Evaluation of Hepatitis C Virus Glycoprotein E2 for Vaccine Design: an Endoplasmic Reticulum-Retained Recombinant Protein Is Superior to Secreted Recombinant Protein and DNA-Based Vaccine Candidates

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Hepatitis C virus (HCV) is the leading causative agent of blood-borne chronic hepatitis and is the target of intensive vaccine research. The virus genome encodes a number of structural and nonstructural antigens which could be used in a subunit vaccine. The HCV envelope glycoprotein E2 has recently been shown to bind CD81 on human cells and therefore is a prime candidate for inclusion in any such vaccine. The experiments presented here assessed the optimal form of HCV E2 antigen from the perspective of antibody generation. The quality of recombinant E2 protein was evaluated by both the capacity to bind its putative receptor CD81 on human cells and the ability to elicit antibodies that inhibited this binding (NOB antibodies). We show that truncated E2 proteins expressed in mammalian cells bind with high efficiency to human cells and elicit NOB antibodies in guinea pigs only when purified from the core-glycosylated intracellular fraction, whereas the complex-glycosylated secreted fraction does not bind and elicits no NOB antibodies. We also show that carbohydrate moieties are not necessary for E2 binding to human cells and that only the monomeric nonaggregated fraction can bind to CD81. Moreover, comparing recombinant intracellular E2 protein to several E2-encoding DNA vaccines in mice, we found that protein immunization is superior to DNA in both the quantity and quality of the antibody response elicited. Together, our data suggest that to elicit antibodies aimed at blocking HCV binding to CD81 on human cells, the antigen of choice is a mammalian cell-expressed, monomeric E2 protein purified from the intracellular fraction.

Hepatitis C virus (HCV) is the major cause of chronic hepatitis, which can evolve to cirrhosis, liver failure, or hepatocellular carcinoma (2, 4). There is no vaccine for HCV, and the only available treatment, a combination of alpha interferon and ribavirin, is efficacious in only a minority of patients (33). Given that approximately 200 million chronic HCV infections have been estimated worldwide (52), there is a pressing need to develop new therapies and vaccination strategies. The development of such strategies will be aided greatly by a more complete picture of the structure-function features of HCV proteins.

HCV is an enveloped plus-strand RNA virus of the Flavi- viridae family (24). Its genome is 9.5 kb in length with one open reading frame that is translated as a single polyprotein, which is processed by host and virus proteases into at least three structural and seven presumptive nonstructural proteins with various enzymatic activities (5, 22, 47). Two glycoproteins, E1 and E2, are probably virion envelope proteins, containing multiple N-linked glycosylation sites, and form heterodimers in vitro (23, 32, 35, 45). The coexpressed E1-E2 complex localizes to the endoplasmic reticulum (ER) and lacks complex N-linked glycans (7, 8, 13, 15, 45, 49).

Neutralizing antibodies often play a pivotal role in defeating viral infections, including prominent human pathogens such as influenza virus and hepatitis B virus (9, 28). The assessment of neutralizing antibody responses to HCV has been difficult because HCV does not grow efficiently in cell cultures. To overcome this obstacle, we developed a surrogate assay which measures the ability of antibodies to inhibit the binding of recombinant E2 to its putative cellular receptor CD81 on human cells (neutralization-of-binding [NOB] assay) (44, 46). CD81 is a membrane-associated protein belonging to the family of tetraspanins (30). Its large extracellular loop (LEL) binds E2 with a $K_d$ of 1.8 nM (42), and this interaction appears necessary and sufficient for binding of bona fide HCV particles (44). Importantly, chimpanzee sera containing anti-envelope antibodies, which are capable of preventing HCV infection in vivo, inhibit the binding of HCV to CD81 in vitro, suggesting that this interaction is relevant to infection (44).

Our research has focused primarily on comparing vaccine formulations of HCV E2, which is an obvious candidate for inclusion in a subunit vaccine because of its potential role in HCV attachment. Thus, targeting antibodies to HCV E2 could be a viable strategy for disrupting the HCV-CD81 interaction. Despite the inherent difficulties in studying HCV infection and the lack of a clear correlate of protection, there is evidence that neutralizing antibodies can be protective. Studies performed with human immunoglobulin (Ig) preparations have suggested some degree of efficacy in preventing the transmission of HCV in the transfusion setting, after liver transplants, and in sexual transmission (17, 27, 43). Relevant data on the existence of HCV-specific neutralizing antibodies also come from experi-
mental vaccination studies in chimpanzees, because of the risk to the animals besides humans susceptible to infection. When vaccinating with recombinant envelope proteins (E1-E2 heterodimer), chimpanzees developed high titers of anti-E2 antibodies in serum, as well as antibody production in mucosal lymphoid tissues (ELISA) and NOB assay, and were completely protected from subsequent challenge with the homologous virus (6, 46).

As with any chronic viral infection, there is a growing interest in eliciting not only antibody but also cytotoxic T-lymphocyte (CTL) responses to HCV. Therefore, we have begun to investigate the use of E2 as an adjuvant or component of a vaccine based solely on recombinant proteins. DNA vaccines have been reported to prime good antibody responses, but, unlike subunit vaccines, they offer the advantage of maximizing CTL responses (12, 41). Their efficacy has been successfully demonstrated in animal models for a wide variety of viral pathogens including hepatitis B virus, human immunodeficiency virus, and influenza virus (10, 29, 51). However, despite its advantages and success in rodent models, DNA vaccination suffers from several drawbacks in that it is still largely in the realm of basic research and nonsecreted antigens are particularly weak stimulators of antibody responses (3).

Here we compared the quantity and quality of anti-E2 antibody responses elicited by various forms of recombinant HCV E2 proteins or by DNA vaccines. Our findings indicate that E2 was returned from recombinant CHO cells as described previously (46, 49). For purification of secreted E2 (S-E2), CHO cell conditioned medium was concentrated 23-fold by ultrafiltration and subjected to flowthrough chromatography on a type I ceramic hydroxyapatite (HAP) (Bio-Rad) column equilibrated in 1 M NaCl–2% Triton X-100, 100 mM Tris-HCl [pH 8.0], 1 mM EDTA, protease inhibitors. The fragments were cloned into the unique NotI site of the pAC-E2 constructs. CHO protein was purified from pooled transfectants without further cloning. Cells were resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholate) containing protease inhibitors. E2 was immunoprecipitated with serum of chimpanzee LS59, which was a nonsecreted form of E2. E2 was eluted with 150 mM NaCl–10 mM phosphate (pH 6.2). I-E2 was eluted with 150 mM NaCl–10 mM acetate (pH 5.0)–0.03% SDS and boiled for 2 min. Equal parts were incubated with or without endothelial cell factor for 20 h at 37°C in the presence of protease inhibitors and then trichloroacetic acid precipitated. After SDS-PAGE under reducing conditions, the E2 antigen was detected by Western blotting (9). The tpa-E2 insert was transcribed into the mammalian activating (TPA)-derived signal sequence (46). The TPA-E2 fragment was transferred into the Smal and NotI sites of the pAC-FN polylinker by standard molecular cloning techniques, resulting in pAC-E2-E1. For the construction of DNA vaccines encoding ER-retained E2, the following strategy has been used. 

**DNA constructs.** The vector pAC-FN used for DNA vaccination was described in detail elsewhere (J. M. Heile, S. Abrignani, and G. Grandi, unpublished data). Briefly, expression of the gene of interest is driven by the human cytomegalovirus immediate-early promoter and enhanced by the IRES element inserted behind the NC NcoI site, which is a particularly weak stimulator of antibody responses (3). The antigen of choice for eliciting the best antibody response in terms of both quantity and quality.

**MATERIALS AND METHODS**

**Cloning, expression, and purification of E2 protein.** E2364–661 and E2364–715 were expressed from recombinant CHO cells as described previously (46, 49). For purification of secreted E2 (S-E2), CHO cell conditioned medium was concentrated 23-fold by ultrafiltration and subjected to flowthrough chromatography on a type I ceramic hydroxyapatite (HAP) (Bio-Rad) column equilibrated in 1 M NaCl–2% Triton X-100, 100 mM Tris-HCl [pH 8.0], 1 mM EDTA, protease inhibitors). The solution was loaded on a Galanthus nivalis lectin (Vector Laboratories, Burlingame, Calif.) affinity column equilibrated in 1 M NaCl–2% Triton X-100–50 mM Tris-HCl [pH 8.0]. The column was washed with 1 M NaCl–0.1% Triton X-100–20 mM NaCl–10 mM phosphate (pH 6.2). I-E2 was eluted with 150 mM NaCl–10 mM phosphate (pH 6.2). To purify the different molecular weight forms of E2, the S-E2363–715 and the I-E2363–715 eluate were adjusted to 30 mM phosphate (pH 7.0), and fractionated on a Superdex 200 (Pharmacia) gel filtration column equilibrated with phosphate-buffered saline (PBS) (pH 7.0)–1 mM EDTA. Aliquots of collected fractions were trichloroacetic precipitated for analysis. Fractions containing monomers, dimers, and trimers of S-E2363–715 and E2364–715 were pooled and concentrated as indicated above.

**Deglycosylation of internal E2363–715 and carbohydrate analysis.** I-E2364–715 was digested with α-mannosidase or endo-β-N-acetylglucosaminidase H (endo H) (Boehringer Mannheim). The exoglycosidase α-mannosidase efficiently removes free mannose (Man) residues that are α1–6 Man or α1–2 Man linked and less efficiently removes α1–3 Man-linked residues. Endo H cleaves between the two N-acetylglucosamine (GlcNAc) residues of N-linked core units, leaving one GlcNAc residue linked to asparagine. Samples were incubated with or without enzyme overnight at 37°C in 50 mM citrate (pH 5.0). α-Mannosidase digestion was monitored by SDS-PAGE, and then 100 mM borate (pH 9.5) was added to stop the reaction. The samples were then dialyzed against 10 mM phosphate (pH 8.0) to remove digested monosaccharides. Endo H digested carbohydrates were removed by Sephacryl T200 chromatography. Insufficient carbohydrate content analyses, digested E2 was treated with 3 M trifluoroacetic acid for 6 h at 100°C to release the monosaccharides. Following lyophilization, samples were derivatized at pH 8.0 with 30 mM phenylmethylsulfonyl fluoride and extracted into chloroform:methanol (2:1). The samples were dried under liquid chromatography, and the monosaccharide content was determined by using an external calibration profile and an internal talose standard.

**In vitro analysis of constructs.** CHO cells were stably transfected with the E2 DNA vaccines for analysis. Selection was achieved by biotin-streptavidin expression of E2 together with a bivalent streptavidin gene. An mRNA expression system for the E2 sequence via an internal ribosomal entry site. neo was amplified by PCR from pcDNANeo (Invitrogen) and cloned in BamHI and blunt-ended Ncol sites of pcDNA-3a (+) (Novagen), with Ncol providing the ATG start codon. The truncated Ncol fragment was excised with FseI and NotI and blunt ended for insertion into the NotI site of the pAC-E2 constructs. CHO DG44 cells were grown in Ham’s F12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.02% 2-mercaptoethanol. Subconfluent monolayers grown in 100-mm diameter dishes were transfected with 20 µg of plasmid DNA by calcium phosphate coprecipitation. Precipitates were incubated with cells for 6 h at 37°C and then given a 3-min shock with 15% glycerol in PBS. Cells were grown for 48 h without selection, divided among 15 plates, and grown for 2 weeks in medium containing 1 mg of Genetec G418 sulfate (Gibco BRL) per ml. E2 protein was purified from pooled transfecteds without further cloning. Cells were resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholate) containing protease inhibitors. E2 was immunoprecipitated with serum of chimpanzee LS59, which was a nonsecreted form of E2 and protected against challenge with homologous HCV (6).

**Antibody titers.** The sera were preincubated with L559 preimmune serum and E2 was precipitated with immune serum. The pellet was resuspended in 100 mM acetic acid (pH 5.0)–0.03% SDS and boiled for 2 min. Equal parts were incubated with or without endothelial cell factor for 20 h at 37°C in the presence of protease inhibitors and then trichloroacetic acid precipitated. After SDS-PAGE under reducing conditions, the E2 antigen was detected by Western blotting. The sera were preincubated with MAbs (MAb 3E51), which recognizes nonconformational E2 (S. Abrignani, D. Rosa, and M. Houghton, unpublished data), followed by horseradish peroxidase-conjugated anti-mouse IgG antiserum (Amersham).

**Immunization of guinea pigs.** Four guinea pigs were purchased from Elmhill, Chumford, Mass. Five animals per group were immunized intraperitoneally (i.p.) with 8 µg of S-E2363–715 or I-E2363–715 in MF59 adjuvant on days 0, 30, and 90, and bled on day 110. Groups of eight to 10-week-old female C57BL/6 mice (Charles River, Calco, Como, Italy) were injected intramuscularly (i.m.) in both hind legs with either DNA in PBS or protein in MF59-0 adjuvant on days 0 and 35 and bled on day 49. Plasmid DNA was prepared using endotoxin-free purification columns (Quagen, Hilden, Germany). Per mouse and injection, a total of 100 µl of 1-µg/µl E2-DNA (100 µg) or 50-ng I-E2363–715 protein (5 µg) was administered.

**Antibody titers.** Anti-E2 antibody titers were measured by ELISA. For guinea pig sera, mouse mAbs 10007639 were coated with HeLa E1-E2 antigen (0.3 µg/ml) purified as previously described (6). The sera were analyzed with horseradish peroxidase-conjugated anti-guinea pig IgG antiserum (Sigma). Mouse sera were analyzed with CBA C57BL/6J mouse serum coated with CHO I-E2363–715 protein at 0.5 µg/ml, using alkaline phosphate-conjugated anti-mouse IgG (Sigma). Antibody titers were calculated as the dilution which gave an optical density (OD) that equaled the cutoff. The cutoff was established as the mean OD + 3 standard deviations for eight preimmune sera.
alyzed with a FACScan flow cytometer (Beckton Dickinson). The net mean fluorescence intensity (MFI) was calculated by subtracting the value obtained in parallel with chimpanzee E599 or guinea pig preimmune serum. The specificity of E2 binding to CD81 on human cells was confirmed as described previously (44) by competing E2 binding to human cells with soluble human CD81 LEL, which was purified as described previously (42) and incubated at a concentration of 50 μg/ml with E2 prior to incubation with MOLT-4 cells.

NOB assay. Mouse and guinea pig sera were tested for their ability to inhibit the binding of E2 protein to human target cells as previously described (25, 46). Briefly, NOB titers were determined by incubation of MOLT-4 cells with a nonsaturating concentration of biotinylated I-E2715 protein (1.5 μg/ml), which was preincubated with a test serum at different dilutions. Following incubation of the cells with streptavidin-phycocerythrin conjugate (Southern Biotechnology Associates) at 2.5 μg/ml, E2 binding was measured with a FACScan. NOB titers correspond to the reciprocal values of serum dilutions which inhibit 50% of binding.

RESULTS

I-E2 but not S-E2 binds human target cells and elicits NOB antibodies following immunization. The E2 protein is processed from the HCV polyprotein, leading to a polypeptide backbone of 363 residues, spanning residues 384 to 746 (31). aa 716 to 746 serve as a membrane anchor, and their deletion can lead to secretion of the E2 protein when expressed in mammalian cells (34, 48). We previously described stably transfected CHO cell lines expressing E2, targeted to the ER via a TPA-derived leader sequence fused to its N terminus and carboxy-terminally truncated at residues 661 (E2661) or 715 (E2715). Both lines secrete a fraction of the E2 protein and retain part of it in the ER (49). Although E2661 is secreted from CHO transfectants with more efficiency than do truncated versions, we first aimed to purify and analyze the largest secreted E2 protein possible, and we previously reported the purification and binding to human target cells of E2715 (46). This E2 protein had been affinity purified from culture supernatants with MAb 5E5/H7 raised against the HeLa intracellular E1-E2 heterodimer, and we obtained E2 only in extremely small quantities. The low yield achieved with a conformational MAb used for purification led us to suspect that we had actually captured either a minor well-folded fraction or an intracellular fraction that had been released into the supernatant by occasional cell lysis. We then purified both the supernatant fraction (S-E2715) and the intracellular fraction (I-E2715) from the same line without the use of E2-specific antibodies. In Western blots, S-E2715 appeared as a broad band of about 74 kDa whereas I-E2715 appeared as a sharper band of about 60 kDa (Fig. 1A), indicating extensive usage of the 11 N-linked glycosylation sites within E2.

The E2 protein is an ideal vaccine candidate for the generation of antibodies that inhibit the binding of virus to target cells. This antibody subset can be estimated in vitro by the inhibition of antibodies that inhibit the binding of virus to target cells. This antibody subset can be estimated in vitro by the inhibition of antibodies that inhibit the binding of virus to target cells.
gested I-E2 715 with α-mannosidase or with endo H and analyzed the products for the presence of monosaccharides and for their binding activity. α-Mannosidase-digested E2 retains all GlcNAc residues compared to the undigested control, as well as, most probably, the β1→3 GlcNAc-linked and some α1→3 mannose-linked mannose residues, which are not efficiently cleaved by the enzyme (Table 1). Endo H digestion was complete, with approximately half of the GlcNAc residues cleaved off and only trace amounts of uncleaved mannose left. Importantly, the deglycosylated forms and the undigested controls were identical in their capacity to bind CD81 (Fig. 4). The enzymes alone have no binding activity (data not shown). Thus, core glycosylation is not necessary for E2 binding to CD81 and complex glycosylation is likely to mask the E2 binding epitope for its cellular receptor. This also may explain the inability of complex glycosylated S-E2 715 to raise NOB antibodies after immunization. By treating S-E2 with peptide-N-glycosidase F (PNGase F), which removes complex sugars completely, we attempted to restore its binding activity. However, PNGase F-digested S-E2 formed high-molecular-weight aggregates, with the majority of the protein precipitating out of solution. The aggregates that remained in solution did not migrate into an SDS gel. This is presumably due to hydrophobic interactions following removal of all hydrophilic carbohydrates or due to conformational changes through the conversion of asparagine at the N-glycosylation site to aspartate. Therefore, it was

FIG. 2. Binding of various truncated E2 proteins to human cells. S-E2 (circles) and I-E2 (squares) truncated at position 661 (open symbols) or at position 715 (solid symbols) and expressed in CHO cells, were tested for their capacity to bind MOLT-4 cells. Cell-bound E2 is indicated as net MFI as detected by flow cytometry using chimpanzee anti-E1-E2 antiserum (A) or using antiserum raised in guinea pigs against I-E2 715 (B) or S-E2 715 (C). The star represents the effect of 50 μg soluble human CD81 LEL per ml on the binding of I-E2 661.

FIG. 3. Monomers of I-E2 715 bind human cells better than do dimers and trimers. Purified I-E2 715 and S-E2 715 were fractionated further by size exclusion chromatography into different molecular weight forms. (A) The elution profiles of I-E2 715 and S-E2 715 are shown. Individual fractions were monitored by SDS-PAGE under nonreducing conditions (data not shown). The monomeric, dimeric, and trimeric forms of E2 are indicated. The triangles indicate the elution time of size exclusion molecular weight standards. (B) Monomers (squares), dimers (circles), and trimers (diamonds) of I-E2 715 (solid symbols) and S-E2 715 (open symbols) were compared by their capacity to bind MOLT-4 cells. Cell-bound E2 was measured by flow cytometry using chimpanzee anti-E1-E2 antiserum and is indicated as net MFI. The ability of the chimpanzee serum to recognize monomers, dimers, and trimers of I-E2 715 and S-E2 715 was confirmed by staining Western blots of E2 SDS-PAGE gels under nonreducing conditions (data not shown). The effect of 50 μg of soluble human CD81 LEL per ml on the binding of monomeric I-E2 715 (asterisk) and monomeric S-E2 715 (star) is represented.
TABLE 1. Stoichiometry of the carbohydrate content of recombinant I-E2<sub>715</sub><sup>a</sup>

<table>
<thead>
<tr>
<th>Expt</th>
<th>Treatment</th>
<th>Amt (mol/mol of E2) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mannose</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>α-Mannosidase</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Mock treated</td>
<td>61</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Endo H</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Mock treated</td>
<td>59</td>
</tr>
</tbody>
</table>

* Other monosaccharides, including glucose, galactose, galactosamine, xylose, and fucose, were not detected.

not possible to compare completely deglycosylated S-E2 with untreated or endo H-treated I-E2 (data not shown).

Construction and in vitro analysis of ER-retained E2 DNA vaccines. Given the potential benefits of DNA vaccines over recombinant proteins (12), we assessed the ability of different E2 forms to stimulate humoral immunity if delivered in the form of a DNA vaccine. Using the DNA vaccination vector pAC, we constructed five E2-encoding plasmids (Fig. 5). (i) pAC-E2<sub>715</sub> corresponds to the construct used for E2<sub>715</sub> expression in CHO cells in that it encodes the same C-terminally truncated E2, directed into the ER by a TPA-leader sequence fused to its N terminus (aa 384), and driven by the human cytomegalovirus promoter. It was the basis for the other constructs, which all have the same N terminus and were made by modifying the C terminus of E2<sub>715</sub>. (ii and iii) pAC-E2<sub>746</sub> (ii) and pAC-E2<sub>809</sub> (iii) encode two naturally occurring membrane-anchored E2 species differing in their C termini (aa 746 and 809, respectively). The larger product contains a small highly hydrophobic region from aa 747 to 809, known as p7. Both forms are retained in the ER (31, 36, 48). Because the cellular localization of purified E2 antigen is critical, we assumed that ER retention of E2 protein expressed by DNA vaccines would favor the induction of NOB antibodies. (iv and v) Based on the same rationale, we additionally constructed pAC-E2<sub>715DEL</sub> (iv) by adding to the C-terminal lysine (aa 715) the three amino acids DEL to obtain the canonical signal for ER retention of luminal proteins, the amino acid motif KDEL (38), and pAC-E2<sub>730KK</sub> (v) by fusing to the C terminus of the first transmembrane-spanning domain of E2 (aa 730) the cytoplasmic tail of the adenovirus E3/19K protein, which confers ER retention and retrieval to transmembrane proteins (40, 50). The latter was constructed because despite the convincing in vitro data indicating its retention in the ER, full-length E2 has been reported to be present on the plasma membrane of hepatocytes in transgenic mice (26).

We next compared biochemical properties of E2 expressed in CHO cells stably transfected with our five DNA vaccination constructs. We were not able to detect an E2<sub>730KK</sub> protein, suggesting rapid degradation possibly due to incorrect folding. The other forms of E2 could be detected by Western blotting, and, as expected, all four are endo H sensitive, indicating an ER localization (Fig. 6). The proteins expressed from pAC-E2<sub>809</sub> (E2-p7) and pAC-E2<sub>746</sub> (E2) have the same apparent molecular weight, indicating that p7 is cleaved, consistent with previous reports (31, 36, 48). Endo H digestion of these two membrane-anchored E2 forms leads to the same sharp double band, suggesting inaccessibility for efficient cleavage of one
TABLE 2. Comparison of anti-E2 antibody responses elicited by protein or DNA immunization in mice

<table>
<thead>
<tr>
<th>Immunization</th>
<th>ELISA titer</th>
<th>NOB titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-E2715</td>
<td>150,000</td>
<td>500 (8)</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2715</td>
<td>10,000</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E2715DEL</td>
<td>15,000</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E2730KK</td>
<td>3,000</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E2746</td>
<td>6,000</td>
<td>60 (3)</td>
</tr>
<tr>
<td>E2809</td>
<td>7,000</td>
<td>40 (3)</td>
</tr>
</tbody>
</table>

* Assessed on pooled sera (eight mice per group) and expressed as the reciprocal values of serum dilutions.

** Mean values of individual NOB-positive sera (the number of NOB-positive sera per group of eight mice is given in parentheses).

DISCUSSION

The experiments presented here were performed to determine the optimal form of the HCV E2 antigen to include in a vaccine from the perspective of the generation of antibodies. The quality of recombinant E2 protein was assessed by two means: (i) the capacity to bind its putative receptor CD81 on human target cells and (ii) the capacity to elicit antibodies, in particular those that inhibit receptor binding (NOB antibodies). We show that CHO cell-expressed truncated E2 protein, when derived from the core glycosylated intracellular fraction (I-E2) but not from the complex glycosylated secreted fraction (S-E2), binds with high efficiency to human cells and elicits NOB antibodies in guinea pigs. We also show that carbohydrate moieties are not necessary for I-E2 binding to cellular targets and that only the monomeric fraction binds efficiently compared to multimers. Moreover, comparing recombinant I-E2 protein to several E2-encoding DNA vaccines in mice, we found that for both quantity and quality of the antibody response, protein immunization is superior to DNA. Based on these results, we propose that, for the induction of antibody responses, the E2 antigen needed in a future HCV vaccine is a recombinant, monomeric protein purified from the intracellular fraction.

In chimpanzees vaccinated with the E1-E2 heterodimer, high NOB antibody titers correlate with protection from infection with an homologous HCV isolate (46). However, when vaccinated chimpanzees were challenged with a heterologous virus, infection occurred in all animals but 9 of the 10 vaccinees did not develop chronic infection (M. Houghton and S. Abrigani, unpublished data). Antisera from animals immunized with recombinant E1-E2 derived from the HCV-1 strain contained high titers of anti-E2 antibodies that were able to block CD81 binding of recombinant E2 derived not only from HCV subtype 1a but also from subtype 1b. By contrast, anti-E1 antibodies did not block the binding of E1-E2 to human cells. Together, immunization experiments in chimpanzees indicate that a vaccine based on HCV envelope proteins is able to prevent at least chronic HCV infection and that in the E1-E2 heterodimer, it is E2 that can generate antibodies capable of blocking virus binding to target cells. Thus, although sterilizing immunity was not achieved following challenge with heterologous virus, the prevention of chronic infection still holds promise, given that acute HCV infections are often asymptomatic and clinically inconsequential.

In an effort to produce recombinant HCV E2 in CHO cells, we originally focused on S-E2715, whose purification is easier to scale up than is that of the intracellular fraction. Although we found that a supernatant fraction of E2 obtained by affinity chromatography binds human cells (46), we realized that our culture supernatant contained only a minor fraction of biologically active (in our binding assay) E2 protein. In an attempt to define the optimal forms of E2 for binding CD81, we assessed intracellular or secreted fractions of different truncated E2 proteins expressed in CHO cells. We found that both I-E2661 and I-E2715 bind substantially better to CD81 than do S-E2661 and S-E2715. It was found previously that S-E2661 binds well to CD81 (18). However, the same authors recently reviewed this finding and reported that I-E2661 binds CD81 with higher efficiency than does S-E2661 (19). Our results not only confirm this finding but also give a functional correlate to the superior binding of I-E2 to CD81. We demonstrate, using S-E2715 or I-E2715 in immunization studies, that both fractions elicit high anti-E2 antibody titers as assessed by ELISA whereas only the I-E2 fraction raises NOB antibody titers. The finding that deglycosylated I-E2715, binds CD81 on human cells as well as untreated I-E2715 suggests that carbohydrates are not directly involved in the recognition of CD81. They could, however, play a role in the correct folding of E2 in the ER.

Given that robust virus cell entry assays and infection assays are not available, we do not know whether CD81 mediates HCV entry into cells. However, we have evidence that (i) CD81 is very inefficient in mediating internalization of bound ligands (42) and (ii) some hepatoma cell lines can bind recombinant envelope proteins in the absence of CD81 (data not shown), suggesting that human cells contain other molecules which interact with HCV and may mediate entry. Recently, the low-density lipoprotein receptor has been proposed as a possible HCV receptor (37) or, more generally, as a molecule exploited by members of the family *Flaviviridae* to enter human cells (1). However, until reliable infection assays are available, it will always be difficult to sort out the relative importance of candidate HCV receptors.

Little is known about the assembly and maturation of HCV. However, studies with recombinant proteins indicate that non-covalent heterodimers of E1 and E2 reside in the ER and are core glycosylated. They have been proposed as the prebudding form of the HCV envelope glycoprotein complex (11, 14).
When truncated forms of E2 pass through the Golgi and are secreted, they become complex glycosylated. Our results indicate that these modifications almost completely abolish the ability of E2 to bind to CD81 on human cells and to elicit NOB antibody responses. Our view is that these complex sugars mask the binding site, most probably through steric or charge interference. Based on the results reported here, I-E2 probably mimics the native envelope form present in the virus, and thus the viral envelope should not be complex glycosylated. Consistent with this view, HCV could assemble at the ER membrane, bud into the ER, and be released into the extracellular environment by host cell lysis, similar to rotaviruses (16). By this mechanism, complex carbohydrate modifications are prevented because viral particles do not pass through the Golgi. Alternatively, it may be that HCV exits through the Golgi in a form which protects at least the carbohydrates in and around the CD81 binding site from further modifications. For the development of a vaccine against intracellular pathogens (e.g., viruses), it is advantageous to optimize antibody, T-helper cell, and CTL responses, all of which have been achieved by DNA vaccination of experimental animals (12). However, here we show that DNA vaccination elicits low ELISA titers of anti-E2 antibodies and low NOB titers compared to immunization with E2 protein. These data parallel results obtained by others with mice or small primates by using truncated E2 constructs for DNA immunization (20, 21, 39). Although NOB titers do not necessarily correlate with total anti-E2 antibody titers in our experience with human sera (46), we cannot rule out that the low efficiency of NOB antibody production following DNA immunization is a direct consequence of the inability of the same DNA constructs to induce high anti-E2 antibody titers in general. DNA vaccines encoding intracellular proteins elicit poor antibody responses in comparison to secreted proteins (3). E2-encoding DNA vaccines therefore might be limited by their inherent inaccessibility to antibodies. Another possible explanation of the lack of NOB antibody responses is that the uptake of DNA and the expression of E2 in vivo alter the glycosylation pattern, localization, and/or conformation of the E2 protein.

In conclusion, elicited antibody responses are limited when blocking HCV binding to CD81 on human cells, protein immunization is superior to DNA immunization. Cellular responses could potentially be achieved by administering DNA in combination with recombinant protein, and investigating the influence of one to another will be an important step in defining optimal vaccination strategies for HCV.

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