or the same) HCV strain. By contrast, chimpanzees persistently infected with HCV appeared to be protected against superinfection with a different HCV strain, which suggests that viral interference, a mechanism recognized in other types of viral hepatitis (*33*), may also play a role in this setting. However other mechanisms (such as neutralizing antibodies) could also be involved. Although primary infection with HCV afforded partial protection against biochemical hepatitis after rechallenge and periods of viremia were generally shorter, histologic evidence of hepatitis reappeared, and the risk of developing chronic HCV infection was not lower than that seen after primary infection in these chimpanzees. Our data suggest that the risk of reinfection was not related to dose or strain of HCV. Reinfection occurred several times in the same chimpanzees rechallenged with different HCV strains and even in a chimpanzee rechallenged with the same dose of the homologous inoculum. Prince and coworkers reported the reappearance of HCV viremia in chimpanzees rechallenged with the homologous or a heterologous virus (*34*) but they could not rule out exacerbation of the earlier infection because sequence analysis was not performed.

Multiple episodes of transfusion-associated hepatitis of short incubation have been described in individual hemophiliacs and in other individuals at risk of repeated exposure to HCV (*5*). The observation of multiple infections and recurrent episodes of hepatitis with short incubation periods in chimpanzees repeatedly exposed to HCV suggests a similar etiology for the multiple attacks of short incubation hepatitis seen in these patients. The mechanism responsible for the lack of protective immunity against reinfection with HCV is at present unknown. We can postulate that the virus fails to induce an effective neutralizing antibody response or that genetic variation leads to the rapid development of escape mutants that circumvent the immune response. Regardless of the mechanism, the findings reported here have implications for the development of effective HCV vaccines.

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15. The animals were caged individually and maintained under conditions that met all relevant requirements for the use of primates in an approved facility. At the time of the study, all chimpanzees were negative for hepatitis B surface antigen (HBsAg) and had normal hepatic enzyme concentrations. Two chimpanzees (189 and 793) had been experimentally infected with hepatitis B virus (HBV) before and were positive for antibody to HBsAg (anti-HBS) at the beginning of the study. Three of the animals (196, 502, and 963) had not been used before in experiments, and each was negative for HBsAg, anti-HBs, and antibody to HBV core antigen (anti-HBc). Weekly serum samples were monitored for ALT, isocitrate dehydrogenase, and γ-glutamyl transferase activity.
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18. P. Farci *et al.*, *N. Engl. J. Med.* **325**, 98 (1991). Total RNA was extracted from 100 μl of serum or plasma by the guanidinium-phenol-chloroform method. Total RNA was reverse transcribed in a volume of 20 μl, and the resulting cDNA was amplified in a 100-μl reaction volume. The primers used for PCR amplification were derived from the 5′ noncoding region of HCV and consisted of an outer primer pair, 5′-ACTGTCTTCACGCAGAAAGCGTCTAGCCAT-3′ and 5′-CGAGACCTCCCGGGGCACTCGCAAGCACC-3′, and an inner primer pair, 5′-ACGCAGAAAGCGTCTAGCCATGGCGTTAGT-3′ and 5′-TCCCGGGGCACTCGCAAGCACCCTATCAGG-3′ [J. Bukh, R. H. Purcell, R. H. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 187 (1992)]. The reaction was performed for 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 2 min, and DNA extension at 72°C for 3 min. After the first amplification, 10 μl of the PCR product were amplified for 35 cycles with the corresponding inner primer pair. The PCR product was analyzed by electrophoresis on 2% agarose gel and visualized by ultraviolet fluorescence after ethidium bromide staining. To reduce the risk of contamination, the PCR products were analyzed in a laboratory physically separated from the one in which the PCR reaction tubes were prepared. All reagents were prepared in a separate room, and dedicated sets of pipettes were used for the preparation of samples and reagents. In addition, six negative controls were included in each PCR assay and tested in parallel with the samples throughout the entire procedure.
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21. M. Houghton, Q. L. Choo, G. Kuo, European Patent application 88310922.5, publication number 0318216.
22. The set from the NS3 region consisted of an outer primer pair, 5′-CTGCAATACGTGTGTCACCC-3′ and 5′-ACATGCATGTCATGATGTAT-3′, and an inner primer pair, 5′-AGACAGTCGATTTCAGCCTT-3′ and 5′-TTGGTGACTGGGTGCGTCAG-3′; the set from NS4 consisted of an outer primer pair, 5′-GTTCGATGAGATGGAAGAGTG-3′ and 5′-ACCAGGCAGCGTTGACAAGCC-3′, and an inner primer pair, 5′-CTCAGCACTTACCGTACATCG-3′ and 5′-GCCCGCCAAGTATTGTATCCC-3′ (*21*).
23. The first-generation assay for antibodies to HCV (anti-HCV) (*12*) was determined with a commercially available enzyme immunoassay (EIA) according to the manufacturer's instructions (Ortho Diagnostic Systems, Raritan, NJ), that uses the recombinant C100-3 antigen. The second-generation EIA for anti-HCV [I. K. Mushahwar, *Can. Dis. Wkly Rep.* **17S5**, 41 (1991)] (Abbott Laboratories, North Chicago, IL) was obtained as an experimental test that contained recombinant antigens representing the nucleocapsid, as well as the NS3 and NS4 (C100-3) regions. Anti-NS5 was measured with an experimental test kit that contained a recombinant antigen consisting of sequences from the 5′ end of the NS5 gene product. We used two EIAs to test for E2/NS1 antibodies. Both assays were constructed with synthetic peptides representing sequences in this region. One peptide (amino acids 380 to 436) represented the 5′ end of E2/NS1 and contained sequences within the first 25 amino acids, which are known to be hypervariable among HCV isolates. The second peptide (amino acids 643 to 683) was also located within E2/NS1 and represented an epitope from a more conserved domain of this region. Both peptides were based on the HCV-1 prototype (P) sequences (*21*). A third peptide was also constructed, representing amino acids 380 to 436, based on the sequence of a second HCV isolate that differed markedly from HCV-1 in the E2/NS1 hypervariable region. These assays have been used to detect antibodies to E2/NS1 in 68% of individuals with chronic HCV infection (R. R. Lesniewski and I. K. Mushahwar, unpublished data).
24. P. Farci *et al.*, *J. Infect. Dis.* **165**, 1006 (1992).
25. S. Govindarajan, unpublished data.
26. M. Houghton *et al.*, *Hepatology* **14**, 381 (1991). The set of primers we used to amplify and sequence the E2/NS1 region consisted of an outer primer pair, 5′-ATA*AAGCTT*CACCGCATGGCATGGGATAT-3′ and 5′-CAC*GAATTC*GGGGCTGGGAGTGAAGCAAT-3′, and an inner primer pair, 5′-GGT*AAGCTT*ATGGCATGGGATATGATGAT-3′ and 5′-CTG*GAATTC*AAGCAATATACCGGACCACA-3′. Italicized sequences represent restriction site sequences that are not included in the HCV genome (*21*).
27. The PCR products amplified from the E2/NS1 region of the HCV genome were purified by Geneclean (BIO 101, La Jolla, CA). Double-stranded PCR fragments were sequenced by the dideoxynucleotide chain termination method with phage T7 DNA polymerase (Sequenase, U.S.

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35. We thank B. Hollinger for strain R of HCV, P. Lusso for critical comments, and T. Heishman and L. Moore for editorial assistance. Liver biopsies from one chimpanzee were evaluated by the late H. Popper; the rest were evaluated by one of us (S.G.).

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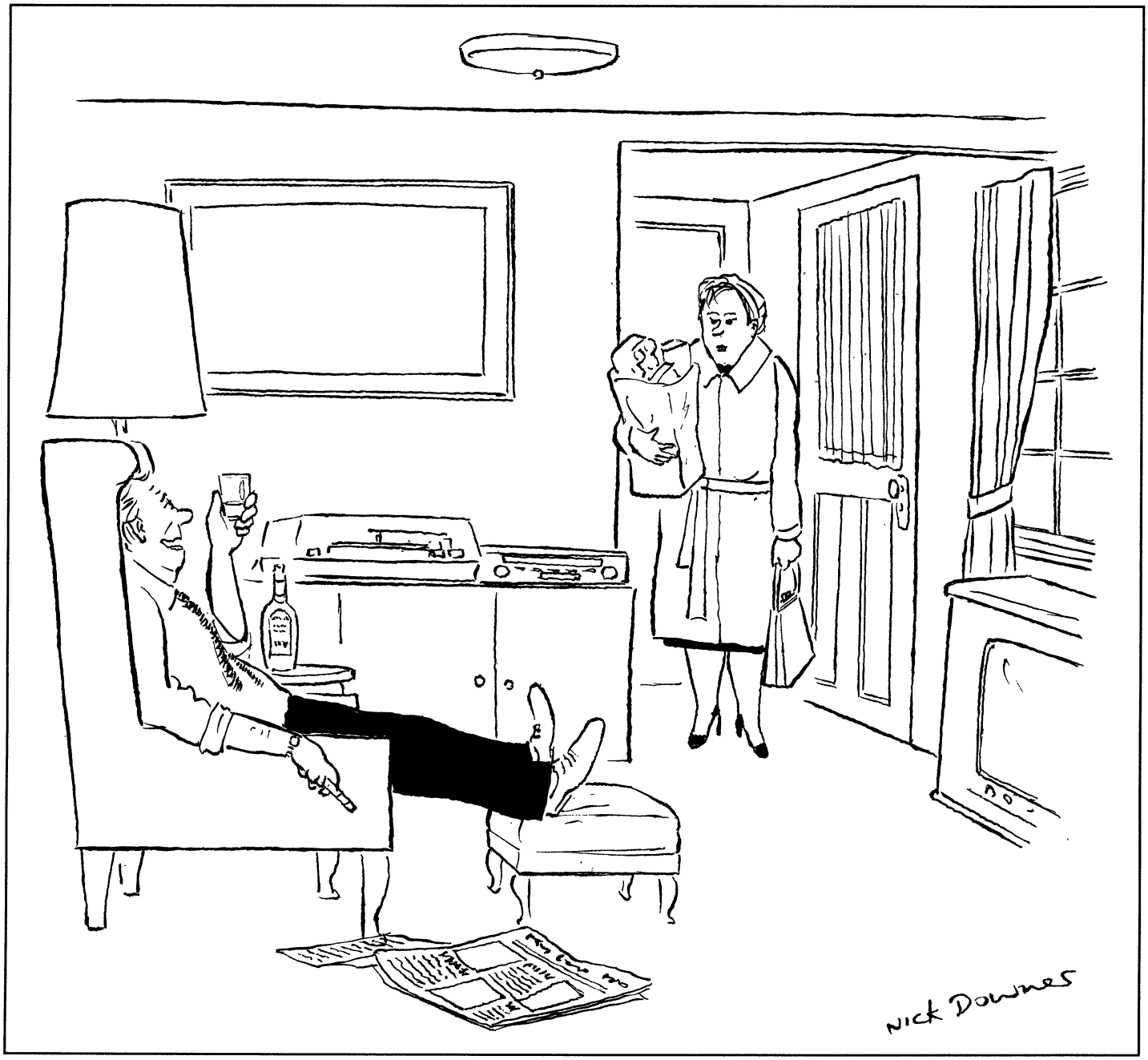

"The universe is 10 billion years old. You don't think that calls for a little celebration?"