Genetic Immunization of Wild-Type and Hepatitis C Virus Transgenic Mice Reveals a Hierarchy of Cellular Immune Response and Tolerance Induction against Hepatitis C Virus Structural Proteins

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Received 14 June 2001/Accepted 17 September 2001

To study the effect of genetic immunization on transgenic expression of hepatitis C virus (HCV) proteins, we evaluated the immunological response of HCV transgenic mice to HCV expression plasmids. FVB/n transgenic mice expressing HCV structural proteins (core, E1, and E2) and wild-type (WT) FVB/n mice were immunized intramuscularly with plasmids expressing core (pHCVcore) or core/E1/E2 (pHCVSt). After immunization, HCV-specific humoral and cellular immune response was studied. Both WT and transgenic mice immunized with either HCV construct produced antibodies and exhibited T-cell proliferative responses against core or envelope. In WT mice immunized with pHCVSt, cytotoxic T-lymphocyte (CTL) activities were detected against E2 but not against core or E1, whereas strong CTL activities against core could be detected in WT mice immunized with pHCVcore. In pHCVSt-immunized, transgenic mice, CTL activities against core or envelope were completely absent, but core-specific CTL activities could be detected in pHCVcore-immunized transgenic mice. A similar pattern of immune responses was also observed in other mouse strains, including a transgenic line expressing human HLA-A2.1 molecules (AAD mice). Despite the presence of a peripheral cellular immunity against HCV, no liver pathology or lymphocytic infiltrate was observed in these transgenic mice. Our study suggests a hierarchy of CTL response against the HCV structural proteins (E2 > core > E1) in vivo when the proteins are expressed as a polyprotein. The HCV transgenic mice can be induced by DNA immunization to generate anti-HCV antibodies and anticore CTLs. However, they are tolerant at the CTL level against the E2 protein despite DNA immunization.

Transgenic models have been developed to study the mechanisms of tolerance and their implications for autoimmune or other immune-mediated diseases. In this regard, transgene-encoded neo-self antigen coupled with the corresponding T-cell receptor transgene has been particularly valuable (4, 20, 23, 35). In addition to central thymic selection, peripheral tolerance mechanisms, including peripheral deletion, anergy, and ignorance have been described (5, 10, 27, 28, 36). In the latter case, it is often possible to break tolerance and induce autoimmunity, leading to immune-mediated tissue injury. Expression of neo-self antigens in the liver presents a particularly interesting scenario because of the putative tolerogenic role of the liver in immune response and the unique anatomy of the liver in which the fenestrated vasculature allows direct access of hepatocytes to circulating T cells (25). This intriguing question has been addressed in several transgenic models in which central and peripheral deletion of reactive T cells appears to confer a robust tolerance to the neo-self antigen expressed in the transgenic liver. In situations whereby peripheral anergy or ignorance induction is operative, tolerance at the T-cell level can be broken by either viral infection or dendritic cell or DNA immunization (23, 31, 35, 37). However, induction of hepatitis still requires adoptive transfer of a large quantity of antigen-specific T cells in most cases (35).

We have previously reported the generation of several transgenic lines expressing hepatitis C virus (HCV) structural proteins (core, E1, and E2) (16). The liver-specific expression of HCV mRNA and proteins could be demonstrated by reverse transcriptase PCR and by Western immunoblotting and immunohistochemistry, respectively. However, the expression level is relatively low. The mice did not exhibit any long-term pathological effects from the expression of the HCV proteins. In this study, we analyze the effects of HCV DNA immunization on both wild-type and transgenic mice and demonstrate an interesting hierarchy of immune response and tolerance induction against the HCV structural proteins.

MATERIALS AND METHODS

Mice. Female FVB/n (H-2b) and BALB/c (H-2d) mice 6 to 8 weeks old were purchased from Charles River Laboratories (Wilmington, Mass.). AAD mice, namely, those expressing the transgene with the α1 and α2 domains from human HLA-A2.1 and the α3 domain of murine H-2Dd in the C57BL/6 background, have been described previously (32) and were obtained from Victor Engelhard of the University of Virginia. A transgenic mouse lineage (AC1-0) expressing HCV 1b structural proteins, core, E1, and E2, under a liver-specific albumin promoter was generated in the FVB/n background as described previously (16).

Plasmids for DNA immunization. For DNA immunization, two different plasmids, pHCVcore and pHCVst expressing the HCV core (amino acids [aa] 1 to 191) and core/E1/E2 polyprotein (aa 1 to 830) under the control of the cytomegalovirus (CMV) promoter (Fig. 1), were constructed by PCR of HCV cDNA from a type 1b strain that was also used for the production of the transgenic
The PCR product was cloned into a CMV expression plasmid, WRG7020, which is an efficient vector for DNA immunization in mice (30). These plasmids have been shown to direct the expression and proper processing of the HCV structural proteins in transfected HuH7 hepatoma cells. All plasmid DNAs were purified with an endotoxin-free plasmid extraction kit (Qiagen, Chatsworth, Calif.) and were dissolved in phosphate-buffered saline at a concentration of 2 μg/μl. For DNA immunization, mice were first injected with 0.25% bupivicaine into both quadriceps muscles and 24 h later were inoculated with 50 μl of the plasmid DNA into each of the quadriceps. Booster injections were given following the same protocol every 21 days for three times. All mice were bled by retro-orbital puncture prior to each immunization and 2 weeks after the last immunization. The sera were assayed for anticore and anti-E1/E2 antibodies.

Recombinant HCV proteins. Full-length core protein of HCV 1b was purified from Sf9 insect cells infected with the recombinant baculovirus bvHCV.S at a multiplicity of infection of 5. The infected Sf9 cells were lysed with buffer containing 50 mM Tris-HCl, 50 mM NaCl, 0.5% NP-40, 1 mM EDTA, and 1× protease inhibitor cocktail (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The lysate was centrifuged at 20,000 × g to pellet cellular debris, and the supernatant was applied to preparative sodium dodecyl sulfate (SDS)–14% polyacrylamide gel electrophoresis (PAGE) using Model 491 Prep Cell (Bio-Rad, Richmond, Calif.). Collected fractions were assayed by immunoblotting with C1 anticoic monoclonal antibody; positive fractions were pooled, concentrated with Centricon 3 (Millipore, Bedford, Mass.), and subjected to a repeated preparative SDS-PAGE for further purification. The resulting core protein is more than 95% pure on an SDS-PAGE gel. Recombinant truncated HCV core protein was purified using the QIAexpress System (Qiagen). Purification of HCV E1 and E2 proteins from BHK-21 cells infected with a recombinant vaccinia virus expressing the HCV structural proteins (wHCV.S) has been described elsewhere (3).

Establishment of syngeneic cell lines expressing HCV structural proteins. To establish stably transfected syngeneic cell lines expressing HCV proteins, core (aa 1 to 192), E1 (aa 137 to 383), E2 (aa 367 to 830), and core/E1/E2 (aa 1 to 830) expression plasmids (pEF-core, pEF-E1, pEF-E2, and pEF-St) were separately constructed by PCR. An AUG start codon and a stop codon were introduced by the PCR primers at each end. The PCR product was inserted into an EF-1 promoter-driven expression plasmid by replacing the enhanced green fluorescent protein gene of the pEF-EGFP plasmid; this plasmid was derived from the EGFP-N1 plasmid (Clontech, Palo Alto, Calif.) from which the CMV promoter was replaced with the EF-1 promoter from the pEF321-Neo plasmid (provided by Tatsuo Miyamura, Tokyo, Japan) (17). The P815 (H-2d) and SQSV cell lines (H-2q) (from Margaret Koziel, Beth Israel-Deaconess Hospital, Boston, Mass.) (18) were transfected with various HCV plasmids or the pEF-EGFP plasmid and were selected by Geneticin (Sigma, St. Louis, Mo.). Selected cells were screened by immunofluorescence and then confirmed by Western immunoblotting with antibodies against core, E1, or E2. Positive clones were expanded as stimulator and/or target cells for cytotoxic T-lymphocyte (CTL) assay.
Anti-HCV ELISA, T-cell proliferation, and cytokine assays. The enzyme-linked immunosorbent assay (ELISA) for detection of antico or anti-E1/E2 antibodies has been described earlier (3). Spleens were removed from immunized mice 2 weeks after the last boost. After preparation of single-suspension cells, spleen cells were cultured in triplicate for 5 days by using a U-bottomed 96-well plate at 2 × 10^5 cells/well in 200 μl of RPMI 1640 containing 10% fetal bovine serum, 4 mM t-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. The cells were stimulated with recombinant core or envelope protein at 1 μg/ml. Non-specific activation was obtained using 1 μg of phytohemagglutinin (Sigma)/ml as positive stimulation. On day 6, [3H]thymidine was added (1 μC/ well) and the cells were incubated for an additional 18 h. The [3H]thymidine incorporation into DNA was measured after harvesting the cells. Incorporation of radioactivity was corrected for background activity. To determine cytokine production, the effector cells stimulated with 1 μg of recombinant proteins/ml were cultured as described above at 5 × 10^5/well and the supernatants were harvested on day 3. Gamma interferon (IFN-γ) and interleukin-4 levels were measured by Quantikine M (R&D Systems, Minneapolis, Minn.).

CTL assay. Splenocytes derived from immunized mice (10^7/well) were stimulated in six-well culture plates for 7 days with 5 × 10^5 irradiated (10,000 rad) stably transfected cells expressing pEF-ST as stimulator cells in RPMI 1640 that contained 10% fetal bovine serum, 4 mM t-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Culture medium was supplemented with 10% T-Stim (Collaborative Biomedical Products, Bedford, Mass.) on days 2 and 5. After restimulation of these effector cells for another week with the same stimulator cells and irradiated (3,000 rad) spleen cells from the FVB/N mouse as feeder cells, the CTL activity was determined in a standard 51Cr release assay using U-bottomed, 96-well plates containing 3,000 51Cr-labeled target cells per well. For FVB/N and BALB/c mice, stably transfected syngeneic cells expressing various HCV structural proteins were used as target cells. Target cells (10^5) were pulsed with 100 μg of 51Cr for 1 h, washed three times, and added to the plates containing different number of effector cells in a final volume of 200 μl. Effector and target cells were cocultured in duplicate, and the supernatants were harvested for analysis after 5 h of incubation. The percentage of specific 51Cr release was calculated as 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). Spontaneous release was determined from target cells incubated without effector cells, and maximum release was determined in the presence of 1% SDS.

ELISPOT assay. The ELISPOT assay for IFN-γ has been described previously (21). Either recombinant proteins (core or E1/E2 protein) or peptides are incubated with splenocytes to determine the HCV-specific CD4 or CD8 responses, respectively. A peptide (aa 131 to 140; sequence ADLMGYIPLV) representing an A2-restricted epitope in the core region was used (32). Since the previously identified two E2 restricted epitopes were not conserved in our HCV strain, we screened a panel of overlapping peptides spanning the E2 region of our strain (K. Murata and T. J. Liang, unpublished results). One of the strongly reactive peptides (aa 614 to 622; RLWHYPCTI) was used for assaying E2-specific CTL activities in the AAD mice. The spots, as counted by KS Elispot-Axioplan 2I (Zeiss, Thornwood, N.Y.), are expressed as per 10^6 cells.

Histological evaluation and adoptive transfer. Tissues were fixed in formalin buffered with phosphate-buffered saline. Sections of paraffin-embedded tissue were cut at 5-μm thickness and stained with hematoxylin and eosin. Wild-type FVB/N mice were immunized with pHCVSt plasmid four times. Splenocytes were harvested after immunization and were stimulated with syngeneic cells expressing core/E1/E2 for 1 week and were assayed for CTL activities. Stimulated splenocytes (n = 10^7) were then injected into transgenic mice, and serum and liver were harvested 1 week later for alanine aminotransferase and histological analysis, respectively.

Statistical analysis. Comparisons of the mean antibody levels and proliferative and CTL activities between groups of mice were analyzed using the Student t test. All tests were two-tailed, and differences were considered significant when P was < 0.05. Percentages of positive responses between groups were compared using Fisher’s exact test.

RESULTS

Antibody response. Wild-type FVB/N mice and HCV transgenic mice were immunized four times with the plasmid vector, pHCVcore, or pHCVSt. Two weeks after the last DNA immunization, the wild-type FVB/N mice showed a weak humoral immune response with low levels of antico and/or antienvelop antibodies in only a fraction of the immunized animals (Fig. 2, top panel). Immunized transgenic mice demonstrated higher titers of antibody response, the reason for which is not clear at present. However, similar to the wild-type mice, only a fraction of animals had detectable humoral response (bottom panel).

T-cell proliferative response. To analyze the presence of cellular immune response, splenocytes were isolated from immunized mice 14 days after the last immunization and were assayed for proliferative T-cell response using either core or E1/E2 as stimulating antigens (Fig. 3). pHCVcore-immunized wild-type mice demonstrated little or no response against the core antigen, whereas several wild-type mice immunized with the pHCVSt construct exhibited positive activities against either the core (three of six) or envelope proteins (three of six). Two of five transgenic mice immunized with pHCVSt had a weakly detectable proliferative response against the core protein, but the overall difference from the wild-type response was not statistically significant. However, the pHCVSt-immunized transgenic mice were completely nonresponsive to either core or envelope proteins. Analyses of cytokine secretion of the proliferative assays showed IFN-γ production to be predominant (Fig. 3 legend) in positive samples, consistent with a TH1 response by intramuscular DNA immunization (9).

CTL response. Splenocytes from immunized mice were stimulated with syngeneic cells expressing core, E1, and E2 for 14 days and were assayed for CTL activities. Typical CTL assays of each group against core-, E1-, E2-, or core/E1/E2-expressing cell lines were shown in Fig. 4A. The pHCVcore-immunized
Our study showed that genetic immunization of FVB/n mice could elicit both humoral and cellular immune responses, al-
beit the magnitude of the response was not strong (results summarized in Table 1). Our results are consistent with other publications on HCV DNA immunization in various strains of mice (11, 12, 14, 22, 30). However, we observed a hierarchy of cellular immune response, particularly that of CTL, against the HCV structural proteins (E2/core/E1) when the proteins are expressed as a polyprotein in the immunizing plasmid. This hierarchy is probably not a result of difference in the expression level of the core protein between the constructs expressing either the core alone or core/E1/E2 polyprotein, because comparable core expression is observed between the two constructs in tissue culture transfection study (not shown). However we cannot completely eliminate a possible expression difference in vivo. Furthermore, E1 expressed either alone (not shown) or together with the other structural proteins appears to be a poor target for CTL induction.

Previous genetic immunization studies using HCV structural genes have not specifically analyzed the difference in cellular immune response among the individual HCV structural proteins. In one study, Saito et al. immunized BALB/c mice with six different constructs expressing different structural genes and studied HCV-specific antibody and cellular responses (30). They noted that only constructs containing the core (either alone or together with E1/E2) elicited CTL response against target cells expressing HCV structural proteins. Since the construct expressing E1 and E2 did not induce any CTL response, the authors concluded that the dominant response was likely against the core. However, CTL responses against the individual core and envelope proteins were not tested specifically in the core/E1/E2-immunized mice. In addition, the E1/E2 construct used in their study did not contain the C-terminal hydrophobic region of E2 (aa 706 to 809), which was included in our construct and might contain important CTL epitopes. Using a full-length E1/E2 construct for DNA immunization in FVB/n mice, we observed humoral and cellular immune responses against the envelope proteins similar to those for the pHCVSt construct (not shown), suggesting that the coexpression of core does not alter the immunogenicity of the envelope proteins. This hierarchy of cellular immune response against the HCV structural proteins is not mouse-strain-specific, be-

FIG. 4. CTL responses in wild-type and HCV transgenic mice immunized (Imm.) with HCV plasmids. Immunized mice were sacrificed 2 weeks after immunization, and splenocytes were harvested for CTL assay as described in Materials and Methods. (A) Representative results from each immunization are shown at various effector-to-target (E:T) ratios. (B) Results from all the immunized mice at an effector-to-target ratio of 30 are shown. The results are expressed as % specific lysis, and greater than 10% specific lysis is considered positive (dotted line). For core- and St-immunized wild-type mice: *, P = 0.001 for comparison of mean values and P = 0.005 for comparison of percentage of positive responses of the core CTL activities. §, P = 0.005 when the mean values of core CTL activities are compared for core- and St-immunized transgenic mice. For results of pHCVSt immunization of wild-type and transgenic mice: †, P < 0.0001 when the mean values are compared and P = 0.002 when the percentages of positive response of the E2 CTL activities are compared. C/E1/E2, core/E1/E2.

TABLE 1. Summary of immune responses after DNA immunization in wild-type and HCV transgenic mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Immunization</th>
<th>Antibody response</th>
<th>TH response</th>
<th>CTL response</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Core</td>
<td>E1/E2</td>
<td>Core</td>
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<tr>
<td>Wild type</td>
<td>HCVcore</td>
<td>+/-</td>
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<td>HCVSt</td>
<td>+/-</td>
<td>+/-</td>
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<tr>
<td>Transgenic</td>
<td>HCVcore</td>
<td>+/-</td>
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*+, >50% of mice are positive; +/-, <50% of mice are positive; -, none of the mice are positive.
cause both BALB/C and FVB/n strains as well as AAD (HLA-A2.1 in the background of C57BL/6) mice responded similarly. Furthermore, analysis of CD8+ response using individual CTL epitopes in the AAD background suggests that this difference is not a result of possibly more CD8+ epitopes in the larger E2 protein. Hierarchy or immunodominance of class I-restricted T-cell responses to virus has been described (39), and the phenomenon is probably multifactorial (6). The explanation for this hierarchy in HCV structural proteins is not apparent at present. It has been shown that immunodominant determinants can suppress subdominant determinants in vivo (15). It is also possible that the core protein, when expressed together with the envelope proteins, may have a different fate in the antigen presentation pathway. These possibilities await further experimentation.

Does this observation have any relevance to cell-mediated immunity during HCV infection in humans? While it is clear that CTL response can be detected against the core in infected persons with either acute or chronic infection (2, 19, 26, 29), very few studies focused on the induction of cellular response against the envelope proteins, probably because of the highly variable sequences in this region. In one study, a strong CD8+ CTL response against the hypervariable region can be detected early during acute HCV infection and sequence variations in this region can lead to antigenism (33). Similar antigenism has also been reported for CD4+ T-helper epitopes (13). These authors postulated that this might be a mechanism for viral escape and persistence. Recent publications have shown that active cellular immune responses against the E2 are frequently present in persons infected with HCV (8, 38). A similar observation can be inferred from a study of cell-mediated immunity during acute infection of chimpanzees, in which several CTL epitopes were identified in the E2 but not in the core region and the strength of CTL responses against these epitopes was associated with viral clearance (7). The relevance to natural infection notwithstanding, our observation on the hierarchy of immune response could have implications in designing constructs for DNA vaccination trials in primates or humans. For example, if induction of core-specific, cell-mediated immunity is important, a core-only construct should be designed. This may also be relevant to other HCV polyprotein constructs, which may induce a set of immune responses qualitatively different from those induced by constructs expressing individual proteins.

The transgenic mouse model has been used to study immunological tolerance and immune response against viral antigens. In one hepatitis B virus transgenic model, DNA immunization with the hepatitis B virus expression construct broke immunological tolerance and induced hepatitis (24). In another model, immunological tolerance at the CTL level could only be broken by dendritic cell immunization, but no hepatitis was observed (31, 37). Hepatitis could be induced only by adoptive transfer of HBV-specific CD8+ CTL clones into these mice (1). In our transgenic mice expressing HCV structural proteins, antibodies against either the core or envelope proteins could be induced by DNA immunization. The transgenic mice did not appear to be tolerant to the core protein at the T-cell level. On the other hand, either CD4 or CD8 cellular immunity against the envelope proteins appeared to be tolerant and could not be broken by DNA immunization in the transgenic mice. It is particularly intriguing that the core protein in the context of polyprotein expression is both a poor immunogen as well as a weak toleragen. This observation is consistent with the present concepts of antigen presentation and tolerance induction (34).

Despite the induction of core-specific antibodies and cellular immune response, the transgenic mice exhibited no inflammatory cell infiltrate or pathology in the liver. Adoptive transfer of splenocytes with strong CTL activities also did not cause any hepatocellular injury. It is possible that a low-level expression of HCV antigens in our transgenic mice was insufficient to induce homing of these HCV-specific T cells into the liver. A similar observation was reported in a recent report in which liver-specific expression of a CTL epitope-containing viral antigen (lymphocytic choriomeningitis virus) was not sufficient to result in hepatitis despite the presence of peripheral T cells that were specific for the epitope (35). Only by lymphocytic choriomeningitis virus superinfection and adoptive transfer of a large number of virus-specific T lymphocytes did hepatocellular injury occur. Therefore, a much more vigorous immune response is necessary for targeting to the liver. This "immune avoidance" behavior of viral antigens expressed in the liver may partially explain the persistence of HCV infection. To completely eliminate HCV infection, it may be necessary to activate and target endogenous HCV-specific immune response specifically to the liver. Such a concept may underpin the success of future immunotherapy for hepatitis C.

ACKNOWLEDGMENTS

J.S. and K.M. have contributed equally to this work.

We thank Margaret Koziel, Stephen Feinstone, and Marion Major for providing reagents and helpful advice. We are also grateful to Jay Berzofsky and Victor Engelhard for providing the AAD mice.

J.S. was partially supported by a fellowship from Jikei University,