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DNA immunization of mice and macaques with plasmids encoding hepatitis C virus envelope E2 protein expressed intracellularly and on the cell surface

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Abstract

We analyzed the humoral immune response elicited by hepatitis C virus (HCV) E2 protein expressed in vivo after injection of plasmid DNA into mice and rhesus macaques. Three plasmids were used for immunization: a plasmid containing the entire sequence of the E2 and p7 genes (pE2); a plasmid encoding a truncated form of the E2 protein targeted to the cell surface (pE2surf); a control plasmid (pDisplay) lacking an HCV insert. Each plasmid was injected intramuscularly into 5 mice and intraepidermally (via gene gun) into 5 mice. Immunization was repeated three times at three week intervals. Five macaques were injected intramuscularly (two with pE2, two with pE2surf and one with pDisplay) and immunization was repeated after 8 weeks. All mice immunized via gene gun with pE2 or pE2surf developed anti-E2. The animals immunized with pE2surf developed an earlier and stronger humoral immune response than those immunized with pE2. Only 2 of the mice injected by the intramuscular route, both immunized with pE2surf, developed detectable anti-E2. One of the two macaques immunized with pE2 and both macaques immunized with pE2surf developed anti-E2; the humoral immune response was much stronger in the animals immunized with pE2surf. Our results suggest that presentation of HCV E2 on the cell surface may increase its immunogenicity while preserving its ability to react with antibodies generated during a natural infection. Published by Elsevier Science Ltd.

Keywords: Hepatitis C virus; DNA vaccine; Surface-expressed E2; Non-human primates

1. Introduction

Hepatitis C virus (HCV) is one of the main etiological agents of chronic liver disease worldwide [1]. About 170 million people globally (4 million people in the US) are chronically infected with HCV [2]. Individuals with chronic HCV infection are at increased risk for the development of liver cirrhosis and hepatocellular carcinoma [1]. Despite the near elimination of HCV transmission via blood transfusion, it is believed that approximately 25,000 new cases of HCV infection occur every year in the US [2] and the majority of these lead to chronic infection. Therefore, the development of an effective HCV vaccine is highly desirable.

HCV is a positive-sense single-stranded RNA virus that belongs to the *Flaviviridae* family [1]. The ≈9.6 kb genome contains a single long open reading frame encoding a polyprotein that is cleaved into at least 10 structural and nonstructural proteins. The structural proteins consist of the capsid protein and two envelope glycoproteins (E1 and E2). The envelope proteins exhibit a high degree of genetic heterogeneity [3–6]; a region in the aminoterminal end of E2 has been shown

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to be extraordinarily heterogeneous (hypervariable region 1 (HVR1)) [7,8]. It is not known whether p7, which is found as a fusion with E2 as well as a cleaved species, is a structural or nonstructural protein [9].

Natural infection with HCV seems not to elicit protective immunity: studies in thalassemic children, as well as studies in experimentally infected chimpanzees, have shown that reinfection with HCV can occur [10,11]. Yet, it has been shown that antibodies with neutralizing activity exist. It was first demonstrated that chronic phase plasma from patient H, with postransfusion hepatitis C, could neutralize HCV from the patient's acute phase plasma, in vitro [12,13]. Next, it was demonstrated that antibodies directed against the HVR1 of the E2 protein had neutralizing activity in vitro [14,15]. Finally, it has been shown that infusion of immune globulin containing HCV antibodies markedly prolonged the incubation period of acute hepatitis C in chimpanzees, although it did not prevent HCV infection [16].

The above mentioned studies have made it clear that the development of an effective vaccine against HCV will be a great challenge. The success of a vaccine probably depends on the production of antibodies with adequate neutralizing activity. However, the lack of a reliable cell culture system for HCV represents a major obstacle for the study of virus neutralization. An experimental vaccine produced from expressed envelope glycoproteins has been shown to induce protection in chimpanzees against a low dose challenge with an homologous strain (HCV-1) [17]. However, challenge with a closely related heterologous strain (HCV-H77) resulted in infection [18]. Furthermore, multiple viral species of HCV coexist as a quasispecies in an infected individual. When such a quasispecies is transmitted to a vaccinated individual, minor variants of the quasispecies that have escaped the vaccine-induced antibodies may emerge [14,19]. Also, the existence of different genotypes with a low degree of homology within the envelope proteins diminishes the hope of identifying conserved neutralization epitopes [20]. Thus, it is likely that a polyvalent vaccine will be needed to generate broadly reactive neutralizing antibodies.

In recent years, vaccination with DNA has been shown to elicit protective immune responses against several pathogens [21–27], including human immunodeficiency virus and flaviviruses [28,29]. DNA vaccination can induce humoral and cellular immune responses and, therefore, its potential might include both prevention of infection and immunotherapy of chronic infections. One of the advantages of DNA vaccination is its versatility for studying the immunogenicity of multiple constructs containing different coding sequences, while avoiding the necessity of protein expression and purification. In addition, the expression in the host of the foreign proteins encoded by DNA sequences facilitates a folding and presentation of the antigens that might better approach that occurring in the natural infection [30].

The analysis of the immune response of mice to the structural proteins of HCV generated by injection of DNA constructs has become an important approach for vaccine development [31–36]. In this study, we have evaluated the humoral immune response in mice and rhesus macaques immunized with DNA constructs encoding the HCV E2 protein. In contrast to previous studies in which the HCV E2 protein was expressed intracellularly or in secreted form, we have demonstrated expression of the E2 glycoprotein on the cell surface and have compared the humoral immune response elicited by this new form of the E2 glycoprotein with the immune response elicited by the intracellular form.

2. Material and methods

The original source of the HCV constructs used in this study was an acute phase plasma sample (H77) from patient H, who had posttranfusion hepatitis C [37]. For detection of E2 glycoprotein by immunoblot and immunofluorescence, we used plasma (H79) from patient H, obtained in the chronic phase two years after the onset of HCV infection.

2.1. Plasmid construction, amplification and purification

We prepared two expression vectors with HCV inserts downstream from the CMV immediate-early enhancer-promoter. The HCV proteins encoded by these plasmids had sequences identical to those of an infectious cDNA clone of strain H77 (genotype 1a) [38]. One construct (pE2) contained the signal sequence located at the carboxy terminus of E1 and the entire E2 and p7 genes of HCV (aa 364–809) in the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) (Fig. 1). The other construct (pE2surf) contained a truncated form of E2 (aa 384–715) lacking the carboxy-terminal hydrophobic domain. The truncated E2 sequence was cloned, in frame, into the pDisplay expression vector (Invitrogen) between a leader sequence which targeted the HCV protein to the secretory pathway that determines posttranslational modifications and the transmembrane domain of the platelet-derived growth factor receptor (PDGFR), which anchored the HCV protein to the plasma membrane (Fig. 1).

The inserts of the two HCV constructs were amplified by PCR (using Advantage KlenTaq Polymerase mix (Clontech, Palo Alto, CA)) from plasmids

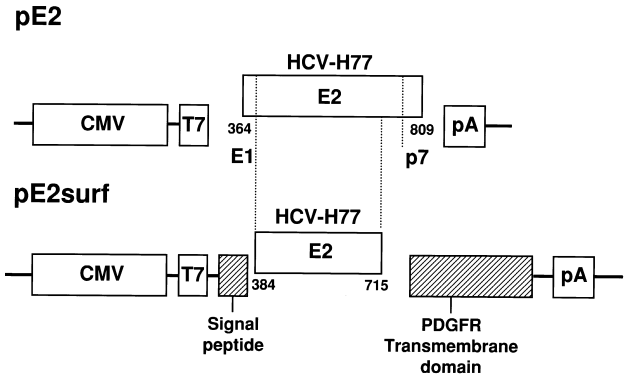

Fig. 1. Construction of pE2 and pE2surf. For pE2, the signal sequence within E1 and the entire sequence of E2 and p7 (aa 364–809) were cloned into the expression vector pcDNA3.1. For pE2surf, DNA encoding a truncated form of E2 lacking 31 carboxyterminal amino acids and p7 (aa 384–715) was cloned into pDisplay. This fragment was cloned in-frame between a signal peptide sequence (to direct the protein to the secretory pathway) and the platelet-derived growth factor receptor (PDGFR) transmembrane domain sequence (for expression of the protein on the cell surface) included in the vector. CMV: human cytomegalovirus immediate-early promoter/enhancer. T7: T7 promoter/priming site. pA: polyadenylation signal.

containing the full-length sequence of strain H77, as previously described [39]. The E2-p7 region of HCV was amplified using a sense primer containing an *Nhe*I restriction site (5′CGTC*GCTAGC*ATGGTGGGGGAACTGGGCGAAGGTCCTGG 3′) and an antisense primer containing a *Hind*III restriction site and two termination codons immediately following the carboxy terminus of p7 (aa 809) (5′ ACGCGT*AAGCTTTTACTA*TGCGTATGCCCGCTGAGGCAACGCC 3′). The PCR products were digested with *Nhe*I and *Hind*III (New England Biolabs, Beverly, MA) and cloned into the digested expression vector pcDNA3.1, by using T4 ligase (Promega, Madison, WI). The truncated form of E2 was amplified using a sense primer containing a *Bgl*II restriction site (5′ ACGCGT*AGATCT*GAAACCCACGTCACCGGGGGAAATGCC 3′) and an antisense primer containing a *Pst*I restriction site (5′ ACGCGT*CTGCAG*CTTAATGGCCCAGGACGCGATGCTTG 3′). The PCR products digested with *Bgl*II and *Pst*I (Promega) were cloned into the digested expression vector pDisplay. Clones containing the correct insert were grown at 37°C in the presence of ampicillin. DNA was prepared from 100 ml of bacterial cultures with the modified alkaline lysis method (Endofree Plasmid Purification Kit, Qiagen, Hilden, Germany) to remove endotoxins. Sequence analysis (both strands of plasmid DNA) confirmed that the HCV constructs encoded the authentic HCV protein.

2.2. Expression of HCV E2 protein in mammalian cells

COS-7 and Huh7 cells were cultured in the presence of Dulbecco's modified Eagle's medium (GIBCO/BRL, Gaithersburg, MD) containing 10% bovine serum (Bio Whittaker, Walkersville, MD) and penicillin–streptomycin (Sigma, St. Louis, MO). Cells were transfected at 60–80% confluency with pE2, pE2surf or pDisplay (without HCV sequences) using Superfect Reagent (Qiagen), according to the manufacturer's instructions. After about 48 h cells were tested for the expression of HCV glycoprotein E2 by Western blot analysis and indirect immunofluorescence.

2.3. Western blotting

Transfected cells grown in 60 mm dishes were lysed (lysis buffer: 1% NP40, 1 mM EDTA, 50 mM Tris–HCl, pH 7.5) for 20 min at 4°C. After a short centrifugation (14,000 rpm for 5 min at 4°C), the cell lysates were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes (Novex, San Diego, CA) by using a Trans-Blot cell (Bio Rad, Hercules, CA). Membranes were incubated with plasma H79 (1:750 dilution) overnight at 4°C. Duplicate membranes were incubated with plasma (1:750 dilution) from an anti-HCV negative blood donor. Following standard washing procedures, membranes were incubated with a goat anti-human immunoglobulin conjugated to horseradish peroxidase (dilution 1:5000) (Pierce, Rockford, IL) for 1 h at room temperature. After washing, the membranes were incubated with substrate (Supersignal Chemiluminescent Substrate, Pierce) and exposed to films.

2.4. Immunofluorescence

Cells grown in 4-well tissue culture chambers were screened for the expression of E2 glycoprotein after transfection with pE2, pE2surf or pDisplay. For detection of intracellularly expressed E2, cells were fixed and permeabilized with cold acetone for 5 min. Thereafter, cells were incubated for 40 min at room temperature with a 1:100 dilution of either plasma H79 or plasma from an anti-HCV negative blood donor. After washing, cells were incubated with a fluorescein-isothiocyanate (FITC)-conjugated goat anti-human IgG (1:100 dilution) (Pierce) for 30 min at room temperature. After washing, slides were mounted and examined for immunofluorescence. Staining for cell surface-expressed E2 was performed as described above, except that cells were neither fixed nor permeabilized (live cells). Cell surface-expressed E2 was also

characterized by staining cells with the conformation-sensitive monoclonal antibody H53 [40] (kindly provided by Dr. J. Dubuisson) at a 1:200 dilution. In this case, we used FITC-conjugated goat anti-mouse IgG (Sigma) as the secondary antibody.

2.5. DNA immunization of mice and rhesus macaques

30 female BALB-C mice (6 weeks old) and 5 adult macaques (*Macaca mulatta*) (3 females (L143, T119, T120) and 2 males (H392, H403), approximately 4 years old) were used in this study. The housing, maintenance, and care of the animals were in compliance with all relevant guidelines and requirements.

Mice were divided into six groups of 5 mice each: groups A, B and C were immunized by intramuscular injection of plasmid DNA and groups D, E and F were immunized intraepidermally by gene gun. Groups A and D mice were negative controls and were immunized with pDisplay, groups B and E were immunized with pE2 and groups C and F were immunized with pE2surf. All mice were immunized at weeks 0, 3 and 6. Mice were bled at weeks 0, 3, 6, 8, 11 and 14. Each hindleg muscle of mice from the intramuscular group was injected with 25 μl of sterile saline containing 25 μg of plasmid DNA (50 μg DNA/mouse). For the gene gun injections, DNA 'bullets' were generated. Briefly, 200 μg of plasmid DNA was vortexed gently with 100 mg of 1.6 μm gold particles (Bio-Rad) in a solution of calcium chloride and spermidine. DNA was allowed to precipitate onto the particles at room temperature for 15 min. The particles were washed three times in cold 100% ethanol to remove unbound DNA and resuspended in ethanol at a particle concentration of 7 mg/ml. The DNA gold particles were deposited onto the inside wall of Tefzel tubing (2.3 mm, I.D., McMaster-Carr) by rotation and allowed to dry. The tubing was cut into 1.25 mm lengths to make the bullets and stored desiccated at −20°C. Mice were injected with DNA-coated particles from four bullets in well-separated sites on the shaved abdomen, using an Accell gene delivery device at a pressure of 400 psi of helium. Each mouse received approximately 8 μg of DNA per immunization.

Rhesus macaques were injected intramuscularly into the biceps *femoris* muscle with 1 ml of endotoxin-free PBS containing 1 mg of DNA. Two animals (H403, H392) were immunized with pE2, two (L143, T119) with pE2surf and the remaining animal (T120) with pDisplay. DNA immunization by the intramuscular route was repeated after 8 weeks; animals were bled weekly.

2.6. Detection of specific HCV antibodies

Anti-E2 was detected with an experimental EIA (Abbott Laboratories, North Chicago, IL) [41]. The EIA uses polystyrene beads, coated with recombinant HCV E2 antigen (genotype 1a) that was expressed in Chinese hamster ovary cells. Sera to be tested were diluted 1:41 in specimen diluent (50 mM Tris–HCl, 10% fetal calf serum, 0.2% Triton X, 0.1% sodium azide, pH 7.5). After washing, samples were incubated with horseradish peroxidase-labeled anti-mouse or anti-monkey antibodies. Following washing, chromogen was added and optical densities were read at 492 nm in a quantum II spectrophotometer (Abbott Laboratories). The positive cut-off was established at 3 times the optical density value of samples from negative control mice or monkeys. For titration of anti-E2, a 1:41 dilution of sera in specimen diluent was progressively diluted and tested as described above. Sera from mice with the highest anti-E2 titer for each group were assayed by ELISA to determine the dominant antibody isotypes (Pharmigen, San Diego, CA).

Sera obtained following vaccination of the mice (week 14) and macaques (week 10) were diluted 1:50 and tested for reactivity against the E2 glycoprotein by immunofluorescence in Huh7 cells transfected with pE2, pE2surf or pDisplay, as described above.

2.7. Peptide synthesis and epitope mapping

A series of 52 peptides (16 mers; the last peptide being an 18 mer) beginning at aa 384 of HCV and encompassing the entire consensus sequence of the E2 and p7 proteins of H77 was synthesized (Pioneer Peptide Synthesis System Multiple Peptide Synthesis accessory; Perseptive Biosystems). Each peptide overlapped the adjacent peptide by 8 amino acids. All synthesized peptides were purified by HPLC.

Pre-vaccination sera and post-vaccination sera from mice (week 14) and macaques (week 10), as well as plasmas H77 and H79 obtained from patient H, were tested for reactivity against the 52 peptides (1:350 dilution for mice, 1:150 for H77, H79 and 1:50 for macaques) by standard ELISA (Lofstrand Laboratories, Rockville, MD). All tests were performed in duplicate. The positive cut-off value was established at 3 times the optical density value of the negative control sample (pre-vaccination sera for mice and macaques, sample H77 for patient H).

2.8. Statistical analysis

The Fisher's exact test was used to compare frequencies for categorical variables and the Mann–Whitney test to analyze differences for quantitative variables.

3. Results

3.1. Expression of HCV E2 glycoprotein in mammalian cells

We constructed two HCV expression vectors: pE2, which contained sequences of the entire E2 and p7 genes of HCV preceded by the signal sequence within E1 (aa 364–809) and pE2surf, which contained a truncated form of E2 (aa 384–715) that excluded the last 31 amino acids of E2 as well as p7 (Fig. 1). In pE2surf, the carboxy-terminal hydrophobic domain of E2 was replaced by the transmembrane domain of the PDGFR (Fig. 1). The HCV proteins encoded by these plasmids had amino acid sequences identical to those of an infectious cDNA clone of strain H77 (genotype 1a) [38]. The ability of pE2 and pE2surf to express E2 glycoprotein in mammalian cells was tested following transfection of the plasmids into Huh7 and COS-7 cells. About two days after transfection, cells were lysed and the lysates were submitted to SDS-PAGE. Because of the extra protein sequences added to the truncated E2surf, E2 and E2surf had a similar size. Immunostaining with chronic-phase plasma (H79) from patient H showed a band of the predicted size

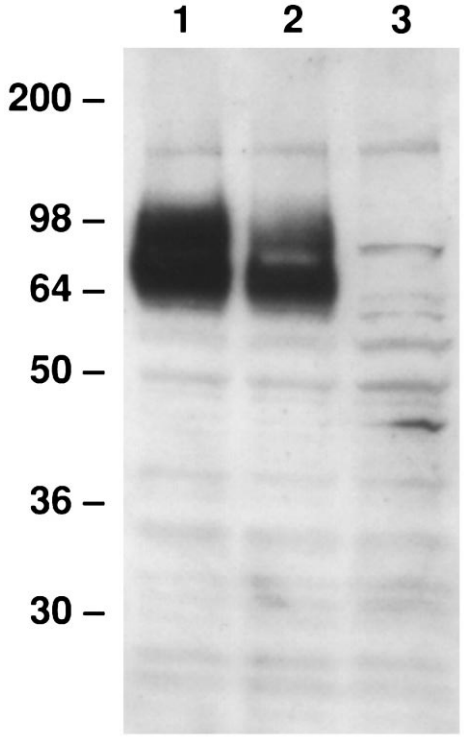

Fig. 2. Western blotting analysis of expressed HCV E2 glycoprotein. 2 days after transfection of Huh7 cells with plasmids pE2, pE2surf or pDisplay, cells were lysed and the lysates were submitted to SDS-PAGE. When stained with anti-HCV positive plasma H79, cells transfected with pE2 (lane 1) and pE2surf (lane 2) showed a specific protein of the expected size (≈68 kDa), which did not appear in cells transfected with pDisplay (lane 3).

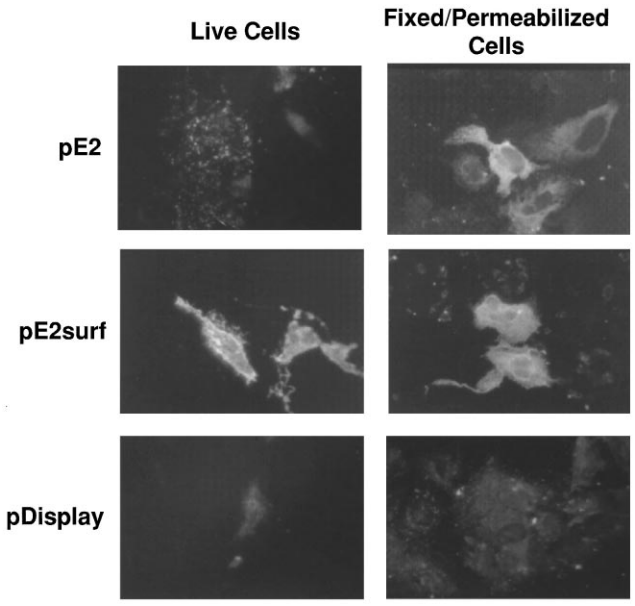

Fig. 3. Indirect immunofluorescence for detection of expressed HCV E2 glycoprotein. Two days after transfection of Huh7 cells with plasmids pE2, pE2surf or pDisplay, cells were examined by indirect immunofluorescence after staining with anti-HCV positive plasma H79. When staining was performed on fixed and permeabilized cells, cells transfected with pE2 and pE2surf both showed intracytoplasmic expression of HCV E2 glycoprotein. When staining was performed on live cells, only cells transfected with pE2surf showed expression of E2 glycoprotein on the cell surface.

(≈68 kDa) in lane 1 and lane 2 (cells transfected with pE2 or pE2surf, respectively), that was absent from lane 3 (cells transfected with the control plasmid pDisplay) (Fig. 2). We did not detect significant differences between the levels of expression of pE2 and pE2surf in Huh7 cells by end-point dilution Western-blot assays. Therefore, both E2 constructs expressed a similar amount of a protein that was immunoreactive with antibodies from a patient chronically infected with HCV.

The cellular location of the expressed E2 glycoproteins was determined by indirect immunofluoresence microscopy. About two days after transfection, cells were stained with the H79 plasma. When cells were treated with acetone, HCV E2 glycoprotein was detected in the cytosol of cells transfected with pE2, as well as in cells transfected with pE2surf, but not in cells that had been transfected with the control plasmid pDisplay (Fig. 3). When live cells were stained (no fixation/permeabilization steps), the E2 glycoprotein was detected on the surface of cells transfected with pE2surf, but not on cells transfected with pE2 or with the control plasmid (Fig. 3). Therefore, truncated E2 was expressed on the cell surface and recognized by human antibodies produced during a natural infection. In addition, the E2 on the surface reacted in the immunofluorescence assay with the conformation-sensitive E2-reactive monoclonal antibody H53, which

recognizes a form of E2 thought to be properly folded [40].

3.2. Specific antibody response to HCV E2 glycoprotein in mice and macaques

BALB-C mice were injected intramuscularly and intraepidermally with pE2, pE2surf or pDisplay at weeks 0, 3 and 6 and tested for antibodies against E2 by ELISA at weeks 0, 3, 6, 8, 11 and 14 and by immunofluorescence at week 14. 2 of the 5 mice inoculated intramuscularly with the surface-expressed E2 (pE2surf) developed anti-E2 antibodies (Fig. 4C). None of the animals that were inoculated intramuscularly with the intracellular form of E2 (pE2) or the control plasmid (pDisplay) developed anti-E2 antibodies (Fig. 4A and B). Thus, only 2 of the 10 mice vaccinated intramuscularly developed anti-E2. In contrast, all 10 animals vaccinated via gene gun with pE2 or with pE2surf developed anti-E2 antibodies (Fig. 4E and F) ($p < 0.01$). None of the control mice developed anti-E2, as measured by ELISA (Fig. 4D) or immunofluorescence (data not shown). There was an excellent correlation between anti-E2 detection by ELISA and by immunofluorescence; a discordant result was obtained only in mouse 5 from group E, which tested negative by ELISA but positive by immunofluorescence. IgG2 was the predominant isotype in mice from the three groups that developed anti-E2.

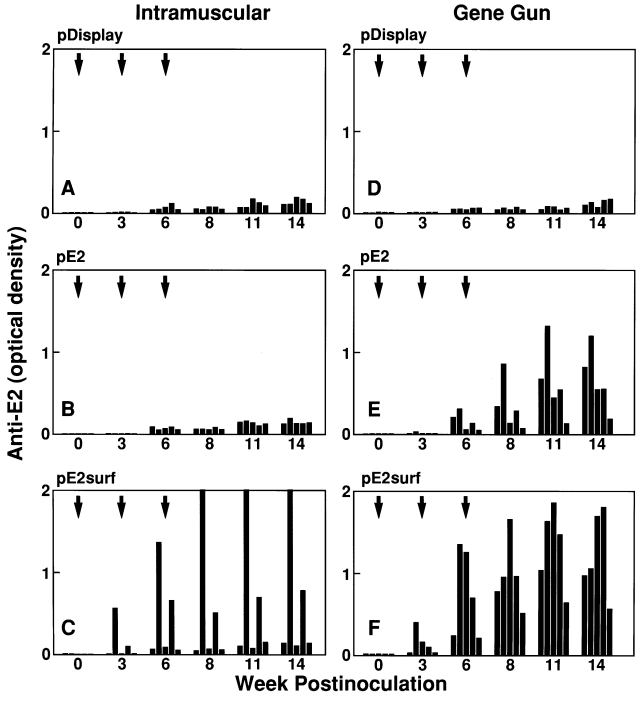

Fig. 4. Anti-E2 response in BALB-C mice after DNA vaccination with pDisplay, pE2 or pE2surf. Intramuscular injection: groups A, B and C. Gene gun: groups D, E and F. Arrows: time of vaccination. Anti-E2 optical density values of the 5 mice included in each group are depicted at each time point.

In mice vaccinated via gene gun, the surface-expressed E2 glycoprotein elicited an earlier and stronger immune response compared to the intracellular form of E2. We found that 3 of 5 animals were positive for anti-E2 after the first immunization with pE2surf and all of them had anti-E2 after the second immunization. In contrast, animals vaccinated with the intracellular form of E2 did not develop antibodies before the second immunization and only 2 animals were positive for anti-E2 after the second immunization. The ELISA optical density values were significantly higher in the group vaccinated with the surface-expressed E2 compared to the group vaccinated with the intracellular E2 at week 8 ($p = 0.03$) and the differences reached near statistical significance at weeks 6 and 11 ($p = 0.055$ in both cases). The relative anti-E2 titers after completing the immunization schedule are shown in Table 1. Again, mice inoculated with pE2surf had higher titers of anti-E2 at all time points compared to the animals inoculated with pE2. However, these differences did not reach statistical significance, probably because only a few animals were included in each group.

Similar results were obtained in rhesus macaques, which were immunized only by intramuscular injection. Neither of the two animals immunized with pE2 developed anti-E2, as detected by ELISA, but one animal was positive by immunofluorescence. Significantly,

Table 1
Relative ELISA anti-E2 titers in mice vaccinated via gene gun[a]

	Reciprocal titer of anti-E2 at indicated week postvaccination		
	8	11	14
Group E: mice immunized with pE2			
1	656	2624	1312
2	1312	2624	1312
3	41	656	656
4	164	656	656
5[b]	20.5	20.5	20.5
Mean	214	571	433
Group F: mice immunized with pE2surf			
1	1312	2624	1312
2	656	1312	656
3	656	5248	2624
4	2624	5248	2624
5	656	1312	1312
Mean	1000	2630	1513

[a]Relative anti-E2 titers by end-point dilution. Mean values are expressed as geometric mean titers.

[b]Mouse 5 from group E, negative by ELISA, was assigned an arbitrary value of one half of the screening dilution (20.5). The cut-off OD was 3 times higher than the OD of a pool of negative control mouse sera.

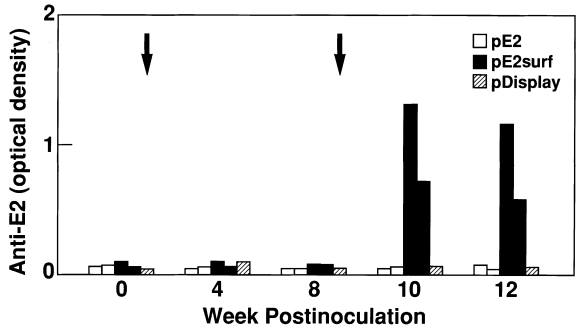

Fig. 5. Anti-E2 response in rhesus macaques after DNA vaccination with pDisplay, pE2 or pE2surf. Anti-E2 optical density values of the 5 macaques are depicted at each time point. Empty bars: immunization with pE2; filled bars: immunization with pE2surf; hatched bars: immunization with pDisplay.

both animals immunized with pE2surf developed anti-E2, which was detected both by ELISA (Fig. 5) and by immunofluorescence. Anti-E2 were detected only after the second immunization.

3.3. Mapping of linear epitopes in E2

To identify linear epitopes eliciting an immune response in the vaccinated BALB-C mice, we collected serum from mice prior to and following vaccination and compared them for reactivity against 52 overlapping peptides (consensus amino acid sequence) of the E2-p7 region of strain H77 (Fig. 6). Human plasma samples H77 (negative for anti-HCV) and H79 (positive for anti-HCV) were used as a reference for epitope mapping.

One major linear epitope was recognized by chronic phase plasma (H79) of patient H; this epitope was represented by peptide 520 (aa 520–535). Interestingly, peptide 520 was recognized as a major linear epitope in 4 of the 10 anti-E2 positive mice vaccinated via gene gun (mouse 2 from group E vaccinated with pE2 and mice 3, 4 and 5 from group F vaccinated with pE2surf). Sera from two additional anti-E2 positive mice (mouse 1 from group E and mouse 2 from group F) recognized several other linear epitopes. In the remaining 6 mice that developed anti-E2 antibodies, major linear epitopes could not be identified (data from mouse 5 of group E, which was anti-E2 positive by immunofluorescence and not by ELISA are not shown). Sera from the vaccinated mice that were negative for anti-E2 by ELISA or immunofluorescence did not react specifically with any of the peptides (data not shown). We did not detect antibodies to the linear epitopes representing the HVR1 region of HCV (Fig. 6).

A major linear epitope was recognized by serum of one macaque (T119) vaccinated with pE2surf; this epitope was represented by peptide 640 (aa 640–655). Sera from the remaining monkeys did not react with any of the peptides (data not shown).

4. Discussion

In the present study, we analyzed the humoral immune response against the HCV E2 glycoprotein following DNA immunization in mice and macaques. We were able to direct a recombinant E2 glycoprotein to the cell surface and showed that this form of E2 was more immunogenic than an intracellular form. Anti-E2 is detected in a large proportion of patients chronically infected with HCV [42–44]; these antibodies do not seem to confer protection against reinfection [11]. This might be explained by low titers of neutralizing antibodies elicited by natural HCV infection, or by the existence of neutralization escape mutants [12, 19]. However, since generation of a strong humoral immune response against the envelope proteins of HCV is believed to be essential for the success of a vaccine, any strategies that increase immunogenicity must be evaluated.

It is well established that the intact E2 glycoprotein expressed alone or with E1 (E1–E2 heterodimer) is retained in the endoplasmic reticulum [9]. The E2 protein extends to residue 746 and deletions at the C-terminal domain lead to its secretion [45]. We targeted the E2 protein to the cell surface by replacing the 31 C-terminal amino acids with the transmembrane domain of a protein (PDGFR) normally transported to the plasma membrane. Our findings are consistent with a recent report in which it was shown that the carboxy-terminal 29 amino acids of the E2 protein of HCV constitute the anchor signal and that the E2 protein can be targeted to the cell surface if this domain is replaced by a membrane anchor [40].

Previous studies have shown that DNA immunization can generate antibodies against the structural proteins of HCV [31–36, 46]. The major focus in these studies has been the E2 protein because it is believed that E2 contains important neutralization epitopes [14, 15, 17–19, 47]. In two studies, secreted forms of the envelope E2 protein were expressed by removing the hydrophobic domain of the protein [35, 36]. However, a surface-expressed form of the HCV E2 protein has not been tested as an immunogen. It is known that soluble protein antigens induce only a moderate signaling of B-cells, whereas cell-bound antigens can induce strong signaling [48] and therefore a better activation of B-cells. Indeed, epitopes exposed on the cell surface of recombinant bacteria may induce antibody responses in a T-cell independent manner, by direct activation of B-cells [49, 50]. Previous research indicates that antigens presented on the cell surface are often more immunogenic than intracellular or secreted

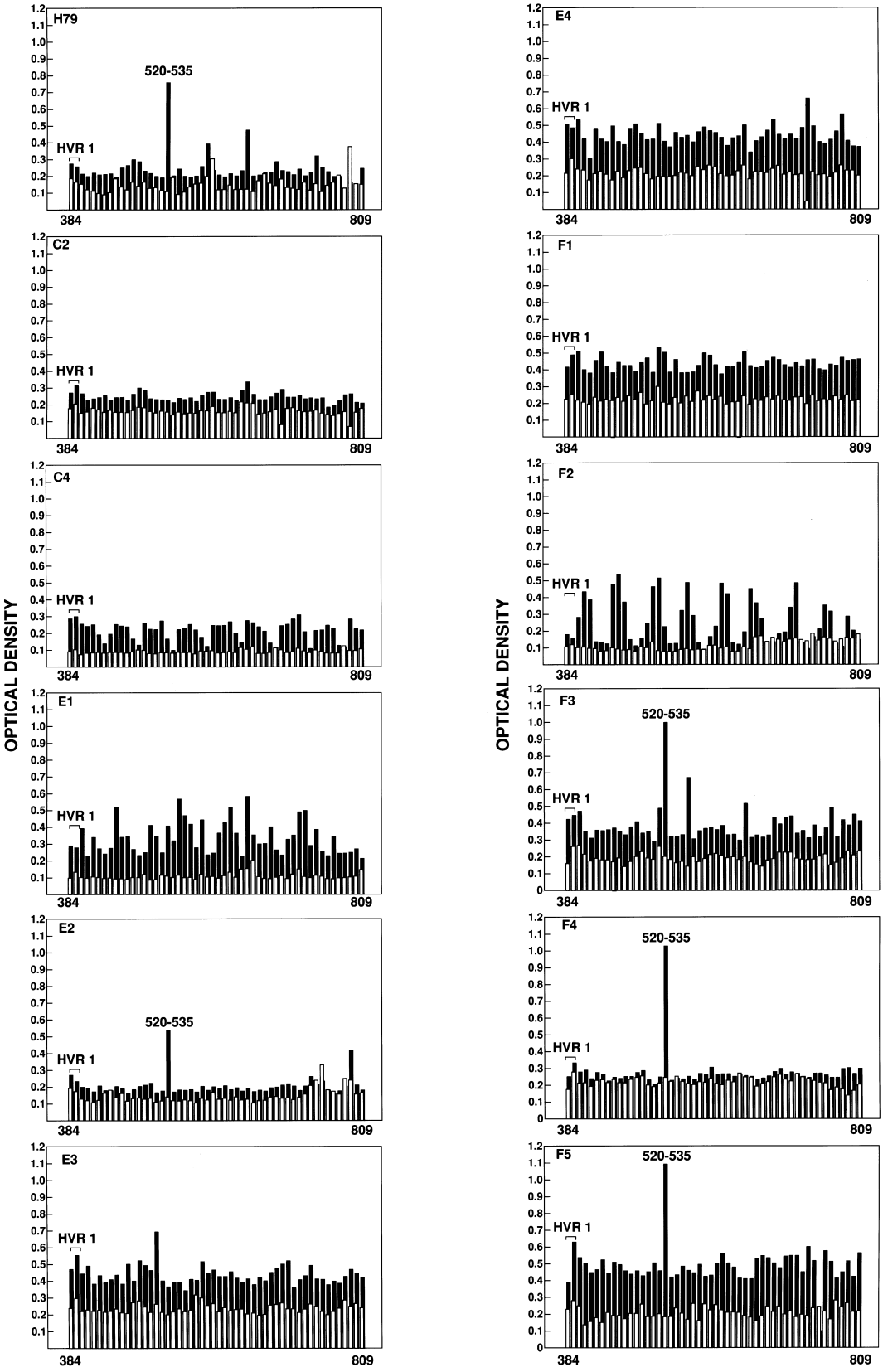

Fig. 6. Mouse antibodies against synthetic peptides of the HCV E2 glycoprotein. Overlapping peptides (16 mer) comprising amino acid positions 384 to 809 were tested in an ELISA with prebleed sera (empty bars) and postvaccination sera (filled bars). Mice are identified by a capital letter, which indicates the vaccination group, and a number from 1 to 5, which coincides with the location of the animals at each time point in Fig. 4. As a reference, plasma samples from patient H (H77 and H79) were tested for linear epitopes. The position of HVR1 and the major reactive epitope described in the text are indicated.

forms [50–52]. We observed that cell surface-expressed HCV E2 consistently elicited higher titers of anti-E2 compared to the intracellular form of E2 when tested in mice. In addition, the majority of animals immunized with the cell surface-expressed form of E2 developed anti-E2 after the first immunization; in contrast, two or three immunizations were necessary to generate anti-E2 in mice immunized with the intracellular form of E2. More importantly, we were able to reproduce these results in a non-human primate model: rhesus macaques immunized with the plasmid encoding the cell surface-expressed E2 developed a much stronger humoral immune response than did those immunized with the plasmid encoding the intracellular form.

It is not known if the surface expression of E2 permits authentic folding of this protein. Heterodimers of E1–E2 are believed to form a functional subunit of HCV virions [9]. Although the ectodomain of viral membrane proteins is often sufficient for oligomerization [53, 54], it was recently shown that the replacement of the E2 transmembrane domain by an anchor signal abolished the formation of E1-E2 complexes [40]. The protein conformation and antigen presentation of the E2 protein is also dependent on its sequence and possibly on its glycosylation. We used an authentic amino acid sequence from an infectious HCV cDNA clone [38]. However, changing the secretory pathway of E2 by targeting the protein to the cell surface might change its glycosylation pattern. Despite these potential obstacles, a conformation-sensitive monoclonal antibody [40] recognized the surface-expressed form of E2 and therefore, it is likely that the E2 on the cell surface was correctly folded.

It was also interesting that a major linear epitope recognized by human plasma H79 and from a significant proportion of HCV infected patients [55] retained its antigenicity in the majority of mice vaccinated with the surface-expressed E2 (via gene gun). Reactivity against such an epitope may, however, not be relevant for vaccine development, since it is found in individuals who are chronically infected with HCV. Neither sera from the immunized mice or macaques nor plasma from patient H recognized peptides encompassing the HVR1. Possible explanation for this included lack of antibodies against this region or titers that were too low to be detected with our assay. Technical reasons such as the length and/or the degree of overlap among adjacent peptides used in the assay have to be taken into consideration; it is noteworthy that plasma H79 did react weakly with a longer peptide encompassing 21 amino acids of the HVR1 [14] (data not shown). Finally, the presence of antibodies against a conformational (and not linear) epitope within the HVR1 can not be excluded.

There was a clear discrepancy between results of assays detecting antibodies to linear versus conformational epitopes. Sera from some mice with high ELISA titers of anti-E2 did not recognize any of the overlapping peptides that represented the E2-p7 protein amino acid sequence, indicating that the majority (if not all) of the antibodies produced were directed against conformational epitopes. Although fewer animals were tested, we observed similar results in macaques. We did not determine whether anti-E2 generated by our DNA vaccine constructs had neutralizing activity because we have not been able to obtain reproducible results with the current in vitro neutralization tests for HCV. It may be more relevant to repeat the experiments in chimpanzees, which can then be challenged with the appropriate virus.

Regarding the differences observed in terms of the route of immunization in mice, other studies have shown a low rate of anti-HCV seroconversion using the intramuscular route for DNA vaccination against HCV [33, 36, 46]. Vaccination with a gene gun delivers DNA into the epidermis, a structure that is particularly rich in antigen-presenting cells (Langerhans cells); this fact might explain the generation of a more potent immune response [30]. These differences may be important when poorly immunogenic proteins are used for vaccination. However, the fact that two mice immunized by intramuscular injection developed high titers of anti-E2 argues against a poor immunogenicity of the construct and points to the possibility of technical difficulties in performing the intramuscular inoculations. Interestingly, a major linear epitope recognized in mice vaccinated by gene gun was not recognized in the two mice from the intramuscular group that developed anti-E2. Although the number of animals was small, these results suggest the possibility of a different qualitative immune response depending on the route of DNA delivery, possibly associated with a different presentation of antigen. Further studies are needed to analyze the quantitative and qualitative differences in the antibody response with respect to the injection route. This is especially important for vaccination of non-human primates, as large amounts of DNA are needed to elicit an immune response by intramuscular injection and other routes of immunization need to be explored. We used the intramuscular route to immunize rhesus macaques because we had no experience in using the gene gun in primates and primarily because of the good results obtained in primates with the intramuscular route in our animal facility [56].

In summary, we have shown that the HCV E2 protein can be targeted to the cell membrane. A plasmid encoding a truncated form of the E2 protein expressed on the cell surface was more immunogenic than one encoding intracellular E2, when tested in mice and macaques. The further development and testing of such DNA vaccine candidates in the chimpanzee model is therefore warranted.

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