JOURNAL OF VIROLOGY, Sept. 1995, p. 5798–5805 Vol. 69, No. 9
0022-538X/95/$04.00+0

DNA-Based Immunization with Chimeric Vectors for the Induction of Immune Responses against the Hepatitis C Virus Nucleocapsid

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Received 9 March 1995/Accepted 17 May 1995

Vectors expressing the first 58 amino acids of the hepatitis C virus (HCV) nucleocapsid alone or as a fusion protein with the middle (pre-S2 and S) or major (S) surface antigens of hepatitis B virus (HBV) were constructed. Intramuscular immunization of BALB/c mice with the chimeric constructs in the form of naked DNA elicited humoral responses to antigens from both viruses within 2 to 6 weeks postinjection. No anti-HCV responses were obtained in mice immunized with the vector expressing the HCV sequence in the nonfusion context. Sera from chimera-injected mice specifically recognized both HCV capsid and HBV surface antigens in enzyme-linked immunosorbent assay and immunoblot testing. Anti-HCV serum titers formed plateaus of approximately 1:3,000; these remained stable until the end of the study (18 weeks postinfection). Anti-HBV immune responses were found to be lower in the chimera-injected animals (<200 mIU/ml) than in those immunized with the native HBV vector (>2,000 mIU/ml). This is the first report of the use of DNA-based immunization for the generation of immune responses to an HCV protein. In addition, these findings show that it is possible to elicit responses to viral epitopes from two distinct viruses via DNA immunization with chimeric vectors.

Hepatitis C virus (HCV) has been shown to be the major cause of parenterally acquired viral non-A, non-B hepatitis (1). In most cases, the virus causes a persistent infection, and previous studies clearly indicate an association between chronic infection with HCV and the development of chronic liver diseases, cirrhosis, and hepatocellular carcinoma (20, 33, 38). The prevalence of the virus throughout the world varies between 0.4 and 2%, although levels as high as 10 to 20% have been reported in Egypt (15). Improvements in diagnostic techniques for the detection of HCV antibodies (Abs) have reduced the risk of infection through contaminated blood products. However, community-acquired infection is still common, particularly in high-risk groups, with a significant proportion of cases in the United States associated with no known risk factors (1). Therefore, a vaccine to protect against infection by HCV remains necessary.

The genome of HCV (9.5 kb; positive-strand RNA), similar in organization to those of the flavi- and pestivirus groups, is expressed as a single polypeptide from a long open reading frame, which is subsequently cleaved by viral and cellular proteases. The structural proteins consist of the core, forming the nucleocapsid, and at least two glycosylated envelope proteins, E1 and E2 (5, 16). The sequence of the viral envelope exhibits both genotype and subtype variation at the amino acid level (17), with the presence of a hypervariable region in the E2 domain (31, 32, 46). This region, which contains linear Ab-binding epitopes, appears to be a target for human immune responses and has been suggested as a possible site through which the selection of immune escape mutants can occur (47). By contrast, the nucleocapsid is well conserved between genotypes (3, 18), and both cytotoxic T-lymphocyte (CTL) and B-cell determinants have been mapped to this antigen (2, 21, 22, 30, 42). CTL generated against internal viral antigens are thought to be important for an effective immune response and can lead to cross-protection against different viral strains. Protective immunity has been successfully induced in animals by using the nucleoprotein of hepatitis B virus (HBV) (19, 29) and rabies virus (11), suggesting that the HCV nucleocapsid could also be used as an effective vaccine antigen.

Traditional methods of protection against biological agents have relied on attenuated or killed vaccines presenting viral proteins directly to the immune system as particles or recombinant antigens. Such vaccines can have limitations, particularly in the generation of CTL responses. Recently, immunization techniques in which naked DNA encoding specific antigens is introduced directly into mammalian tissue have been developed (48). Because of in vivo expression of the immunogen, such gene inoculations can lead to the presentation of antigens in a more natural form, thereby mimicking that observed during the viral infection. This technique has been used to elicit immune responses in animals against several viral agents, including human immunodeficiency virus (44, 45), bovine herpes virus 1 (6), and HBV (8, 25). Importantly, DNA immunization with vectors expressing the nucleocapsid (36, 43) and hemagglutinin (14, 37) genes of influenza virus or glycoprotein of rabies virus (49) have been shown to be protective in mice.

The aim of this study was to compare different vectors for the presentation of the HCV nucleocapsid in the generation of immune responses by using DNA-based immunization. We used a system expressing the HCV nucleocapsid alone or as a fusion with the HBV surface proteins (HBsAg); these constructs have been previously shown to be effective for the presentation of foreign epitopes in immunization using in vitro-produced particles (10, 27). In addition, naked DNA en-

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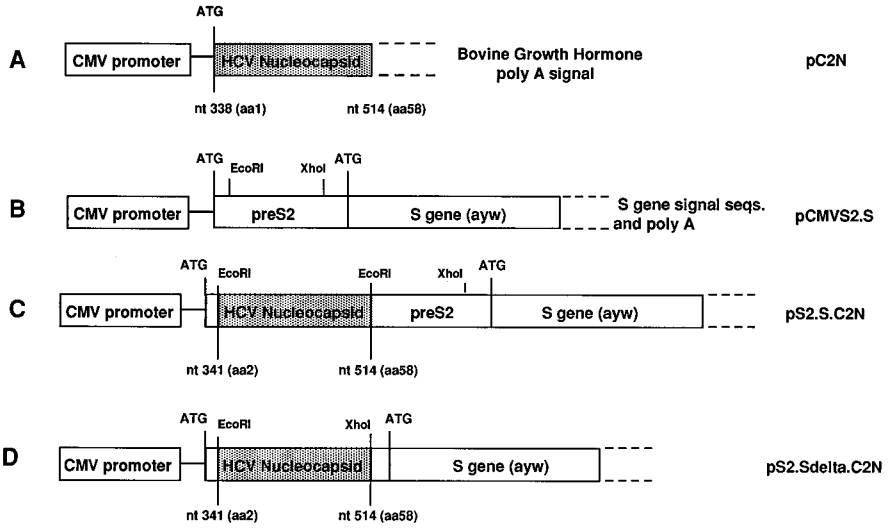


FIG. 1. Expression vectors for HBV and HCV antigens. pC2N (A) expresses the first 58 aa of the HCV nucleocapsid. The nucleotide sequence was cloned from a vector encoding nt 1 to 5404 of the HCV genome (H strain), conserving the native capsid ATG and translation initiation context. pCMVS2.S (B) expresses the pre-S2 and S antigens from the two in-frame ATG codons. The segment encompassing nt 341 to 514 (aa 2 to 58) of the HCV nucleocapsid was cloned by PCR amplification from the same plasmid used to make pC2N into the *Eco*RI or the *Eco*RI and *Xho*I sites of pCMVS2.S to create the chimeric vectors pS2.S.C2N (C) and pS2.Sdelta.C2N (D).

coding HBsAg has been shown to elicit high humoral and CTL responses when used to immunize mice intramuscularly (7, 9).

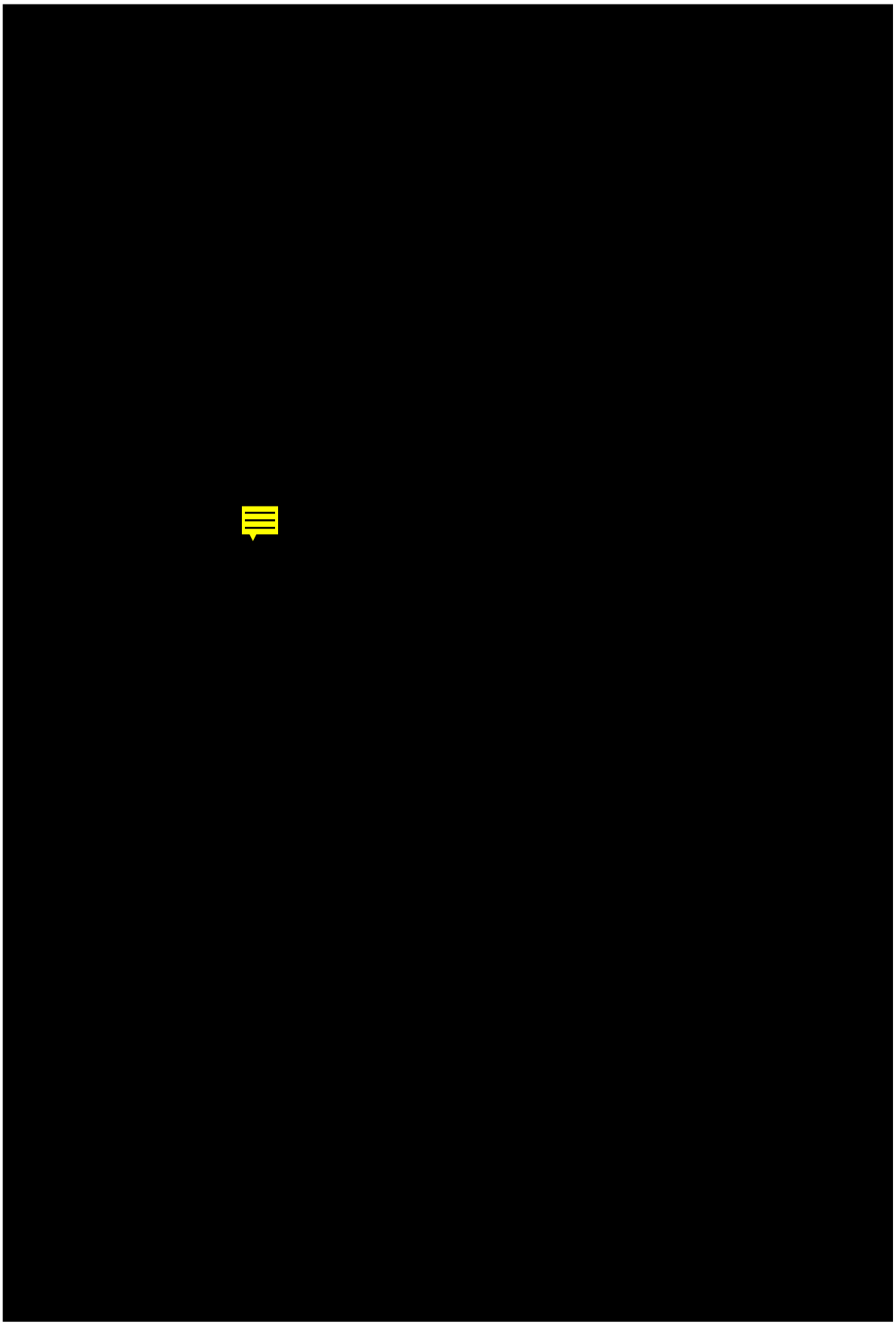
For the purpose of this study, we constructed vectors expressing the first 58 amino acids (aa) of the HCV nucleocapsid alone or fused to the pre-S2 and/or S genes of HBV. This region contains the major human B-cell determinants of the capsid protein (30) as well as human CTL epitopes (2, 22) and contains no internal ATG codons which would interfere with the expression of the HBV proteins in the fusion contexts. We report here the use of chimeric expression vectors for the successful generation of immune responses to a portion of the capsid of HCV which, when expressed in a nonfusion context, elicited no HCV-specific Abs. We also demonstrate that it is possible to generate immune responses to antigens from two distinct viruses, namely, the HBsAg and the HCV nucleocapsid, via intramuscular injection of naked DNA.

Construction of expression vectors. Throughout this report, the term "chimeric vectors" is used to describe constructs expressing determinants from both HBV and HCV, and "nonchimeric" refers to those expressing proteins from either HBV or HCV. All vector constructions and analyses were carried out by using standard techniques (39).

Nonchimeric vectors pC2N, expressing aa 1 to 58 of the HCV nucleocapsid, and pCMVS2.S (25), expressing the major (S) and middle (pre-S2 and S) proteins of HBV (Fig. 1A and B, respectively), were used to assess the effects of the fusion context on the presentation of determinants from both viruses. Chimeric vectors which expressed the first 58 aa of the HCV capsid as a fusion with the HBV surface proteins under the control of the cytomegalovirus promoter were constructed. The first chimera (pS2.S.C2N; Fig. 1C) expressed the capsid at the amino terminus of pre-S2. In this context, there were 15 nucleotides (nt) of pre-S2 upstream of the HCV sequence, encoding 5 aa including the initiator methionine. The second chimera (pS2.Sdelta.C2N; Fig. 1D), in which most of the pre-S2 region was replaced by the HCV sequence, contained the same 15 nt upstream of the capsid but only 27 nt, encoding 9 aa, of pre-S2 3′ terminal. The two chimeric vectors expressed identical HCV capsid protein sequences as fusions with either the pre-S2 and S proteins (pS2.S.C2N) or S alone (pS2.Sdelta.C2N). The pre-S2 region was removed in the pS2.Sdelta.C2N construct to determine whether this strongly immunogenic domain had any masking effect on the HCV capsid immune response. Equally efficient expression of fusion and nonfusion proteins from all vectors was confirmed by in vitro translation using the Promega TNT T7 coupled reticulocyte lysate system (data not shown).

Cellular expression of HBV and HCV proteins. The distribution of both HBV and HCV proteins in transfected cells was analyzed by immunostaining. Diet cells, a mouse hepatocyte cell line transformed with the simian virus 40 large T antigen and containing unexpressed HBV sequences (a gift from C. Pourcel [34]), were maintained as monolayers in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Petri dishes (100-mm diameter) were seeded with 10^6 cells 24 h before transfection with 20 μg of DNA, using a calcium phosphate transfection system (Gibco BRL), and analyzed for specific antigen expression at 48 h posttransfection. Cells were fixed with 2% formaldehyde–phosphate-buffered saline (PBS) and permeabilized with 1% Triton–10% sucrose–1% fetal calf serum in PBS. Expression of proteins was detected by double labelling of cells with an anti-HCV capsid mouse monoclonal antibody (a gift from M. Jolivet, BioMerieux, Marcy l'Etoile, France) and an anti-HBsAg rabbit polyclonal Ab (Behring). Tetramethyl rhodamine isothiocyanate and fluorescein isothiocyanate anti-mouse immunoglobulin (Ig) and anti-rabbit Ig conjugates were used as secondary antibodies.

Despite the efficient expression of the HCV capsid from pC2N in vitro, no cellular staining of the antigen was observed in transfected cells, although control experiments using a chloramphenicol acetyltransferase-expressing vector (pCAT; Invitrogen Corp.) confirmed successful transfection (data not shown). This finding suggested that the HCV transcript or protein was unstable within the cellular environment. By con-



trast, both HBsAg and HCV capsid proteins were detected in cells transfected with pS2.S.C2N and pS2.Sdelta.C2N (Fig. 2A to D). The use of the two conjugates demonstrated that these proteins were expressed in the same cells and showed identical localization, suggestive of the presence of a fusion protein. This possibility was further confirmed by immunoblotting of cell lysates with an anti-HCV capsid Ab which detected polypeptides of the expected sizes in the glycosylated and nonglycosylated forms, indicating that the HCV capsid sequence does not affect the glycosylation of the HBV proteins in these fusions (data not shown).

Strong expression of HBsAg was observed in the cytoplasm of cells transfected with the vector pCMVS2.S (Fig. 2F). These cells were negative for HCV capsid expression (Fig. 2E). No fluorescence signals were observed when preimmune rabbit serum was used as the primary antibody (Fig. 2G) or in cells transfected with the vector pcDNA3 (Fig. 2H).

Comparisons between cells transfected with the chimeric and native vectors showed a difference in the fluorescence patterns obtained for HBsAg. The signal in cells expressing this protein alone (Fig. 2F) was more diffuse than that observed for the fusion proteins (Fig. 2B and D), in which the antigen appeared more punctate and showed a greater association with the cellular membranes. Some nuclear localization of the fusion proteins was also observed, suggesting an effect of the HCV capsid sequence on the distribution of the HBsAg in the transfected cells.

Cytoplasmic patterns and nuclear staining similar to those reported here have been previously observed in cells expressing the capsid alone (23, 40). Several potential nuclear localization signals have been identified within the capsid (40), one of which (aa 38 to 43) is contained within the capsid sequence included in our chimeric vectors. Such a signal could contribute to the redistribution of antigens within the cells, thereby affecting their secretion or presentation.

The presence of fusion proteins carrying the HCV determinant was demonstrated in the culture supernatant of cells transfected with the chimeric vectors by using an HCV capsid capture enzyme-linked immunsorbent assay (ELISA) using anticapsid monoclonal Ab-coated plates and an anti-HBsAg polyclonal Ab to detect captured protein (data not shown). Analyses using a commercial ELISA employing a mixture of anti-HBsAg monoclonal Abs (MONOLISA AgHBs; Diagnostic Pasteur, Marnes-la-Coquette, France) detected secreted HBsAg in culture supernatants of cells transfected with the vectors pS2.S.C2N and pS2.Sdelta.C2N, although at reduced concentrations and at later time points compared with pCMVS2.S. Analysis of equivalent cellular extracts indicated similar differences in HBsAg concentrations within the cells (data not shown). These observations strongly suggest an effect of the HCV capsid on the HBV antigens possibly altering the presentation of these proteins such that they are no longer recognized by the monoclonal Abs used in the detection assay.

Seroconversion to virus-specific Abs in DNA-immunized mice. All DNA preparations were generated by using Qiagen purification columns (Diagen, Hilden, Germany). Vectors were injected into the tibialis anterior muscles of 9- to 12-week-old male BALB/c mice (Charles River, Saint-Aubin-lès-Elbeuf, France) as described by Davis et al. (8). Half of the immunized mice were given three additional injections of DNA at 2-week intervals. Mice anesthetized with sodium pentobarbital (0.1 ml of a 0.75% [wt/vol] solution in 0.9% [wt/vol] physiological saline per 10 g of body weight) were bled from the eye at 2- to 4-week intervals, and the sera were analyzed for Abs to HBsAg and HCV nucleocapsid. Additional mice were injected intravenously with 75 μg of purified *Escherichia coli*-expressed capsid (30) three times at 3-week intervals. These animals were sacrificed at 12 weeks postinjection (p.i.), and sera were collected for comparative studies.

Sera were tested for HBsAg Abs by using the MONOLISA anti-HBsAg second-generation assay, which uses a mixture of HBsAg (pre-S2 and S) from the ad and ay subtypes (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). ELISA testing and epitope mapping of Abs to HCV nucleocapsid were carried out with purified *E. coli*-expressed capsid protein or specific peptides as previously described (30).

In this report, the term "seroconversion" defines the production of detectable HCV- or HBV-specific Abs in immunized mice.

(i) Anti-HCV Ab responses. Serum samples from mice injected with the vector pC2N were tested up to 14 weeks p.i., but no anti-HCV nucleocapsid responses were detectable. By contrast, 100% of the mice injected with the chimeric vectors seroconverted to anti-HCV capsid Ab within 6 weeks p.i. (Fig. 3A), indicating that the incorporation of the capsid into a chimeric context stabilized its expression and improved presentation to the mouse immune system.

(ii) Anti-HBsAg Ab responses. All of the mice immunized with the vector pCMVS2.S seroconverted to anti-HBsAg Ab within 2 weeks p.i. in both the boosted and nonboosted groups (Fig. 3B). This finding contrasts with the slower response rates observed in the animals injected with the chimeric vectors; in particular, only 70% of those injected with the vector pS2.Sdelta.C2N developed detectable Abs.

Comparisons of the responses to the two viruses suggest that the slower response rates to HBsAg in the chimera-injected mice were not due to an overall slower immune reaction in these groups or ineffective antigen expression from these vectors but were due to a change in the conformation or presentation of the HBsAg expressed from these constructs.

Quantitative determination of Ab levels in DNA-injected mice. Analyses of quantitative levels of circulating Ab illustrate more clearly the differences in the responses to the different expression vectors.

(i) Anti-HCV Ab responses. The anticapsid responses observed in the mice immunized with the chimeric vectors are shown in Fig. 4 as mean reciprocal end point titers determined by ELISA. In this case, mice given booster injections mounted higher responses to both vectors earlier than those given single injections of DNA (titers of 1:3,000 to 1:4,000 versus 1:1,000 to 1:1,500 at 6 weeks p.i.). The mean titers of mice from boosted groups did not increase after 6 weeks p.i., suggesting that the final injection of DNA did not contribute to a further enhancement of the response. In contrast, the serum titers in animals given single injections of DNA, although initially lower overall than in those given additional injections, rose within the same time period from 1:1,000 to between 1:2,000 and 1:4,000. At 18 weeks p.i., all mean titers for HCV capsid Ab in all of the

FIG. 2. Immunofluorescence analysis of HBsAg and HCV capsid expression in transfected Diet cells. At 48 h posttransfection, cells were fixed and permeabilized as described in the text and incubated with both anticapsid (1:500) and anti-HBV (1:200) Abs. Specific Ab binding was detected by using a mixture of tetramethyl rhodamine isothiocyanate (TRITC; 1:20; Nordic) and fluorescein isothiocyanate (FITC; 1:40; Dako) as indicated. Transfections were performed with pS2.S.C2N (A and B), pS2.Sdelta.C2N (C and D), pCMVS2.S (E and F), pS2.S.C2N (preimmune rabbit serum was used as the primary antibody) (G), and pcDNA3 (Invitrogen) (H).

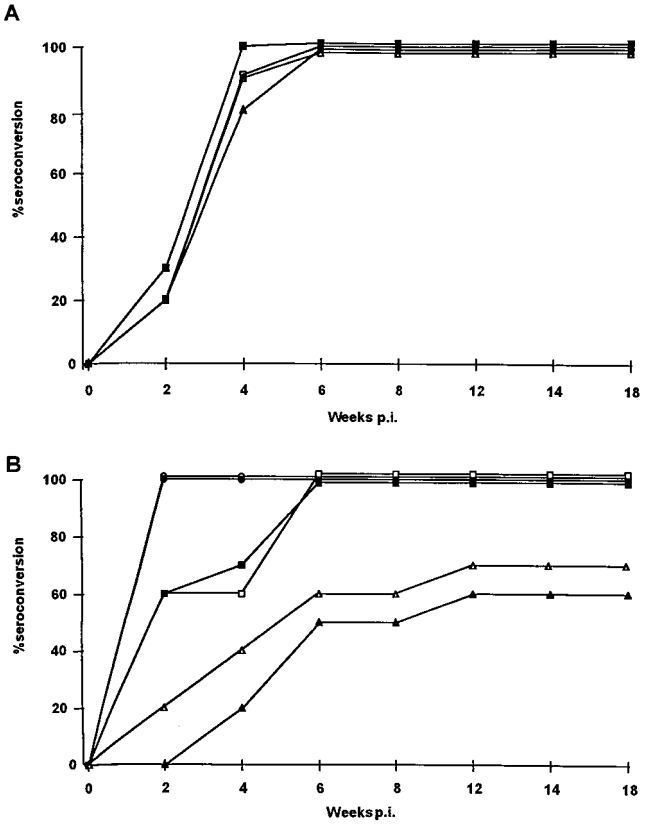


FIG. 3. Percentage seroconversion of mice following DNA injection. (A) Anti-HCV capsid Ab responses; (B) anti-HBsAg Ab responses. Boosted and nonboosted animals are represented by filled and open symbols, respectively. ■, pS2.S.C2N boosted (n = 10); □, pS2.S.C2N (n = 10); ▲, pS2.Sdelta.C2N boosted (n = 10); △, pS2.Sdelta.C2N (n = 10); ●, pCMVS2.S boosted (n = 5); ○, pCMVS2.S (n = 5).

immunized groups were at comparative levels, suggesting that in the long term, the generation of anticapsid Ab is not necessarily improved by the administration of multiple injections. The anti-HCV Ab titers obtained in the DNA-immunized mice were comparable to those obtained from the animals injected with purified capsid protein (data not shown).

The major HCV capsid determinants recognized by the sera from mice taken 6 weeks after injection with the chimeric vectors were mapped by peptide ELISA. Sera from DNA and protein injected mice bound most strongly to a peptide spanning aa 2 to 45. This peptide was mapped more specifically to residues 21 to 45 for sera from mice injected with pS2.S.C2N (data not shown). No specific binding to the shorter peptide was obtained for sera from mice injected with pS2.Sdelta.C2N, possibly because of lower Ab titers present in these mice at this time.

(ii) Anti-HBsAg Ab responses. Figure 5 shows the levels of anti-HBsAg Ab in sera from injected mice expressed as milli-international units per milliliter. The graphs show mean Ab levels in sera from mice immunized with each vector, for single and multiple injection groups.

Both groups immunized with the vector pCMVS2.S showed a steady increase in Ab levels, forming plateaus at 6 and 14 weeks, respectively (Fig. 5C). The responses in the group given a single injection, although lagging behind those of the mice given multiple injections, reached higher levels at 14 to 18 weeks p.i. (an average of 4,000 mIU/ml, compared with 2,000 mIU/ml), suggesting that for this vector, additional injections are not necessary for the generation of higher Ab titers. These data contrast with the anti-HBsAg responses in mice injected with the chimeric vectors (Fig. 5A and 5B), in which the mean levels were considerably reduced in all groups. Additional immunizations improved the responses to HBsAg, although the mean values were still only 30 to 50% (500 to 1,000 mIU/ml) of those observed for the native vector. The lower responses to pS2.Sdelta.C2N than to pS2.S.C2N could be due to the absence of the pre-S2 sequence in this vector, as Abs to this region are also assayed by the ELISA used in this study.

Differences in Ab titers of between 5- and 10-fold were observed between individual mice within the same groups for all antigens, using both chimeric and nonchimeric vectors, demonstrated by the error bars in Fig. 4 and 5. However, no correlation could be made between high or low HBV responses to chimeric vectors and a correspondingly high or low anti-HCV capsid response.

Comparisons between the two chimeric vectors indicate that for both viral determinants, the Ab responses to pS2.S.C2N were slightly superior to those against pS2.Sdelta.C2N, which lacks the pre-S2 sequence. This finding indicates not only that the response to the capsid is unaffected by that to the pre-S2 region but that this sequence contributes to the improved presentation of the HCV antigen to the immune system. The absence of the pre-S2 sequence in the vector pS2.Sdelta.C2N may have contributed to the reduced anti-HBV responses observed in the injected mice. However, the removal of the

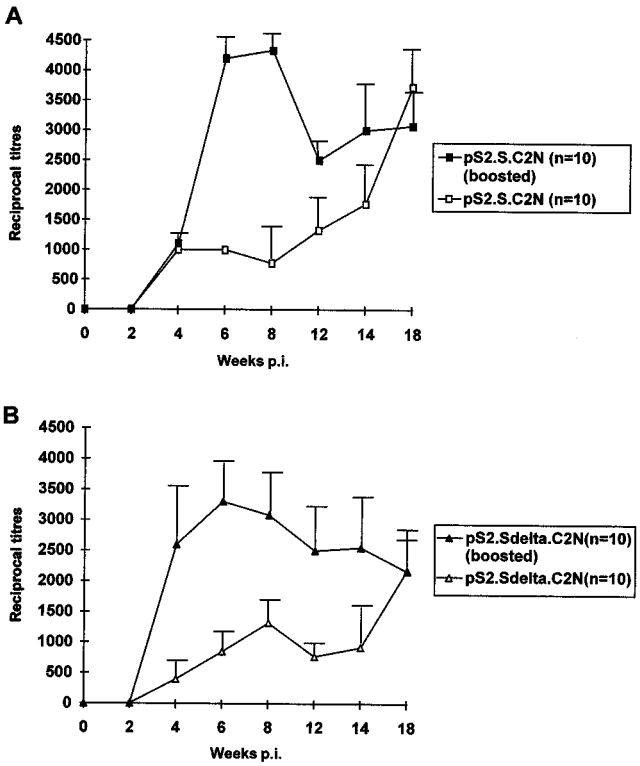


FIG. 4. ELISA titers of anti-HCV capsid Abs in inoculated mice. Results are shown as the reciprocal of the serum dilution equivalent to three times the optical density (OD) of sera from mice injected with the vector pcDNA3 against time (weeks) p.i. (A) pS2.S.C2N; (B) pS2.Sdelta.C2N. Boosted and nonboosted animals are represented by filled and open symbols, respectively. For clarity, error bars indicating the standard errors of the means are shown in the positive sense only.

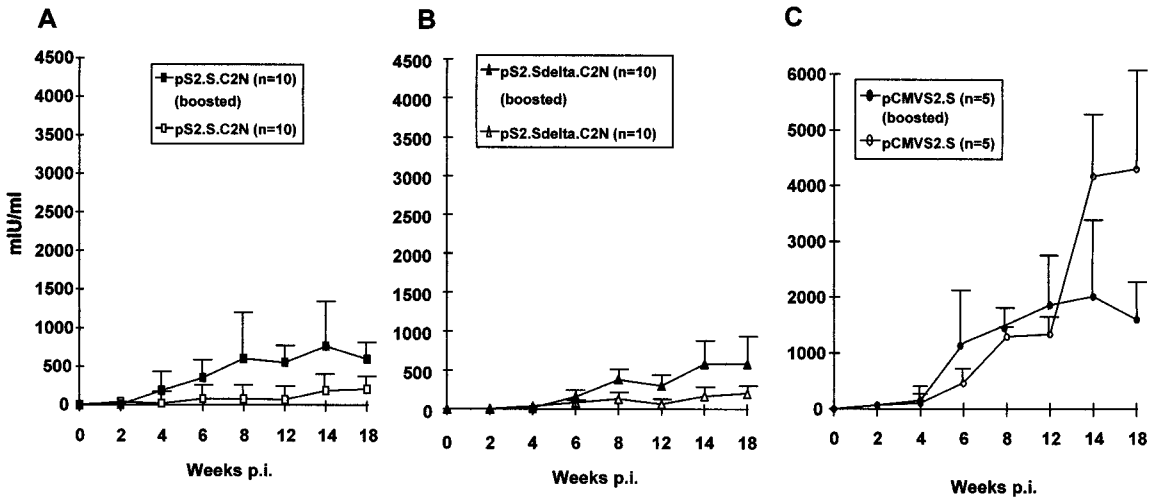


FIG. 5. Quantitative assessment of anti-HBsAg Abs in inoculated mice. Results are expressed as mean milli-international units per milliliter against time (weeks) after the first injection. (A) pS2.S.C2N; (B) pS2.Sdelta.C2N; (C) pCMVS2.S. Boosted and nonboosted animals are represented by filled and open symbols, respectively. For clarity, error bars indicating the standard errors of the means are shown in the positive sense only.

pre-S2 sequence from the vector pCMVS2.S and its effect on the induced anti-HBV immune responses in DNA-immunized mice have been addressed by Michel et al. (25) and shown to lead to no decrease in the anti-HBsAg titers obtained.

The HCV sequence used in this study clearly alters the form and distribution of HBsAg in transfected cells from a diffused pattern confined to the cytoplasm to a more aggregated or complexed form with some nuclear localization. We also observed that the lower levels of HBsAg detected in cells transfected with the chimeric vectors correlate with those observed in the supernatant, indicating that this finding is not solely due to nonsecretion of particles. The in vivo data from mice immunized with these vectors (i.e., lower anti-HBsAg Ab titers and slower seroconversion rates) reflect the observations made in vitro. These observations are not considered to be due to inefficient expression from the chimeric vectors compared with that obtained from the native vector for several reasons. (i) No start codons which could have lead to aberrant translation initiation exist in any reading frame within the capsid sequence used. (ii) In vitro translation indicated comparatively efficient expression of proteins from all vectors. (iii) Immunofluorescence analysis suggests that similar levels of protein expression occur in all transfected cells in vitro, and immunoblotting indicates that full-length, glycosylated fusion proteins are produced from the chimeric vectors. Previous reports of studies using recombinant HBV particles (10, 27) suggest that the inserted sequence can have an effect on the presentation of the HBsAg in chimeric proteins which is more dependent on the nature than on the length of the foreign epitope. This seems to be the case with our chimeric constructs. Efforts are currently under way in this laboratory to improve the anti-HBV responses from such vectors while still maintaining the high anti-HCV responses.

Immunoblotting analysis of sera from DNA-immunized mice. The specificity of sera from immunized mice was assessed by immunoblotting as previously described (30), using extracts from cells infected with a baculovirus-HCV recombinant expressing the capsid, E1, and E2 proteins of HCV (Fig. 6A) or HBV particles purified from patient sera (Fig. 6B).

(i) Anti-HCV Ab. Rabbit polyclonal anticapsid serum (Fig. 6A, lane 5) detected a specific band of 22 kDa from baculovirus recombinant-infected cells, representing the HCV capsid. This antigen was recognized by anti-pS2.S.C2N and anti-pS2.Sdelta.C2N sera (lanes 2 and 3, respectively). No signals were obtained with sera from mice injected with pCMVS2.S or pcDNA3 (lane 1 or 4, respectively) or when the sera were blotted against extracts from cells infected with wild-type baculovirus (lanes 6 and 7).

(ii) Anti-HBsAg Ab. Sera from pCMVS2.S DNA-immunized

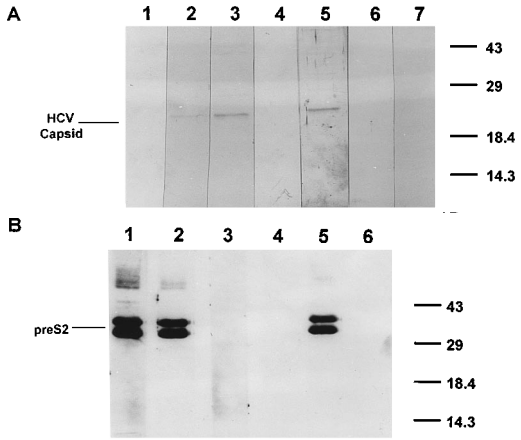


FIG. 6. (A) Immunoblots of sera from injected mice against extracts from cells infected with a baculovirus-HCV recombinant. Injected vectors: pCMVS2.S (lane 1), pS2.S.C2N (lane 2), pS2.Sdelta.C2N (lane 3), and pcDNA3 (lane 4). A rabbit anticapsid polyclonal antiserum (1:50) was used as a positive control (lane 5). Lanes 6 and 7 represent sera from pS2.S.C2N- and pS2.Sdelta.C2N-injected mice blotted against extracts from cells infected with wild-type baculovirus. The 22-kDa HCV capsid antigen is indicated on the left. (B) Immunoblots of sera from injected mice against purified HBV particles. Injected vectors: pS2.S.C2N (boosted) (lane 1), pS2.S.C2N (nonboosted) (lane 2), pS2.Sdelta.C2N (boosted) (lane 3), pS2.Sdelta.C2N (nonboosted) (lane 4), pCMVS2.S (lane 5), and pcDNA3 (lane 6). The glycosylated and nonglycosylated pre-S2 proteins detected by the sera are indicated on the left. Sera from positive mice were pooled and used at a 1:30 dilution. Anti-mouse or anti-rabbit IgG peroxidase-conjugated serum was used as the secondary antibody. Positive reactions were detected by using either 3,3-diaminobenzidine tetrahydrochloride (0.2 mg/ml; 0.05% [vol/vol] H_2O_2) (A) or the Amersham ECL Western blotting (immunoblotting) analysis system (B). Proteins were separated by sodium dodecyl sulfate–13% polyacrylamide gel electrophoresis (24). Positions of molecular weight markers are shown at the right in kilodaltons.

mice recognized only the pre-S2 antigen (Fig. 6B, lane 5) because of the linear nature of these epitopes compared with the conformational characteristics of the S antigen (28). Sera from mice immunized with pS2.S.C2N (lanes 1 and 2) gave a pattern the same as that for sera from pCMVS2.S-injected mice. The pS2.Sdelta.C2N construct lacks the majority of the pre-S2 coding region; therefore, sera from this group produced no signal against the HBV particles (lanes 3 and 4).

Isotyping of anti-HCV capsid sera. At 6 weeks p.i., sera from mice immunized with the chimeric vectors were tested to determine the major anticapsid isotype by using a mouse hybridoma subtyping kit (Boehringer Mannheim). This isotype was found to be predominantly IgG2a for both vectors, with trace amounts of IgG2b and IgG3 in individual mice with high anticapsid titers (data not shown). This finding was compared with that obtained for sera from mice immunized with purified *E. coli*-expressed capsid protein taken at 12 weeks p.i. The sera from these animals were found to contain predominantly subtype IgG1, with some IgG2a. Sera taken at 2 weeks p.i. were also tested for IgA and IgM antibodies but were found to be negative (data not shown). These results indicate a different type of immune response in mice when immunized with naked DNA rather than protein. The relevance of isotype differences in the natural infection and for the development of a protective immune response remains to be established. However, these differences may be important in the context of intramolecular or intrastructural T-cell help involving internal antigens which could be exploited in vaccine strategies (28).

Discussion. It is still unclear whether infection with HCV can generate a protective immune response in humans or chimpanzees (12, 35). Successful immunization against homologous HCV strains has been reported for studies using chimpanzees and purified recombinant E1-E2 complexes (4), although the efficacy of such a vaccine against heterologous strains of the virus has yet to be demonstrated. In vitro neutralization of HCV with human plasma from an HCV-positive patient prior to inoculation of seronegative animals (13) has been shown to prevent HCV infection. However, the neutralizing Abs were detected in the patient only early after infection, suggesting that the Ab repertoire does not remain stable because of the presence of circulating quasispecies. A similar observation was made by Shimizu et al. (41), who employed an in vitro assay using serial serum samples from a chronically infected patient. Although these more recent studies do not address the possibility of reactivation of specific neutralizing Ab upon later challenge with similar viruses, taken together with the numerous reports of variability in the envelope regions (31, 32, 47), these data bring into question the suitability of these antigens in immunization programs.

Immunization trials using the more conserved HCV capsid have yet to be reported, although the success of a vaccine containing this antigen may depend on the generation of adequate CTL responses in the host. DNA immunization is particularly useful for the induction of this type of immune response. The presence of a CTL response to this HCV antigen will need to be confirmed if the antigen is to be considered a viable component of a candidate vaccine. Such studies are currently under way in this laboratory together with the analyses of vectors containing additional capsid sequences, in fusion and nonfusion contexts, which include other identified CTL epitopes. This report also indicates the feasibility of using chimeric vectors and DNA immunization for the generation of responses to determinants from more than one virus.

We thank C. Pourcel and M.-L. Michel for useful discussion and advice. We thank M. Maisonnas, I. Bordes, P. Berthillon, and C. Garrigou for superb technical assistance.

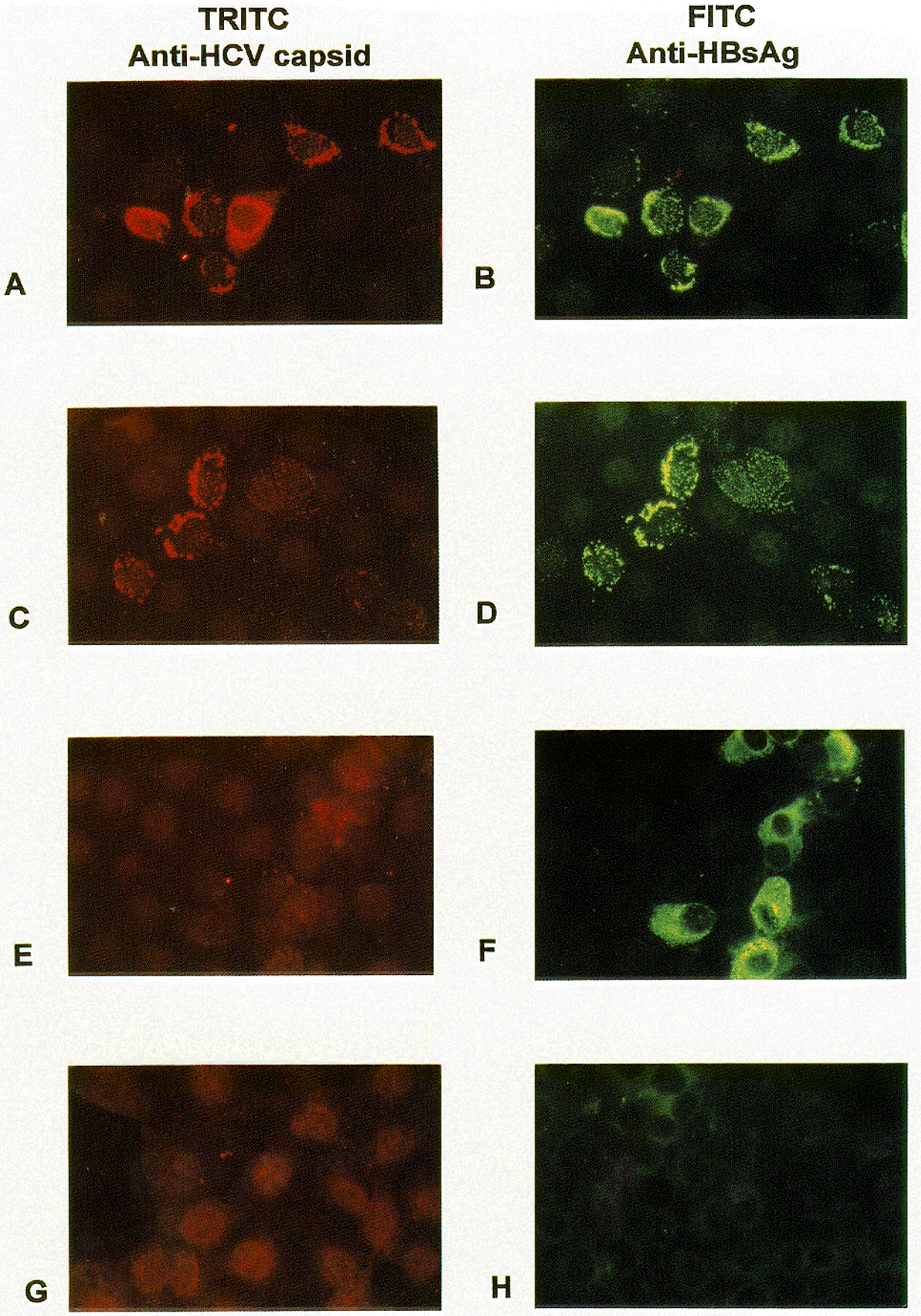
This work was partly financed by l'Association pour la Recherche sur le Cancer. M.E.M. is a recipient of a fellowship from Fondation Merieux.

REFERENCES

1. **Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, and Q. L. Choo.** 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med. **321:**1494–1500.
2. **Battegay, M., J. Fikes, A. M. Di Bisceglie, P. A. Wentworth, A. Sette, E. Celis, W.-M. Ching, A. Grakoui, C. M. Rice, K. Kurokohchi, J. A. Berzofsky, J. H. Hoofnagle, S. M. Feinstone, and T. Akatsuka.** 1995. Patients with chronic hepatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2.1 molecules. J. Virol. **69:**2642–2470.
3. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:**8239–8243.
4. **Choo, Q., G. Kuo, R. Ralston, A. Weiner, D. Chien, G. Van Nest, J. Han, K. Berger, K. Thudium, C. Kuo, J. Kansopon, J. McFarland, A. Tabrizi, K. Ching, B. Moss, L. B. Cummins, M. Houghton, and E. Muchmore.** 1994. Vaccination of chimpanzees against infection by the hepatitis C virus. Proc. Natl. Acad. Sci. USA **91:**1294–1298.
5. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, R. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**2451–2455.
6. **Cox, G. J. M., T. J. Zamb, and L. A. Babiuk.** 1993. Bovine herpesvirus 1: immune responses in mice and cattle injected with plasmid DNA. J. Virol. **67:**5664–5667.
7. **Davis, H. L., B. A. Demeneix, B. Quantin, J. Coulombe, and R. G. Whalen.** 1993. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. Hum. Gene Ther. **4:**733–740.
8. **Davis, H. L., M. L. Michel, and R. G. Whalen.** 1993. DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody. Hum. Mol. Genet. **3:**1847–1851.
9. **Davis, H. L., R. Schirmbeck, J. Reimann, and R. G. Whalen.** DNA-mediated immunization in mice induces a potent MHC class I-restricted cytotoxic T lymphocyte response to hepatitis B surface antigen. Hum. Gene Ther., in press.
10. **Delpeyroux, F., N. Chenciner, A. Lim, Y. Malpiece, B. Blondel, R. Crainic, and R. E. Streeck.** 1986. A poliovirus neutralization epitope expressed on hybrid hepatitis B surface antigen particles. Science **233:**472–475.
11. **Dietzschold, B., H. H. Wang, C. E. Rupprecht, E. Celis, M. Tollis, H. Ertl, and H. Koprowski.** 1987. Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. Proc. Natl. Acad. Sci. USA **84:**9165–9169.
12. **Farci, P., H. J. Alter, S. Govindarajan, D. C. Wong, R. Engle, R. R. Lesniewski, S. M. Desai, R. H. Miller, N. Ogata, and R. H. Purcell.** 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science **258:**135–140.
13. **Farci, P., H. J. Alter, D. C. Wong, R. H. Miller, S. Govindarajan, R. Engle, and R. H. Purcell.** 1994. Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. Proc. Natl. Acad. Sci. USA **91:**7792–7796.
14. **Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson.** 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. USA **90:**11478–11482.
15. **Hibbs, R. G., A. L. Corwin, N. F. Hassan, M. Kamel, M. Darwish, R. Edelman, M. R. Rao, A. S. Khalifa, S. Mokhtar, N. S. Fam, E. M. Kladions, and S. B. Bassily.** 1993. The epidemiology of antibody to hepatitis C in Egypt. J. Infect. Dis. **168:**789–790. (Letter.)
16. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno.** 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc. Natl. Acad. Sci. USA **88:**5547–5551.
17. **Houghton, M., A. J. Weiner, J. Han, G. Kuo, and Q. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **14:**381–387.
18. **Inchauspe, G., S. Zebedee, D. H. Lee, M. Sugitani, M. Nasoff, and A. M. Prince.** 1991. Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. Proc. Natl. Acad. Sci. USA **88:**10292–10296.
19. **Iwarson, S., E. Tabor, H. C. Thomas, P. Snoy, and R. J. Gerety.** 1985. Protection against hepatitis B virus infection by immunization with hepatitis B core antigen. Gastroenterology **88:**763–767.
20. **Katkov, W. N., J. L. Dienstag, H. Cody, A. A. Evans, Q. L. Choo, M.**

Houghton, and G. Kuo. 1991. Role of hepatitis C virus in non-B chronic liver disease. Arch. Int. Med. **151:**1548–1552.

21. **Kita, H., T. Moriyama, T. Kaneko, I. Harase, M. Nomura, H. Miura, I. Nakamura, Y. Yazaki, and M. Imawari.** 1993. HLA B44-restricted cytotoxic T lymphocytes recognizing an epitope on Hepatitis C virus nucleocapsid protein. Hepatology **18:**1039–1044.
22. **Koziel, M. J., D. Dudley, N. Afdhal, Q. Choo, M. Houghton, R. Ralston, and B. D. Walker.** 1993. Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognize epitopes in the core and envelope proteins of HCV. J. Virol. **67:**7522–7532.
23. **Kumar, U., D. Cheng, H. Thomas, and J. Monjardino.** 1992. Cloning and sequencing of the structural region and expression of putative core gene of hepatitis C virus from a British case of chronic sporadic hepatitis. J. Gen. Virol. **73:**1521–1525.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriphage T4. Nature (London) **227:**680–685.
25. **Michel, M. L., H. L. Davis, M. Schleef, M. Mancini, P. Tiollais, and R. G. Whalen.** DNA-mediated immunization to the hepatitis B surface antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. Proc. Natl. Acad. Sci. USA, in press.
26. **Michel, M. L., M. Mancini, Y. Riviere, D. Dormont, and P. Tiollais.** 1990. T- and B-lymphocyte responses to human immunodeficiency virus (HIV) type 1 in macaques immunized with hybrid HIV/hepatitis B surface antigen particles. J. Virol. **64:**2452–2455.
27. **Michel, M. L., M. Mancini, E. Sobczak, V. Favier, D. Guetard, and E. M. Bahraoui.** 1988. Induction of anti-human immunodeficiency virus (HIV) neutralizing antibodies in rabbits immunized with recombinant HIV-hepatitis B surface antigen particles. Proc. Natl. Acad. Sci. USA **85:**7957–7961.
28. **Milich, D.** 1988. T- and B-cell recognition of hepatitis B viral antigens. Immunol. Today **9:**380–386. (Review.)
29. **Murray, K., S. A. Bruce, P. Wingfield, P. van Eerd, A. de Reus, and H. Schellekens.** 1987. Protective immunisation against hepatitis B with an internal antigen of the virus. J. Med. Virol. **23:**101–107.
30. **Nasoff, M. S., S. L. Zebedee, G. Inchauspe, and A. M. Prince.** 1991. Identification of an immunodominant epitope within the capsid protein of hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**5462–5466.
31. **Ogata, N., H. J. Alter, R. H. Miller, and R. H. Purcell.** 1991. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**3392–3396.
32. **Okamoto, H., M. Kojima, S. Okada, H. Yoshizawa, H. Iizuka, T. Tanaka, D. A. Peterson, Y. Ito, and S. Mishiro.** 1992. Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology **190:**894–899.
33. **Paterlini, P., F. Driss, B. Nalpas, E. Pisi, D. Franco, and P. Berthelot.** 1993. Persistence of hepatitis B and hepatitis C viral genomes in primary liver cancers from HBsAg-negative patients: a study of a low-endemic area. Hepatology **17:**20–29.
34. **Paul, D., M. Hohne, C. Pinkert, A. Piasecki, E. Ummelmann, and R. L. Brinster.** 1988. Immortalized differentiated hepatocyte lines derived from transgenic mice harboring SV40 T-antigen genes. Exp. Cell Res. **175:**354–362.
35. **Prince, A. M., B. Brotman, T. Huima, D. Pascual, M. Jaffery, and G. Inchauspe.** 1992. Immunity in hepatitis C infection. J. Infect. Dis. **165:**438–443.
36. **Raz, E., D. A. Carson, S. E. Parker, T. B. Parr, A. M. Abai, G. Aichinger, S. H. Gromkowski, M. Singh, D. Lew, M. A. Yankauckas, S. M. Baird, and G. H. Rhodes.** 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. Proc. Natl. Acad. Sci. USA **91:**9519–9523.
37. **Robinson, H. L., L. A. Hunt, and R. G. Webster.** 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine **11:**957–960.
38. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, S. Koi, M. Onji, Y. Ohta, Q.-L. Choo, M. Houghton, and G. Kuo.** 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **87:**6547–6549.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Shih, C., S. J. Lo, T. Miyamura, S. Chen, and Y. Wu Lee.** 1993. Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. J. Virol. **67:**5823–5832.
41. **Shimizu, Y. K., M. Hijikata, A. Iwamoto, H. J. Alter, R. H. Purcell, and H. Yoshikura.** 1994. Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. J. Virol. **68:**1494–1500.
42. **Shirai, M., H. Okada, M. Nishioka, T. Akatsuka, C. Wychowski, R. Houghten, C. D. Pendleton, S. M. Feinstone, and J. A. Berzofsky.** 1994. An epitope in hepatitis C virus core region recognized by cytotoxic T cells in mice and humans. J. Virol. **68:**3334–3342.
43. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745–1749.
44. **Wang, B., J. Boyer, V. Srikantan, L. Coney, R. Carrano, C. Phan, M. Merva, M. Agadjanan, L. Gilbert, K. E. Ugen, W. V. Williams, and D. B. Weiner.** 1993. DNA inoculation induces neutralizing immune responses against human immunodeficiency virus type 1 in mice and nonhuman primates. DNA Cell Biol. **12:**799–805.
45. **Wang, B., K. E. Ugen, V. Srikantan, M. Agadjanyan, K. Dang, Y. Refaeli, A. I. Sato, J. Boyer, W. V. Williams, and D. B. Weiner.** 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **90:**4156–4160.
46. **Weiner, A. J., M. J. Brauer, J. Rosenblatt, K. H. Richman, J. Tung, K. Crawford, G. Saracco, Q. L. Choo, M. Houghton, and J. H. Han.** 1991. Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology **180:**842–848.
47. **Weiner, A. J., H. M. Geysen, C. Christopherson, J. E. Hall, T. J. Mason, G. Saracco, F. Bonino, K. Crawford, C. D. Marion, K. A. Crawford, M. Brunetto, P. J. Barr, T. Miyamura, J. McHutchinson, and M. Houghton.** 1992. Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. Proc. Natl. Acad. Sci. USA **89:**3468–3472.
48. **Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner.** 1990. Direct gene transfer into mouse muscle in vivo. Science **247:**1465–1468.
49. **Xiang, Z. Q., S. Spitalnik, M. Tran, W. H. Wunner, J. Cheng, and C. J. Ertl.** 1994. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology **199:** 132–140.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