Live and Killed Rhabdovirus-Based Vectors as Potential Hepatitis C Vaccines

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A highly attenuated, recombinant rabies virus (RV) vaccine strain-based vector was utilized as a new immunization strategy to induce humoral and cellular responses against hepatitis C (HCV) glycoprotein E2. We showed previously that RV-based vectors are able to induce strong immune responses against human immunodeficiency virus type I (HIV-1) antigens. Here we constructed and characterized three replication-competent RV-based vectors expressing either both HCV envelope proteins E1 and E2 or a modified version of E2 which lacks 85 amino acids of its carboxy terminus and contains the human CD4 transmembrane domain and the CD4 or RV glycoprotein cytoplasmic domain. All three constructs stably expressed the respective protein(s) as indicated by Western blotting and immunostaining. Moreover, surface expression of HCV E2 resulted in efficient incorporation of the HCV envelope protein regardless of the presence of the RV G cytoplasmic domain, which was described previously as a requirement for incorporation of foreign glycoproteins into RV particles. Killed and purified RV virions containing HCV E2 were highly immunogenic in mice and also proved useful as a diagnostic tool, as indicated by a specific reaction with sera from HCV-infected patients. In addition, RV vaccine vehicles were able to induce cellular responses against HCV E2. These results further suggest that recombinant RVs are potentially useful vaccine vectors against important human viral diseases.

Key Words: hepatitis C; vaccine; rabies virus; rhabdovirus; CTL; seroconversion; humoral response.

INTRODUCTION

Hepatitis C virus (HCV) is the primary etiological agent of non-A, non-B hepatitis affecting approximately 170 million people worldwide (Alter, 1997). The majority of patients (70%) develop chronic hepatitis and a third of these cases progress to liver cirrhosis. All infected individuals have an increased risk of hepatocellular carcinoma (Aihara and Miyazaki, 1998). Current treatments include alpha interferon (INF-α) therapy alone or in conjunction with ribavirin, but these treatments are expensive, show a relatively low rate of response, and carry the potential for significant side effects (Fried and Hoofnagle, 1995). Therefore, the development of a protective HCV vaccine is a high priority. The task of developing an HCV vaccine is a difficult one for several reasons. First of all, the broad genetic heterogeneity of the virus has resulted in six known genotypes (Simmonds et al., 1994), and the virus can exist in any given individual as a population of closely related variants known as quasispecies. Additionally, viral persistence may be correlated with the emergence of variants which have the ability to evade the immune system (Major et al., 1999; Weiner et al., 1992b). Lastly, protection from reinfection may not occur even if an acute HCV infection has been cleared by the immune system (Farci et al., 1992; Prince et al., 1992).

HCV is a small, enveloped positive strand RNA virus of the Flaviviridae family (Clarke, 1997). The 9.6-kb genome consists of a 5' nontranslated region (NTR) which contains an internal ribosome entry site (IRES) to begin translation of the viral polyprotein (Le, Siddiqui, and Maizel, 1996), which is cleaved by both host and viral proteases to yield four structural and six nonstructural (NS) proteins (Reed and Rice, 1998). The genome encodes two envelope glycoproteins, E1 and E2, which are released from the polyprotein via signal peptidase cleavages (Grakoui et al., 1993). Both proteins are largely modified by N-linked glycosylation and are thought to be type I integral transmembrane proteins with C-terminal hydrophobic anchor domains. Expression of both the glycoproteins in mammalian cell lines illustrates their retention in the endoplasmic reticulum (ER) with no surface expression detectable (Duvet et al., 1998). The E2 glycoprotein contains two hypervariable regions (HVR), with HVR1 located at amino acid positions 390–410, and HVR2 located at positions 474–480 (Weiner et al., 1991). Antibodies directed against the HVR1 of E2 have been implicated in controlling HCV infection (Kato et al., 1993). In addition, the HVR1 of E2 contains both B cell and
cytotoxic T lymphocyte (CTL) epitopes. Furthermore, antibodies specifically directed at this region reportedly blocked viral attachment in susceptible cells further implicating E2 as responsible for viral attachment to the host cell (Kojima et al., 1994; Leroux-Roels et al., 1996; Lesniowski et al., 1995). However, there are conflicting data regarding the clinical importance of sequence variation in the HVR1 of E2. One study indicates that the evolutionary dynamics of HCV quasispecies predict whether an infection will resolve itself or become chronic (Farcì et al., 2000), whereas another study concludes that variations within the HVR1 of E2 does not correlate with disease progression (Forns et al., 2000b). Overall, the characteristics of the E2 glycoprotein render it an attractive target for a protective vaccine.

To date, HCV vaccine studies involving E2 have utilized several strategies in a murine model including purified recombinant antigens, DNA immunization (Gordon et al., 2000), DNA priming in conjunction with recombinant viruses such as Semliki Forest virus and canarypox (Pancholi et al., 2000; Vidalin et al., 2000), DNA priming with recombinant protein boosting (Song et al., 2000), replication-deficient recombinant adenovirus (Makimura et al., 1996), and plasmid DNA immunization (Inchauspe, 1999). Each of these strategies has experienced limited success in producing both a humoral and cellular immune response. Overall, E2 generates a potent antibody response, but a weak CTL response in mice in contrast to the core protein (Saito et al., 2000), whereas another study concludes that CD81, a potential receptor for HCV (Pileri et al., 1998). These findings suggest E1 and E2 of HCV may be important targets for both cellular and humoral responses. We therefore decided to include these proteins in an initial approach to analyze a Rhabdovirus-based vector as a potential HCV vaccine. To generate RV recombinant viruses we used a RV vaccine strain-based vector with a new RV transcription unit, containing a RV stop/start signal and two single sites (Fig. 1). The gene encoding the HCV E1, E2, and p7 proteins to be expressed from SPBN were generated by PCR and cloned between the BsiWI and NheI sites resulting in the plasmid pSPBN-E1E2p7. To analyze if the expression of the HCV E2 on the surface of the infected cell enhances its immunogenicity, two similar RV recombinant viruses containing the gene encoding the ectodomain of HCV E2, lacking its carboxy-terminal 85 amino acids, fused to the transmembrane domain (TMD) and the CD of RV were also PCR amplified. The resulting PCR products were cloned utilizing the BsiWI/Nhel sites to pSPBN resulting in pSPBN-E2CD4 and pSPBN-E2CD4G (Fig. 1). The pSPBN-E2CD4G was constructed on the basis of a previous finding that the RV CD is required for incorporation of a foreign glycoprotein into RV virions (Mebatsion et al., 1996; Mebatsion et al., 1997). An RV viral particle containing HCV E2 would be helpful to examine if recombinant killed RV viruses are able to induce E2-specific seroconversion and therefore are useful as potential HCV vaccines.

As shown previously, RV vectors are able to stably express large foreign genes (McGettigan et al., 2001a; Mebatsion et al., 1996; Schnell et al., 2000), and thus, we did not anticipate problems to recover the recombinant RVs. As expected, infectious RVs were detected in tissue culture supernatants of cells transfected by standard RV recovery protocols for pSPBN, pSPBN-E1E2p7, pSPBN-E2CD4, and pSPBN-E2CD4G (Finke and Conzelmann, 1999). In contrast to the previously constructed recombinant RVs expressing HIV-1 gp160 (Schnell et al., 2000), the recombinant RVs expressing HCV proteins grew at least to the same titers as SPBN, which were at least 10^8 foci-forming units (ffu).
Expression of HCV glycoproteins by recombinant RVs

The HCV envelope proteins E1 and E2 interact to form a noncovalent heterodimeric complex, which is retained in the endoplasmic reticulum (ER), whereas a chimeric HCV E2 protein containing the transmembrane domain (TMD) and cytoplasmic domain (CD) of CD4 is transported to the cell surface (Dubuisson, 2000). To ensure that the replacement of the CD of E2CD4 with that of RVG did not interfere with the surface expression of the recombinant protein, BSR cells were infected with SPBN-E1E2p7 (Fig. 2, Panels A, A9, A0), SPBN-E2CD4G (Fig. 2, Panels B, B9, B0), or SPBN (Fig. 2, Panels C, C9, C0) at an m.o.i. of 0.1 and fixed 48 h later with paraformaldehyde. Infected cells were analyzed directly by immunofluorescence microscopy with a monoclonal antibody directed against HCV E2 (Fig. 2 panels A9, B9, C9) or permeabilized with Triton X-100 for internal staining with an antibody against HCV E2 (Fig. 2 panels A9, B9, C9) or RV N protein (Fig. 2 panels A9, B9, C9). These results indicate that the chimeric E2 protein containing the CD TMD and the RV G CD is transported to the surface of the infected cell. In addition, the finding that the conformation-sensitive monoclonal antibody used for the immunostaining recognized the recombinant HCV E2, suggests that the protein is properly folded.

To analyze the expression and processing of HCV E1 and E2 expressed by the recombinant viruses, lysates from cells infected with recombinant RVs were separated by SDS-PAGE under reducing conditions and analyzed by Western immunoblotting with antibodies directed against HCV E2 or E1. As illustrated in Fig. 3, lane 2, two bands of the expected size for the uncleaved precursor of the E1E2 polyprotein and the cleaved E2 can be detected in cell lysates infected with SPBN-E1E2p7 with an HCV E2 specific monoclonal antibody H-52. A band similar in size to the E2 expressed by SPBN-E1E2p7 in lysates from SPBN-E2CD4 and SPBN-E2CD4G infected cells. In addition, a more diffuse, slow-migrating band, which was not observed for wild-type E2, was detected for both chimeric E2 proteins containing a TMD and CD. Previous experiments by Dubuisson and co-workers suggested that the slower-migrating band of E2 corresponds to molecules having their glycans processed by Golgi enzymes and which are no longer retained in the endoplasmic reticulum (ER) (Cocquerel et al., 2000). It is interesting to note that only the slower migrating form of E2 is incorporated into RV virions (Fig. 4, lanes 5 and 6) supporting the theory that these molecules reach the cell surface, whereas the faster migrating band is retained in the ER. Immunoblotting with the monoclonal antibody A4 directed against HCV E1 (Dubuisson et al., 1994) detected a band of about 27 kD as expected for HCV E1 only in cell lysates infected with the recombinant RV SPBN-E1E2p7 (Fig. 3, lanes 6 and 10), confirming the cleavage of the E1E2p7 precursor protein even in a nonhuman cell line. Infection with all four recombinant RVs was confirmed with a human polyclonal serum directed against RV G protein (data not shown).

Recombinant HCV E2 is incorporated into RV virions

One goal of this study was to determine if it was possible to incorporate chimeric E2 into RV virions. A recombinant virion containing HCV E2 may be useful for both producing E2 antigen for diagnostic use and as a killed vaccine against HCV. To analyze incorporation of the chimeric E2 proteins into RV particles, BSR cells were infected with SPBN, SPBN-E2CD4, and SPBN-E2CD4G with a m.o.i. of 0.1. Three days after infection, virus was purified from the supernatants of infected cells by a 20 to 70% density sucrose gradient. Viral proteins

FIG. 1. Construction of recombinant RV genomes. At the top (A), the SPBN vector derived from the RV vaccine strain SAD B19 is illustrated. Through site-directed mutagenesis and a PCR strategy, a transcription stop/start signal was introduced in addition to four unique restriction enzyme sites (SmaI, PacI, BsiWI, and Nhel). The HCV proteins (blue box) were introduced into pSPBN using the BsiWI and Nhel sites resulting in the plasmids pSPBN-E1E2p7 (B), pSPBN-E2CD4 (C), and pSPBN-E2CD4G (D). E2CD4 and E2CD4G are a truncated version of HCV E2 lacking 85 amino acids at their C terminus, fused to the TMD (green box), and CD of human CD4 (light blue box) or TMD of CD4 and CD of RV G (red box), respectively.
were separated by SDS-PAGE and detected by Coomassie blue staining. The results in Fig. 4 (lanes 1–3) showed equal amounts and the same pattern of the RV proteins for all three recombinant viruses, but no additional protein of the expected size for the HCV E2 could be detected in the viral particles. The lack of detection of E2 may be due to E2 incorporation at low levels or it is running as a more diffuse band than the other RV proteins due to multiple O- and N-linked glycans. We therefore analyzed the recombinant virions by Western blotting with an antibody directed against E2 and readily detected the recombinant E2 in both SPBN-E2CD4 and SPBN-E2CD4G particles (Fig. 4, lanes 5 and 6), whereas no signal was detected for SPBN (Fig. 4, lane 4) or SPBN-E1E2p7 (data not shown). It was surprising that both E2CD4 and E2CD4G were incorporated into RV particles because an earlier finding by Mebatsion et al. (1996) indicated that the RV G CD is a requirement for incorporation of a foreign glycoprotein into RV virions (Mebatsion et al., 1996). This is not the case for HCV E2 and quantification indicated that the content of the recombinant E2CD4 was at least 60% of E2CD4G.

The presence of the RV G CD in the HCV envelope protein expressed by SPBN-E2CD4G was also verified by Western blotting with an antibody specific for the RV G CD. Pilot studies with this antibody showed that recombinant E2CD4G is comigrating with RV G, which made it impossible to distinguish between the two proteins. Because RV G contains only three to four N-linked glycosylation sites but HCV E2 is heavily O- and N-glycosylated, we decided to remove the N-glycan chains by digestion of the RV virions with \( \text{N} \)-glycosidase F. As illustrated in Fig. 4, lanes 7–9, the anti-RV G antibody detected a band of similar size and intensity in the expected size of deglycosylated RV G, whereas two prominent additional bands are only detected in virions containing E2 envelope protein with the RV G.

Reactivity of recombinant RV virions with human sera

We next analyzed whether the chimeric HCV E2 in the recombinant particles is displayed in such a way that it can be recognized by sera of HCV-positive patients. Recombinant HCV E2 is mostly produced by transfection...
cells with plasmids encoding naive E2 or a truncated form of HCV E2 which is secreted in the tissue culture supernatant. In both cases, only small amounts of protein are produced. On the other hand, recombinant RVs are easy to grow and purify and 1 mg RV G protein can be extracted from 1 liter of tissue supernatants of RV infected cells. In addition, RV virions can easily be deactivated before purification and therefore handling infectious material is limited to the growth of the viruses. To analyze the antigenicity of the recombinant RV particles containing HCV E2, ELISA plates were coated with monoclonal antibodies directed against the HCV E1 and E2 glycoproteins as indicated (α-E1, α-E1 + E2, or α-E1).

Recombinant RVs expressing HCV glycoproteins are immunogenic in mice

We next wanted to determine whether recombinant RVs expressing HCV glycoprotein(s) are immunogenic in mice. The immune responses which may protect humans from HCV infection are not well defined, but it is likely that both cellular and humoral responses will be required for protection of infection or clearance of HCV. To analyze the immunogenicity of our RV vector expressing HCV proteins, we infected a group of 10 female BALB/c mice with 1 × 10^7 ffu of SPBN-E2RVG, and a group of five mice with an equal amount of the RV vector SPBN, and left five mice uninfected. Fourteen days postimmunization, all mice were bled and sera analyzed by ELISA using recombinant HCV E2, but no E2-specific antibodies were detected. Our previous experiments with recombinant RVs expressing HIV-1 gp160 indicated that the induction of a humoral response against HIV-1 gp160 required a boost with recombinant HIV-1 gp120 (Schnell et al., 2000). We therefore decided to apply a boost vaccination using killed RV particles derived from SPBN-E2RVG infected cells as a source of recombinant HCV E2. A group of five mice primed with live SPBN-E2CD4G and boosted with killed SPBN particles served as a control. Ten days later, mice were bled and sera analyzed by an HCV-specific ELISA. We expected to detect seroconversion against E2 in the sera of mice primed with live SPBN-E2CD4G and boosted with killed SPBN-E2CD4G, but in only two out of five mice were E2-specific antibodies detected. Of note, no adjuvant was used for the immunization with the killed virions, which may explain why only a portion of the mice developed antibodies directed against HCV E2. To analyze if a second inoculation with the same killed RV virions would induce a higher rate of seroconversion against HCV E2, mice of each group received a second immunization with the same killed virions as used for the first one. Ten days postimmunization, all mice were bled and sera analyzed by ELISA using recombinant HCV E2. We expected to detect seroconversion against E2 in the sera of mice primed with live SPBN-E2CD4G and boosted with killed SPBN-E2CD4G, but in only two out of five mice were E2-specific antibodies detected. Of note, no adjuvant was used for the immunization with the killed virions, which may explain why only a portion of the mice developed antibodies directed against HCV E2. To analyze if a second inoculation with the same killed RV virions would induce a higher rate of seroconversion against HCV E2, mice of each group received a second immunization with the same killed virions as used for the first one. Ten days postimmunization, all mice were bled and sera analyzed by ELISA using recombinant HCV E2. We expected to detect seroconversion against E2 in the sera of mice primed with live SPBN-E2CD4G and boosted with killed SPBN-E2CD4G, but in only two out of five mice were E2-specific antibodies detected. Of note, no adjuvant was used for the immunization with the killed virions, which may explain why only a portion of the mice developed antibodies directed against HCV E2. To analyze if a second inoculation with the same killed RV virions would induce a higher rate of seroconversion against HCV E2, mice of each group received a second immunization with the same killed virions as used for the first one. Ten days postimmunization, all mice were bled and sera analyzed by ELISA using recombinant HCV E2.

FIG. 3. Western blot analysis of HCV proteins expressed by RV. BSR cells were infected with recombinant RVs as indicated (SPBN, SPBN-E1E2p7, SPBN-E2CD4, SPBN-E2CD4G at a m.o.i. of 5. Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with monoclonal antibodies directed against the HCV E1 and E2 glycoproteins as indicated (α-E1, α-E1 + E2, or α-E1).

FIG. 4. Incorporation of HCV proteins in recombinant RVs. Purified particles of SPBN, SPBN-E2CD4, or SPBN-E2CD4G were separated by SDS-PAGE and visualized by Coomassie blue staining (CB, lanes 1, 2, 3) or transferred to a nitrocellulose membrane before (α-E2) or after digestion with N-glycosidase F (α-RV-G-tail). Blots were probed with monoclonal antibodies directed against the HCV E2 (α-E2, lanes 4, 5, and 6) or a polyclonal rabbit serum specific for the RV G CD (α-RV-G-tail, lanes 7, 8, 9).
later, the mice were bled and E2-specific ELISAs performed. The results in Fig. 6 show that all mice boosted with the killed virions containing the HCV E2 seroconverted, whereas sera from SPBN-E2CD4G primed mice that were boosted twice with killed SPBN virions were negative. These results indicate that two inoculations with inactivated RV virions containing chimeric HCV E2 are able to induce a potent humoral response directed against HCV E2. Of note, priming with the recombinant RV SPBN-E2CD4G did not result in a stronger B cell response against HCV E2 as seen in unprimed or SPBN primed mice.

Last, we sought to determine if immunized mice produced a specific CTL response to HCV E2. In contrast to HIV-1 gp160, limited information is available for specific CTL epitopes of HCV glycoproteins in mice and the responses are mostly weak, even after multiple immunizations. To analyze if a single inoculation with our RV-based vaccine vehicle expressing the HCV glycoproteins E1 and E2 is able to induce a cellular response against HCV E2, 10 female BALB/c mice were vaccinated with \(10^7\) ffu of SPBN-E1E2p7 and spleens were harvested 11 weeks later. Splenocytes were cultured and stimulated for 7 days with an E2-specific peptide (1323), and T-Stim was added as a source of IL-2. On the day of the assay, target cells were pulsed both with and without an E2 peptide (1323) and labeled with Cr\(^{51}\). Effector and targets were incubated together at several ratios for 4 h. Figure 7 illustrates an example of one of two independent experiments. Specific lysis was detected in a broad range of effector:target ratios of 100:1 to 12.5:1. The observed level of cytolysis was lower than shown previously for HIV-1 gp160 or Gag, which is probably due to the used 20mer peptide or a lower frequency of CTL memory cells. Of note, the results indicate that a single inoculation with recombinant RV expressing HCV-E2 in-
FIG. 7. Immunization of mice with SPBN-E1E2p7 induces HCV E2-specific CTLs. Six- to eight-week-old female BALB/c mice were immunized ip with $1 \times 10^7$ ffu of SPBN-E1E2p7. Spleens were harvested 11 weeks after immunization, and cultured and stimulated with the E2 peptide 1323 and IL-2. A standard chromium release assay was performed 1 week after harvesting, against P815 cells pulsed (+ peptide) or not (− peptide) with peptide 1323.

 induces a long-lasting, antigen-specific cellular immune response.

DISSCUSSION

Since the molecular cloning of HCV (Choo et al., 1989), characterization of its genome has rapidly progressed but the development of a vaccine against HCV still represents an impressive challenge for several reasons. Currently, no method exists to propagate HCV in vitro (Flolev et al., 1999; Lohmann et al., 2001), which eliminates the possibility of utilizing attenuated or killed HCV as a vaccine strategy. So far, HCV vaccine studies are based on recombinant proteins (Choo et al., 1994), naked-DNA-expressing HCV protein(s) (Gordon et al., 2000), different viral vectors, and prime/boost combinations of DNA and recombinant viruses (Pancholi et al., 2000; Vidalin et al., 2000). Here, we describe a new approach for a potential HCV vaccine using both killed RV particles containing recombinant HCV E2 and live, replication-competent, RV vaccine strain-based vectors.

For this vaccine approach, three RV were constructed expressing either the HCV envelope proteins E1 and E2 or a modified version of E2 which lacks 56 amino acids of its carboxy terminus and contains the human CD4 TMD and the CD4 or RV glycoprotein CD. Our previous results with RV-based vectors expressing HIV-1 envelope and Gag protein indicated great promise for recombinant RVs because these vectors are able to induce both strong humoral and cellular responses against the expressed HIV-1 antigens (McGettigan et al., 2001a, 2001b; Schnell et al., 2000). Because the protective immune response against HCV is not well defined, we decided in our initial HCV vaccine approach to focus on both wings of the immune response. Increasing evidence indicates that cellular immune responses play an important role in self-limited HCV infections and recovery from HCV infection. In general, both CD4+ helper T cells and CD8+ cytotoxic T cells seem to be more frequent and stronger in patients who recover than in patients who develop a chronic infection (Liang et al., 2000). Moreover, one study indicated that the number of IFN-γ producing cells during the first 6 months after the onset of disease is associated with eradication of the HCV infection (Gruener et al., 2000). Our data indicate that a RV vaccine vector is able to induce long-lasting CTL responses against HCV E2, but the specific killing was not as strong as previously seen for HIV-1 Gag or envelope. Our data are consistent with those of other groups who used other HCV vaccine approaches in BALB/c mice and detected only a low percentage of specific CTLs against HCV E2 (Vidalin et al., 2000). More recently, Gordon et al. (2000) characterized a new MHC class I E2-specific epitope for the H-2d haplotype (Gordon et al., 2000), which may be helpful for further studies of cellular responses against HCV E2 in BALB/c mice. Of note, our results clearly indicate that RV-based vectors are potent to induce E2-specific CTLs. We are currently analyzing our vaccine approach in an HLA-A2.1 transgenic mouse model that should allow the use of previously characterized peptides for human HLA-A2.1-restricted CTLs (Battegay et al., 1995).

In contrast to the cellular response(s), the requirements for an HCV-specific humoral response for a HCV vaccine are more conflicting. Infection of host cells with enveloped viruses is typically mediated by an interaction between the viral glycoprotein(s) in the host-cell derived membrane and a cellular receptor(s) on the host cell. Previous studies indicate that the hypervariable region 1 (HVR1) of E2 binds to the cellular CD81 molecule of the host cell (Flint et al., 1999). Hence, it is probable that host-produced antibodies against E2 would neutralize the attachment and/or fusion of HCV virions to host cells during a natural infection. This finding is in conflict with other results which indicate that the absence of a persistent anti-E2 response correlates with termination of the infection in chimpanzees and humans (Prince et al., 1999). On the other hand, passive immunization in chimpanzees prolonged the incubation period of acute HCV hepatitis and preincubation of HCV with the serum of a chronically infected patient prevented infection (Farci et al., 1994; Krawczynski et al., 1996).

Here, we present a new vaccine strategy to immunize against HCV. We demonstrated that killed RV particles containing HCV E2 proteins are able to induce vigorous B cell responses. The reason for these strong responses could be that a viral glycoprotein displayed on a viral particle is more immunogenic than its soluble form. It has been shown for RV, that soluble G, in contrast to the virion-associated G, fails to protect from lethal RV challenge (Dietzschold et al., 1983). Of note, it is not certain that the observed humoral responses induce by RV viri-
ons would be protective against HCV infection. There is evidence that the humoral response(s) against HCV can create variants, which escape the recognition by antibodies (Farci et al., 1996; Weiner et al., 1992a). A study by Shimizu et al. (1994) determined the in vitro virus-neutralizing effect of serum of a patient with chronic HCV during a follow-up of 14 years in comparison to the infecting virus population. Their findings suggest that virus-specific antibodies can decrease viral infectivity and that immune pressure may lead to the evolution of escape mutants during the course of chronic infection. Nonetheless, these antibodies were mostly directed against the HVR1 within the E2 protein. The recent finding that a recombinant HCV containing an HVR1-deleted E2 protein is still able to cause chronic infection in chimpanzees (Forns et al., 2000a) indicates that antibodies directed against conserved epitopes outside of the E2 HVR1 may be more useful to neutralize virus. Further studies will indicate if killed RV particles containing a HVR1-deleted E2 protein are still able to induce the same immune responses as demonstrated for SPBN-E2CD4G particles.

In summary, we now demonstrate that a recombinant RV expressing HCV envelope proteins can be generated. The HCV proteins were stably expressed and induced a long-lasting cellular response in mice. Moreover, two vaccinations with killed RV virions containing HCV E2 in the viral envelope induced a strong E2-specific B cell response. These results further emphasize the potential use of RV-based vectors as both live and killed vaccines for other viral diseases, and ongoing investigations will indicate if this approach will be useful as preventive or therapeutic vaccines against HCV and HIV-1.

MATERIALS AND METHODS

Plasmid construction

All polymerase chain reactions (PCR) were performed using high-fidelity Vent DNA polymerase (New England Biolabs, Beverly, MA) to minimize the introduction of sequence errors. pSBN was described previously (Schnell et al., 2000) and was the target to introduce a new single restriction site (PacI, bold) downstream of the RV G gene by site-directed mutagenesis (GeneEditor) using the primer 5'-CGTACGAAAATGAAATTCCGAC-3' and the reverse primer 5'-TCAAAGCTAGC-3' containing a BsiWI site (bold), and the ligation was PCR reamplified with the primers RP56 and RP8. The PCR product was cloned utilizing the unique BsiWI and Nhel sites resulting in pSPBN-E2CD4G.

To construct an RV encoding the HCV E2 ED lacking 85 amino acids at its carboxy terminus and containing the CD4 TMD and the RV G CD, the RV G CD was PCR amplified from pSBN (Schnell et al., 2000) using the forward primer RP29 5'-CCC GGGTTAACAGAAGAGTCAATG CATCAGAAG-3' (Hpal, bold) and the reverse primer RP8 5'-CCCTCTAGATTACAGCTGTTGCTCACC-3' (XbaI, bold). The ED of HCV E2, lacking its carboxy-terminal 85 amino acids, fused to the TMD of CD4 was amplified by PCR from pTM1/E2_31RHABDOVIRUS-BASED HCV VACCINE

Recovery of infectious RV from cDNA

For recovery experiments of the recombinant RVs, the previously described RV recovery system was used (Finke and Conzelmann, 1999). Briefly, BSR–T7 cells (Buchholz et al., 1999) which stably express T7 RNA polymerase were transfected with 5 μg of full-length RV cDNA in addition to plasmids encoding for the RV N, P, L, and G proteins using a CaPO4 transfection kit (Stratagene, La Jolla, CA) as instructed by the manufacturer. Three days posttransfection, supernatants were transferred onto fresh BSR cells and infectious RV was detected an additional 3 days later, by immunostaining with FITC against the RV-N protein (Centacor Inc.).

Immunofluorescence microscopy

BSR cells were plated in 6-well plates containing coverslips and infected with a m.o.i. of 0.1 for 48 h. Cells were fixed with 4% paraformaldehyde at room temperature for 20 min. For internal immunostaining, cells were permeabilized with 1% Triton in (phosphor-buffered saline) PBS for 5 min at room temperature. Cells were washed three times with PBS-glycine [10 mM glycine in PBS (pH 7.4)] and incubated with a monoclonal mouse antibody directed against HCV E2 (H53, 1:600) for 1 h at

pSPBN-E1E2p7. A recombinant RV expressing the ectodomain (ED) of HCV E2, lacking 85 amino acids at its C terminus, and fused to the transmembrane domain (TMD) and cytoplasmic domain (CD) of human CD4, was amplified by PCR from pTM1/E2_31RHABDOVIRUS-BASED HCV VACCINE

AcGAGCTCTTTAACAATGGATCAGGATCC-3' containing a BsiWI site (bold), and the reverse primer RP69 5'-CGTACGAAAATGAAATTCCGAC-3' containing a Nhel site (bold) site. The PCR product was digested with BsiWI and Nhel and cloned into pSPBN, which was digested previously with BsiWI and Nhel. The resulting plasmid was entitled pSPBN-E1E2p7.

In summary, we now demonstrate that a recombinant RV expressing HCV envelope proteins can be generated. The HCV proteins were stably expressed and induced a long-lasting cellular response in mice. Moreover, two vaccinations with killed RV virions containing HCV E2 in the viral envelope induced a strong E2-specific B cell response. These results further emphasize the potential use of RV-based vectors as both live and killed vaccines for other viral diseases, and ongoing investigations will indicate if this approach will be useful as preventive or therapeutic vaccines against HCV and HIV-1.

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All polymerase chain reactions (PCR) were performed using high-fidelity Vent DNA polymerase (New England Biolabs, Beverly, MA) to minimize the introduction of sequence errors. pSBN was described previously (Schnell et al., 2000) and was the target to introduce a new single restriction site (PacI, bold) downstream of the RV G gene by site-directed mutagenesis (GeneEditor) using the primer 5'-GGTCCTGGTAGTGCTG-3' and the reverse primer RP75 5'-CTCCTGTTAGTGTGC-3' containing a Nhel site (bold). The PCR product was cloned into pSPBN using the unique BsiWI and Nhel sites resulting in pSPBN-E2CD4G.

To construct an RV encoding the HCV E2 ED lacking 85 amino acids at its carboxy terminus and containing the CD4 TMD and the RV G CD, the RV G CD was PCR amplified from pSBN (Schnell et al., 2000) using the forward primer RP29 5'-CCC GGGTTAACAGAAGAGTCAATG CATCAGAAG-3' (Hpal, bold) and the reverse primer RP8 5'-CCCTCTAGATTACAGCTGTTGCTCACC-3' (XbaI, bold). The ED of HCV E2, lacking its carboxy-terminal 85 amino acids, fused to the TMD of CD4 was amplified by PCR from pTM1/E2_31RHABDOVIRUS-BASED HCV VACCINE

AcGAGCTCTTTAACAATGGATCAGGATCC-3' containing a BsiWI site (bold), and the reverse primer RP69 5'-CGTACGAAAATGAAATTCCGAC-3' containing a Nhel site (bold) site. The PCR product was digested with BsiWI and Nhel and cloned into pSPBN, which was digested previously with BsiWI

AcGAGCTCTTTAACAATGGATCAGGATCC-3' containing a BsiWI site (bold), and the reverse primer RP69 5'-CGTACGAAAATGAAATTCCGAC-3' containing a Nhel site (bold) site. The PCR product was digested with BsiWI and Nhel and cloned into pSPBN, which was digested previously with BsiWI and Nhel. The resulting plasmid was entitled pSPBN-E1E2p7. A recombinant RV expressing the ectodomain (ED) of HCV E2, lacking 85 amino acids at its C terminus, and fused to the transmembrane domain (TMD) and cytoplasmic domain (CD) of human CD4, was amplified by PCR from pTM1/E2_31RHABDOVIRUS-BASED HCV VACCINE

AcGAGCTCTTTAACAATGGATCAGGATCC-3' containing a BsiWI site (bold), and the reverse primer RP69 5'-CGTACGAAAATGAAATTCCGAC-3' containing a Nhel site (bold). The PCR product was cloned into pSPBN using the unique BsiWI and Nhel sites resulting in pSPBN-E2CD4G.

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AcGAGCTCTTTAACAATGGATCAGGATCC-3' containing a BsiWI site (bold), and the reverse primer RP69 5'-CGTACGAAAATGAAATTCCGAC-3' containing a Nhel site (bold) site. The PCR product was digested with BsiWI and Nhel and cloned into pSPBN, which was digested previously with BsiWI and Nhel. The resulting plasmid was entitled pSPBN-E1E2p7.
room temperature and again washed three times with PBS-glycine. After incubation for another 30 min with donkey anti-mouse FITC 1:100 (Jackson ImmunoResearch, West Grove, PA) cells were washed three times with PBS-glycine and analyzed by fluorescence microscopy. A FITC-labeled antibody against RV N (Centocor) was used as described previously (Foley et al., 2000; Schnell et al., 2000).

Western blotting

BSR cells were infected with a m.o.i. of five for 48 h and resuspended in lysis buffer [50 mM Tris, pH 7.4/150 mM NaCl/1% NP-40/1% SDS/1X protease inhibitor cocktail (Sigma, St. Louis, MO)] on ice for 5 min. The suspension was transferred to a microcentrifuge tube and spun for 1 min at 14,000 rpm to remove cell debris. Proteins were separated by 10% SDS/PAGE and transferred to a PVDF-Plus membrane (Osmonics, Minnetonka, MN). Blots were blocked for 1 h [5% dry milk powder in PBS (pH 7.4)]. After blocking, blots were washed twice using a 0.1% PBS-Tween-20 solution and incubated with either monoclonal murine α-E2 antibody (H52, 1:1000) (Flint et al., 1999), monoclonal murine α-E1 antibody (A4, 1:1000) (Dubuisson, 2000) or rabbit α-RV-G tail antibody (1:20,000) (Foley et al., 2000) in 0.1% PBS-Tween for 1 h. Blots were then washed three times with 0.1% PBS-Tween. Secondary antibodies of goat α-mouse or donkey α-rabbit HRP conjugated antibodies (1:5000) (Jackson ImmunoResearch) were added, and blots were incubated for 1 h. Again, blots were washed three times with 0.1% PBS-Tween and washed once with PBS (pH 7.4). Chemiluminescence (NEN) was performed as instructed by manufacturer. For quantification, Hyperfilm ECL film (Amersham Pharmacia Biotech) was preflashed with a Sensitize Preflash Unit as indicated by the manufacturer (Amersham Pharmacia Biotech) and scanned and quantification was performed with NIH Image, version 1.61.

Cytotoxicity assays

Groups of five 6- to 8-week-old female BALB/c mice (Harlan) were inoculated intraperitoneally (ip) with 10^7 ffu of SPBN-E1E2p7. To analyze the induction of specific CTL response against E2, spleens from three mice of each group were aseptically removed and combined, and single cells suspensions were prepared. Red blood cells were lysed with ACK lysing buffer (BioWhitaker), splenocytes were washed twice in RPMI-1640 media containing 10% fetal bovine serum and pulsed with 5 μg/ml peptide1323 [EATYSRCQSGPVITPRCMV, amino acids 592–610 in HCV strain 1a], and 10% T-STIM (Collaborative Biomedical Products) was added as a source of interleukin-2 (IL-2). Cytolytic activity of cultured CTLs was measured by a 4-h assay with ^51Cr-labeled P815 target cells. Target cells were prepared by incubating with 10 μg/ml peptide1323 and 100 μCi ^51Cr for 2 h and washed twice. Target cells were added to effector cells at various E:T ratios for 4 h. The percent specific ^51Cr release was calculated as 100 × (experimental release—spontaneous release)/(maximum release—spontaneous release). Maximum release was determined from supernatants of cells that were lysed by the addition of 5% Triton X-100. Spontaneous release was determined from target cells incubated without added effector cells.

Immunization for humoral responses

To analyze seroconversion against HCV E2, mice were immunized ip with 1 × 10^7 ffu of the respective RV as indicated. For vaccination with killed RV particles, sucrose-purified RV (SPBN or SPBN-E2CD4G) was deactivated by incubation with β-propiolactone (1:1000) overnight at 4°C followed by another incubation at 37°C for 30 min.

Mice were vaccinated/boosted ip with 20 μg of killed RV particles as indicated in the text and figure legends. Ten days after the boost, sera were collected and analyzed by ELISAs for HCV-specific antibodies as described below.

Use of recombinant HCV E2 for screening of seroconversion of vaccinated mice

Ninety-six-well MaxiSorp plates (Nunc) were coated with recombinant E2 (ImmunoDiagnostic Inc.) in coating buffer (50 mM Na2CO3, pH 9.6) at a concentration of 2.5 μg/ml and incubated overnight at 4°C. Plates were washed three times with 0.05% PBS/Tween and blocked with 5% dry milk powder in PBS for 1 h at room temperature. Mouse sera were diluted in 1× PBS, added to the plates, and incubated at room temperature for 1 h. After washing three times with 0.05% PBS/Tween, the secondary antibody (goat α-mouse HRP conjugated, Jackson ImmunoResearch) diluted 1:5000 in 1× PBS was added and the plates were incubated for 30 min at 37°C. OPD substrate (Sigma) was added to the plates after washing three times with 0.05% PBS/Tween. Substrate reaction was stopped by the addition of 50 μl 2M H2SO4 to each well. Plates were read at 490 nm.

Use of E2 proteins derived from purified recombinant virions

Recombinant RVs in the supernatants from SPBN or SPBN-E2CD4RVG infected BSR cells were sucrose-purified and incubated for 30 min with 1% Triton X-100 in PBS. RV ribonucleoprotein (RNP) complex was removed by centrifugation at 16,000 g at 4°C for an hour. Supernatants were removed and used directly to coat ELISA plates or frozen at 80°C. Ninety-six-well MaxiSorp plates were coated with glycoprotein(s) derived from 25 μg purified SPBN or SPBN-E2RVG virions for each plate in coating buffer (50 mM Na2CO3, pH 9.6) and incubated overnight at 4°C. Plates were washed three times with


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