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Vaccination of chimpanzees against infection by the hepatitis C virus

(immunization)

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ABSTRACT **A high incidence of community-acquired hepatitis C virus infection that can lead to the progressive development of chronic active hepatitis, liver cirrhosis, and primary hepatocellular carcinoma occurs throughout the world. A vaccine to control the spread of this agent that represents a major cause of chronic liver disease is therefore needed. Seven chimpanzees (*Pan troglodytes*) have been immunized with both putative envelope glycoproteins [E1 (gp33) and E2 (gp72)] that were copurified from HeLa cells infected with a recombinant vaccinia virus expression vector. Despite the induction of a weak humoral immune response to these viral glycoproteins in experimentally infected chimpanzees, a strong humoral immune response was obtained in all vaccinees. The five highest responders showed complete protection against an i.v. challenge with homologous hepatitis C virus 1. The remaining two vaccinees became infected, but both infection and disease may have been ameliorated in comparison with four similarly challenged control chimpanzees, all of which developed acute hepatitis and chronic infections. These results provide considerable encouragement for the eventual control of hepatitis C virus infection by vaccination.**

The hepatitis C virus (HCV) was characterized in 1989 (1) and has been shown (2, 3) to be the major etiological agent of parenterally transmitted, viral non-A, non-B hepatitis. HCV causes persistent infections in most cases (4) and leads to the development of chronic hepatitis and liver cirrhosis in ≈50% and 10% of cases, respectively (5). A significant proportion of patients with liver cirrhosis will also develop primary hepatocellular carcinoma (6). The prevalence of HCV infection around the world is generally between 0.4 and 2% (7–10), although a much higher level has been reported in Egypt (14%; see ref. 11). Therefore, HCV constitutes a major cause of chronic liver disease throughout the world. With the recent development of recombinant-based diagnostic assays for the detection of circulating HCV antibodies (2, 3), the risk of being infected with HCV after transfusion of blood or cellular components has been substantially reduced (12, 13). However, community-acquired infection is much more common and occurs at various frequencies in high-risk groups such as i.v. drug users, health-care workers, and sexual and household contacts of hepatitis patients, although ≈40% of cases in the United States appear to have no known risk factor for acquisition of infection (14). Thus, the development of an HCV vaccine to prevent transmission within the community is highly desirable.

HCV is distantly related genetically to both the pestiviruses and flaviviruses and, like these relatives, appears to process virion structural proteins from the N-terminal region of the polyprotein precursor encoded by the positive-stranded RNA genome (15). The host signal peptidase mediates the cleavage of a basic, presumed nucleocapsid protein (C; ≈20 kDa) from the N terminus of the polyprotein precursor followed by two glycoproteins (E1, ≈33 kDa and E2, ≈72 kDa), both of which represent potential components of the viral envelope (16–18). A variety of presumed nonstructural proteins (NS2–5) are processed from the downstream region of the polyprotein mediated, in part, by a viral protease encoded within the NS3 domain (18–21). Recently, we have expressed the entire structural gene region of HCV-1 (encompassing the complete C-, E1-, and E2-encoding genes along with part of the downstream NS2-encoding gene) in mammalian cells using a recombinant vaccinia virus (rVV) vector and have been able to copurify the E1 and E2 glycoproteins (gp33 and gp72) under nondenaturing conditions from the endoplasmic reticulum. A fraction of the purified material was shown to exist in the form of a large E1/E2 oligomeric complex (22). We now report on the efficacy of this purified preparation in vaccinating chimpanzees against experimental infection with HCV-1.

MATERIALS AND METHODS

Vaccine Preparation. A *Stu* I–*Bgl* II cDNA restriction fragment of the HCV-1 genome (nt 63 to +2901; aa 1–967; ref. 15) encoding the complete C (20 kDa), E1 (gp33 kDa), and E2 (gp72 kDa) proteins along with a C-terminally truncated NS2 product was cloned into the *Sma* I site of plasmid SC59 downstream of a hybrid early/late vaccinia promoter (S. Chakrabarti and B.M., unpublished work). BSC40 cells preinfected with wild-type WR vaccinia were transfected with the SC59 recombinant and thymidine kinase-negative recombinants, selected, and purified through three rounds of plaque purification (23). Spinner cultures of HeLa cells (10^9 cells per liter) were infected with the rVV at a multiplicity of infection of 1–10 and harvested 24 hr later by pelleting and freezing. After lysis by Dounce homogenization in hypotonic buffer, the pellet was extracted by homogenizing in 2% Triton X-100, and E1 (33 kDa) and E2 (72 kDa) were selectively copurified by successive chromatography on agarose-bound Gralanthus Nivalis-lectin (Vector Laboratories) and fast-flow S-Sepharose cation exchanger (Pharmacia). The yield of E1/E2 from 120 liters of infected HeLa cells was ≈1.5 mg (≥80% pure),

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Abbreviations: HCV, hepatitis C virus; rVV, recombinant vaccinia virus; PBL, peripheral blood lymphocytes; ALT, alanine aminotransferase; CID_{50}, dose that infects 50% of chimpanzees; RT, reverse transcriptase; C protein, nucleocapsid protein; E1 and E2, envelope glycoproteins 1 and 2; NS2–5, nonstructural proteins 2–5.
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which was used to immunize five chimpanzees (*Pan troglodytes*; L357, L653, L534, L559, and L470). Plaque assays verified the absence of live rVV in the purified preparation. Two other chimpanzees (WS176 and WS181) received an identical E1/E2 subunit vaccine derived from a 3′ truncated cDNA fragment (nt −63 to +2718; aa 1–906). There were no differences in the sizes of E1 and E2 derived from either cDNA fragment. Recombinant nucleocapsid (C) subunit was synthesized by directly expressing a cDNA fragment of HCV-1 encoding aa 2–120 in yeast. After extraction in 5 M urea and subsequent dialysis, the C protein was purified to homogeneity by successive chromatography on Q- and S-Sepharose (Pharmacia).

In two chimpanzees (L534 and L470), the above live rVV ($\approx 5 \times 10^8$ plaque-forming units) was administered by scarification and by intradermal inoculation on week −76.

Immunization and Challenge of Chimpanzees. Chimpanzees were injected i.m. with 1–2 ml of recombinant HCV antigens combined with an oil/water micro-emulsified adjuvant according to regimens summarized in Table 1. Two chimpanzees (WS181 and WS176) received 18 immunizations over a 9-mo period, whereas the other five animals received just three injections at ≈0, 1, and 7 mo. Two of these five chimpanzees (L534 and L470) were immunized initially with the live rVV (Table 1). All chimpanzees were challenged 2–3 weeks after the final boost with ≈10 chimpanzee infectious doses (CID_{50}) of HCV-1 administered i.v. in 1 ml of autologous serum obtained before commencement of the entire vaccination schedule. The challenge virus was present in a plasma pool derived from the chronic phase of infection of chimpanzee no. 910 and shown to have a chimpanzee infectious titer of $\approx 10^6$ CID_{50} per ml in 1985 (25). This infectivity titer was confirmed by us in 1992 immediately before challenging the vaccinated chimpanzees. (Six chimpanzees challenged i.v. with 1 ml of a 10^{-4} dilution all became infected, as did two additional chimpanzees challenged i.v. with 1 ml of a 10^{-5} dilution. Of two further chimpanzees challenged i.v. with 1 ml of a 10^{-6} dilution, only one became infected. These chimpanzees were monitored as described below.)

Monitoring of Chimpanzees. *Pan troglodytes* were maintained under standard conditions for the humane care of chimpanzees (26). Published procedures were followed in measuring serum alanine aminotransferase (ALT) levels (27), circulating HCV antibodies (2, 3), and HCV RNA (28) in extracts of plasma (drawn in EDTA), liver biopsies, and peripheral blood lymphocytes (PBL) using PCR. Liver biopsies were also examined in the electron and light microscope for evidence of ultrastructural alterations and inflammatory activity, respectively, as described (27).

RESULTS

A total of four chimpanzees were used as controls for i.v. challenge with ≈10 CID_{50} of HCV-1. HCV RNA was first detected in plasma samples obtained either 1 week (Fig. 1; L521, WS177, and L439) or 2 weeks (L663) after challenge. All of these animals were viremic at the most recently sampled times (≥32 weeks after challenge), although some had been periodically negative in PCR assays of plasma samples (Fig. 1, L521 and L663). Viral RNA was detected in extracts of liver biopsies from chimpanzee L439 obtained 8 and 12 weeks after challenge, although viral RNA was not detected in washed PBLs obtained 12 weeks after challenge, even though HCV RNA was detected in the plasma at this time (Fig. 1). All four control animals experienced acute hepatitis, as evidenced from a significant rise in serum ALT levels within 2–6 weeks after challenge. Peak ALT values were recorded 4–16 weeks after challenge (Fig. 1). Of the three animals from whom liver biopsies were examined in the electron microscope, undulating endoplasmic reticulum, membranous tubules, and rings first appeared within hepatocytes by 1–3 weeks after challenge (Fig. 1). These changes are characteristic of HCV infection of this species (27). Antibodies to the recombinant C25 antigen (a chimeric polypeptide composing parts of the C, NS3, and NS4 domains; ref. 3) appeared in all control animals (by week 11 or 12). In contrast, antibodies to the purified E1 and E2 glycoproteins remained undetectable during the follow-up period (24–36 weeks after challenge; Fig. 1). This low seroconversion rate to the E1 and E2 glycoproteins (and to the C protein) was confirmed in an expanded study of chimpanzees that were all chronically infected with either HCV-1 or the highly related H strain (ref. 29; Table 2) and is in contrast to the very high (≥95%) seroprevalence of these antibodies observed in patients from the United States with parenterally transmitted chronic non-A, non-B hepatitis (30). Even when present in experimentally infected chimpanzees, titers of antibodies to the purified E1 and E2 glycoproteins were substantially lower than those measured in infected blood donors and chronically infected patients (Table 2).

Seven chimpanzees were vaccinated with the purified E1/E2 antigens with different immunization protocols (Table 1). Reactive antibodies were induced in all of these animals but with a wide range of titers (Fig. 1). When challenged with the same dose of HCV-1 used to successfully infect all four control animals, none of the five vaccinees with the highest antibody titers at the time of challenge became viremic at any time during the follow-up period of at least 33 weeks (Fig. 1; L357, L653, L534, L559, and WS176). Plasma samples were consistently negative in PCR assays for viral RNA as were liver and PBL extracts obtained at two different times (Fig. 1; liver and PBL extracts were not tested from WS176). None of the five animals seroconverted to anti-C25 and of the four tested, none showed any hepatocyte ultrastructural changes characteristic of infection (Fig. 1). Serum ALT values remained generally within the normal range (Fig. 1), and there was little evidence for either periportal or parenchymal inflammation of the liver in contrast to the significant levels observed in three of the four control, infected animals (data not shown). Thus, these five vaccinees were effectively

Table 1. Chimpanzee immunization protocols

Chimpanzee no.	E1/E2 (gp33/gp72) subunits, μg	Adjuvant*	Vaccination schedule
WS181 and WS176	3	MF59 and MTP (10 μg)	Every other day between weeks −41 and −37 (15 times)
	30	MF59 and MTP (100 μg)	Weeks −28, −22, and −2
L653	40	MF59 and MTP (100 μg)	Weeks −34, −29, and −2
L559, L357, and L534†	40	MF75 and MTP (100–400 μg)	Weeks −31, −26, and −3
L470†‡	40	MF75 and MTP (200–400 μg)	Weeks −31, −26, and −2

All chimpanzees were challenged with ≈10 CID_{50} of HCV-1 on week 0 by i.v. inoculation.
*The compositions of MF59, MF75, and muramyl tripeptide (MTP) were as described (24).
†L534 + L470 were initially primed with live rVV on week −76.
‡Also coimmunized with 40 μg of recombinant nucleocapsid (C) subunit in same weeks.

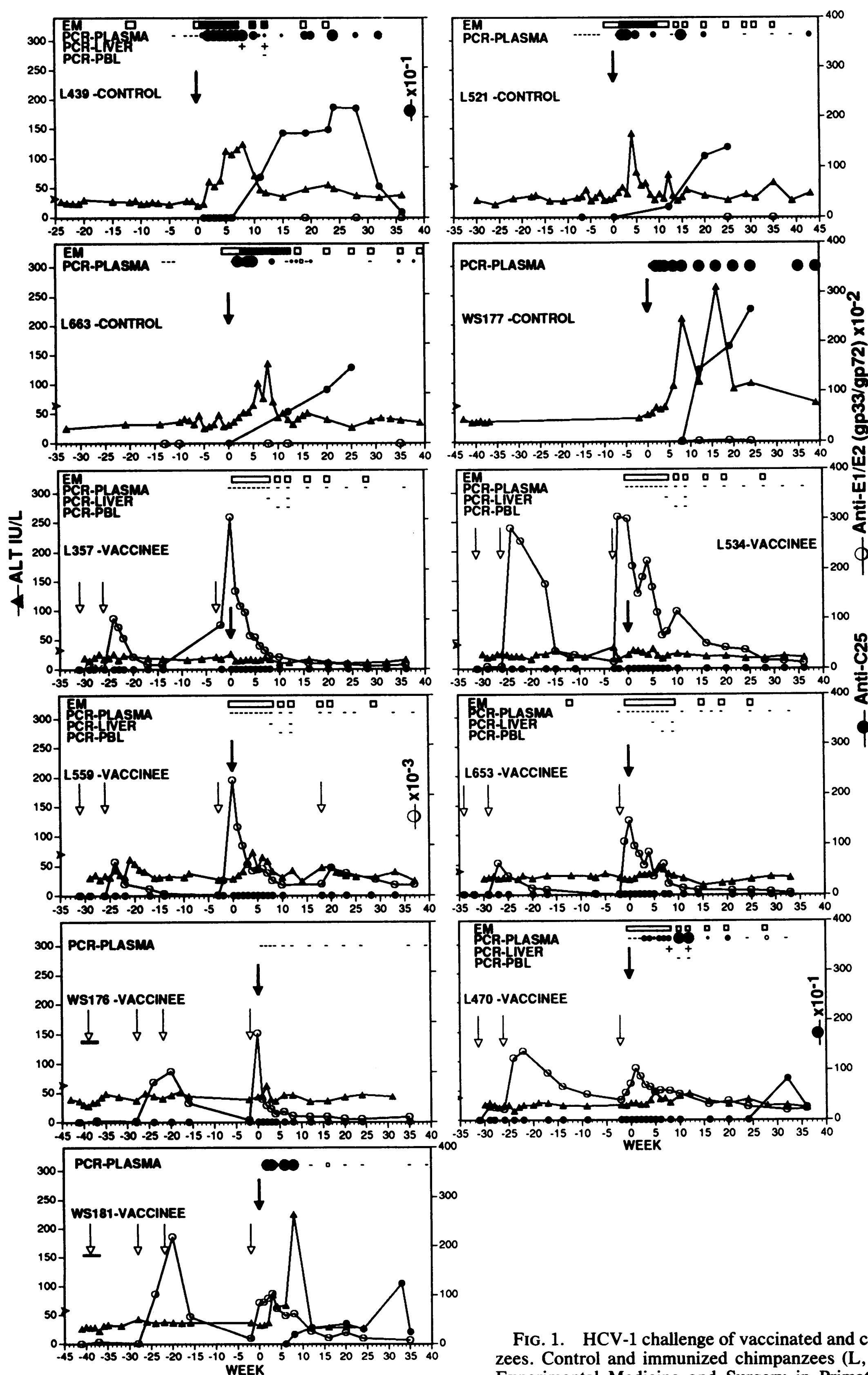

FIG. 1. HCV-1 challenge of vaccinated and control chimpanzees. Control and immunized chimpanzees (L, Laboratory for Experimental Medicine and Surgery in Primates; WS, White Sands) were challenged with ≈10 CID_{50} of HCV-1 and monitored as described. The presence (solid boxes) or absence (open boxes) of hepatocyte ultrastructural changes observed in the electron microscope (EM) is indicated. The approximate relative levels of HCV-1 RNA detected in plasma using reverse transcriptase (RT)-PCR assays are reflected by sizes of the shaded circles (open circles denote borderline positives; minus signs denote undetectable levels). The results of RT-PCR assays of liver and PBL extracts are recorded as either + or −. Note that the scales for anti-C25 and anti-E1/E2 (gp33/gp72) are different in animals L439, L470, and L559, respectively, and are expressed as the product of the ELISA OD reading in the determined linear range multiplied by the serum dilution factor. The arrows in the ALT axes indicate the mean + 3.75 SD of prechallenge values; open vertical arrows notate time of subunit vaccine administration, and the solid vertical arrow denotes viral challenge on week 0. IU/L, international units per liter.

Table 2. Prevalence of HCV antibodies in chimpanzees chronically infected with HCV-1 or HCV-H strains

	Prevalence of HCV protein domain (antigen)						
Antigen	C (C22)	E2 (gp43)	E1 and E2 (gp33/gp72)	NS3 (C33C)	NS4 (C100)	NS5 (NS5)	C, NS3, and NS4 (C25)
Ratio	4/14 (29%)	2/14 (14%)	4/10* (40%)	14/14 (100%)	12/14 (86%)	6/14 (43%)	14/14 (100%)

Antibody assays were done as described (2, 3, 30) on plasma samples from viremic chimpanzees with chronic infections of ≥36 weeks and ≤13 yr duration.

*Titers of positive samples were 23, 30, 177, and 477 (range, 0–477; mean, 71) and compared with titers of between 395 and 39,720 (mean, 4186) seen in infected blood donors and chronically infected patients (n = 25).

protected against experimental infection. In contrast, the two animals exhibiting the lowest antibody response to the vaccine at the time of virus challenge both clearly became infected and seroconverted to anti-C25 as well as to individual nonstructural proteins (L470 and WS181, Fig. 1 and data not shown). However, viremia was delayed to the third week after challenge in L470 and was generally lower than observed in the four control, infected animals (Fig. 1). Also, this animal showed no ultrastructural alterations within hepatocytes, exhibited serum ALT levels that were only minimally elevated (Fig. 1), and developed less liver inflammation than observed in three of the four control, infected animals (data not shown). Both animals have also been negative in the most recent PCR assays for viral RNA (Fig. 1).

DISCUSSION

The striking protection observed against viral challenge in five of the seven vaccinees appeared directly related to the level of the vaccine-induced antibody response. Antibody titers of ≈≥15,000 (Fig. 1) apparently resulted in protection against both infection and disease, from a challenge dose (≈10 CID_{50}) that may be representative of that transmitted in many community-acquired infections of this generally low-titer viral agent. Slight increases in serum ALT levels were observed in some of these protected animals (L559, L653, and WS176), but there were no additional markers of infection present and sensitive RT-PCR assays for viral RNA of plasma, PBL, and liver samples were all negative (Fig. 1). Conceivably, these animals might have undergone a mild, abortive infection. Alternatively, the minimal ALT elevations may have been as a result of performing frequent liver biopsies during this period, either as a direct consequence of the anesthesias and/or as a result of minimal, needle-induced liver damage.

Only one of the five protected animals was initially inoculated with live rVV (Table 1 and Fig. 1 animal L534), and the E1/E2 antigen purification scheme was shown to inactivate the original vaccinia virus used as the expression vector. In addition, two of the five protected animals were immune to vaccinia as a result of a previous inoculation with the live, wild-type WR vaccinia virus (Table 1 and Fig. 1, L357 and L653). Therefore, the observed efficacy of the subunit vaccine was clearly not dependent on priming and/or boosting of the immune response by live rVV.

Two of the seven vaccinees became infected after viral challenge; these were animals that failed to respond well to the final vaccine immunization and which, therefore, had the lowest antibody titers at the time of viral challenge (Fig. 1, L470 and WS181). However, infection was retarded and inhibited in one of these animals, as was the acute phase of hepatitis (Fig. 1, L470). Interestingly, both of these infected vaccinees were negative for viral RNA in the most recent RT-PCR assays, at which time liver functions had returned to normal (Fig. 1). Thus, these animals may have resolved their acute infections. It will be very important to confirm this hypothesis from further follow-up studies of these animals because all eight control chimpanzees experimentally infected with HCV-1 during our studies have developed chronic infections (Fig. 1 and data not shown). Thus, vaccination may stimulate the resolution of acute infection in those cases where primary infection is not prevented.

A wide range in vaccine-induced anti-E1/E2 levels was obtained among the seven animals at the time of viral challenge (Fig. 1; range, 7,300–229,920; mean, 48,391), which were generally higher than the levels observed in infected blood donors and chronically infected patients (range, 395–39,720; mean, 4186; n = 25) and which were considerably higher than the levels found in chimpanzees chronically infected with either HCV-1 or the highly related "H" strain (Table 2; range, 0–477; mean, 71; n = 10). This nonexistent or weak humoral immune response to the virion proteins observed in unimmunized, experimentally infected chimpanzees relative to infected humans (see ref. 30 and Table 2) suggests that the immunogenicity of the vaccine may, in turn, be substantially higher in humans than chimpanzees. In addition, immunogenicity data obtained by using recombinant glycoproteins of herpes simplex virus type 2 suggest that antibodies induced in human volunteers (31) are substantially more stable than in nonhuman primates (32). Thus, the stability of HCV vaccine-induced antibodies may be anticipated to be greater in humans than that observed in our vaccinated chimpanzees in which a rapid turnover was observed (Fig. 1). Future immunogenicity studies in humans will answer these important questions. It is also interesting to note that the weak or nonexistent humoral immune response to E1/E2 observed in our experimentally infected chimpanzees could account for the ready ability to re-infect chimpanzees with homologous viral challenges (33, 34).

Although the HeLa cell-derived vaccine used in the current studies was clearly efficacious, an earlier study involving the immunization of two chimpanzees with a combination of partial E1 (aa 199–330) and partial E2 (aa 404–661) antigens derived from yeast and insect cells, respectively, failed to protect both animals from infection after challenge with either ≈100 or ≈10 CID_{50} of HCV-1. However, the resulting hepatitis in one animal (10 CID_{50} challenge) was milder than that in unimmunized, infected control animals, whereas in the other (100 CID_{50} challenge), chronic infection was not established (M.H., unpublished work). Another previous study involved the immunization of one chimpanzee with live rVV followed by just a single dose of HeLa-derived E1 (gp33) and E2 (gp72). Although the induced antibody response was not high enough to prevent infection after challenge (≈100 CID_{50} of HCV-1), the resulting hepatitis was ameliorated in comparison with that in control, infected animals (M.H., unpublished work). These data indicate the importance of both antigen selection and immunization regimen in vaccine efficacy. The current study reported here did not evaluate efficacy against a challenge of >≈10 CID_{50} of HCV-1. Further investigations are required to address this potentially important issue. It should also be emphasized that the vaccinees in this study were challenged within a few weeks of the last boost at a time when antibody levels were maximal. The effects of challenging at later times on the development of both acute and chronic infection remain important issues for the future.

Another important issue relates to the observed heterogeneity of HCV. At least six related, but nonetheless distinct, genotypes have now been distinguished from phylogenetic analyses, and at least some of these virus types are composed of more than one subtype (35). The primary amino acid sequences of the putative envelope glycoprotein domains differ by up to 50% (at least), suggesting that multivalent vaccines may be required for global protection. However, the results reported here offer substantial encouragement and optimism for achieving effective control of HCV infection.

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