In Vivo Electroporation Enhances the Immunogenicity of Hepatitis C Virus Nonstructural 3/4A DNA by Increased Local DNA Uptake, Protein Expression, Inflammation, and Infiltration of CD3+ T Cells

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In Vivo Electroporation Enhances the Immunogenicity of Hepatitis C Virus Nonstructural 3/4A DNA by Increased Local DNA Uptake, Protein Expression, Inflammation, and Infiltration of CD3+ T Cells

Gustaf Ahlén,* Jonas Söderholm,*† Torunn Tjelle,‡ Rune Kjeken,‡ Lars Frelin,* Urban Höglund,§ Pontus Blomberg,¶ Michael Fons,† Iacob Mathiesen,‡ and Matti Sällberg2*

The mechanisms by which in vivo electroporation (EP) improves the potency of i.m. DNA vaccination were characterized by using the hepatitis C virus nonstructural (NS) 3/4A gene. Following a standard i.m. injection of DNA with or without in vivo EP, plasmid levels peaked immediately at the site of injection and decreased by 4 logs the first week. In vivo EP did not promote plasmid persistence and, depending on the dose, the plasmid was cleared or almost cleared after 60 days. In vivo imaging and immunohistochemistry revealed that protein expression was restricted to the injection site despite the detection of significant levels of plasmid in adjacent muscle groups. In vivo EP increased and prolonged NS3/4A protein expression levels as well as an increased infiltration of CD3+ T cells at the injection site. These factors most likely additively contributed to the enhanced and broadened priming of NS3/4A-specific Abs, CD4+ T cells, CD8+ T cells, and γ-IFN production. The primed CD8+ responses were functional in vivo, resulting in elimination of hepatitis C virus NS3/4A-expressing liver cells in transiently transgenic mice. Collectively, the enhanced protein expression and inflammation at the injection site following in vivo EP contributed to the priming of in vivo functional immune responses. These localized effects most likely help to insure that the strength and duration of the responses are maintained when the vaccine is tested in larger animals, including rabbits and humans. Thus, the combined effects mediated by in vivo EP serves as a potent adjuvant for the NS3/4A-based DNA vaccine. The Journal of Immunology, 2007, 179: 4741–4753.

I
It has been shown that sustained control and clearance of hepatitis C virus (HCV) infection is related to an effective immune response, in particular a T cell response targeted to the nonstructural (NS) 3 protein (1–3). Both CD4+ and CD8+ T cells have been found to be of a key importance in the control and clearance of HCV in model systems (4, 5). It has also been shown that viral persistence may be promoted by immunological escape from T cell recognition (6, 7), but the ability of the virus to undergo escape may be limited by the viral fitness (8). Thus, the key roles played by host T cells and the evolutionary pressure placed on the virus to escape these mechanisms suggests that an enhanced HCV-specific T cell response should favor viral clearance.

The NS3 protein is involved in the processing of the HCV precursor polyprotein as well as in the unwinding of the replicating dsRNA (9). These dual enzymatic functions explain, at least in part, the conserved nature of the protein (8, 10). A feature of HCV replication is the generation of dsRNA. HCV infects the hepatocyte through at least two proposed receptors, CD81 and Claudin-1 (9, 11). Infected cells have a number of sensory systems for detecting the presence of dsRNA generated during viral replication that can trigger an antiviral state in the cell (12, 13). The NS3/4A complex can interfere with this signaling by cleaving the adaptor molecules TRIF (14) and Cardif/IPS-1/VISA/MAVS (15). Thus, the NS3/4A complex is a conserved complex that performs key functions in the viral replication cycle. In addition, the immune response to the NS3 protein in HCV-infected patients who clear the infection has been well characterized (1). These observations support the development of plasmid-based therapeutic vaccines encoding the NS3/4A protein complex.

Current therapies for chronic infections caused by HCV genotype 1 strains, are largely unsuccessful and represent a major unmet need for a therapeutic vaccine (16). New therapies with protease inhibitors targeting the NS3 protease seem promising, although problems with resistance mutations and toxicity have already been reported (17, 18). An NS3/4A-based therapeutic vaccine can, if safe and effective, also be considered as a first line therapy in treating naive subjects and as an add-on to existing combination therapies for the treatment of patients who have failed to sustain a response to conventional therapy. In addition, the vaccine may also prevent development of chronic infection in acutely infected patients or be used as a prophylactic vaccine in naive
individuals, because studies in chimpanzees using a vaccine also including NS3 suggested promising prophylaxis (19).

The theory of therapeutic vaccination is that the hepatic infection may offer a less than optimal priming of T cells (20). Thus, by activating T cells outside the liver via vaccination, one may allow for the complementing or reshaping of the existing T cell repertoire. Although the liver may not always act as a tolerogen (21), inhibiting molecules such as the ligand to programmed death-1 are expressed in the liver and can actively down-regulate activated T cells (22, 23). Thus, the therapeutic vaccination approach needs to be tested in various infectious or transgenic animal models or in humans to have a full understanding of the potential mechanisms.

The present NS3/4A plasmid-based vaccine has been tested in various mouse models and has been found to induce a functional in vivo immune response that is able to enter the liver and clear liver cells that express the HCV NS3/4A protein complex (8, 24–26). However, a major obstacle regarding DNA vaccines is that the immunogenicity drastically decreases when moving to larger animals (27). In vivo electroporation (EP) has been used in humans and large animals to enhance the uptake of both cancer drugs and DNA plasmids (28–31). In vivo EP administered immediately after i.m. injection of DNA plasmid transiently opens pores in the myocyte membranes, and the plasmid is effectively taken up in the nucleus and expressed (28, 32). A consistent finding is that in vivo EP mainly enhances the DNA uptake and the expression of the gene of interest (28, 32). We now characterize further mechanisms by which in vivo EP enhances the immunogenicity of DNA vaccines by using a highly immunogenic, codon-optimized NS3/4A (coNS3/4A) plasmid (25). We show that in vivo EP enhances the immunogenicity of coNS3/4A by both increasing protein expression levels and the duration of expression and by enhancing the infiltration of CD3+ T cells and a local inflammatory response at the site of injection.

Materials and Methods

Animals

Male and female C57BL/6 mice were bred and housed at the Unit for Embryology and Genetics at Karolinska Institutet, Karolinska University Hospital Huddinge, Sweden. The animals were caged at five mice per cage and fed a commercial diet (RM3 (p) PL IRR diet; Special Diet Service) with free access to food and water. All animals were at least 6 wk of age before start of the experiment. New Zealand White rabbits weighing 2.5–3.5 kg, were purchased from commercial vendors and were kept at Visio-\n
Embryology and Genetics at Karolinska Institutet, Karolinska University Hospital Huddinge, Sweden. The animals were caged at five mice per cage and fed a commercial diet (RM3 (p) PL IRR diet; Special Diet Service) with free access to food and water. All animals were at least 6 wk of age before start of the experiment. New Zealand White rabbits weighing 2.5–3.5 kg, were purchased from commercial vendors and were kept at Visio-\n
Plasmid DNA

The SV40-luciferase plasmid (pGL4.13-[Luc2-SV40]; Promega) was produced in-house by standard technologies. The coNS3/4A plasmid (25) was produced using a Good Manufacturing Practice regulations at Vecura AB.

Immunizations

The coNS3/4A DNA vaccine was administered by a single i.m. injection (0.05 ml in mice and 0.3 ml in rabbits) with a 27-gauge needle into the right tibialis anterior (TA) muscle. Doses ranged from 0.5 to 50 μg of DNA in mice and from 70 to 700 μg of DNA in rabbits. Immediately after the injection in vivo, EP was applied using the MedPulser DNA delivery system (Inovio Biomedical) with a 0.5-cm needle array set to deliver two 60-ms pulses of 246 V/cm to the injection site. One two-needle electrode tip (in mice) or one four-needle electrode tip (in rabbits) was used per injection and per animal. The procedure was repeated up to three times in mice and up to five times in rabbits at monthly intervals.

Pharmacokinetics and biodistribution of plasmid DNA and gene expression

The early pharmacokinetics and biodistribution of plasmid DNA and gene expression were first studied in mice. Groups of mice were immunized i.m. in the right TA with either 50 μg of pGL4.13-[Luc2-SV40] plasmid or 50 μg of the coNS3/4A plasmid. The presence of reporter gene expression was determined by bioluminescence in anesthetized mice using the In Vivo Imaging System 100 (Xenogen). The luciferin (Xenogen) substrate was injected and 5–10 min later the mice were analyzed and images and assessment of emitted light were performed with Living Image software (version 2.50; Xenogen).

In parallel groups of mice, the biodistribution of the coNS3/4A plasmid in the striated muscle groups of both hind legs was determined using a quantitative PCR (qPCR). Mice were immunized with 50 μg of coNS3/4A DNA in the TA with or without, an immediate in vivo EP treatment using the Med-\n
Pulser DNA delivery system with a two-needle array giving two 60-s pulses of 246 V/cm. Muscle tissues were removed and total DNA was extracted using an automated procedure for DNA extraction (Magna pure LC; Roche). The levels of plasmid was then quantified by using an automated qPCR procedure (TaqMan; Applied Biosystems) according to the manufacturer’s instructions with the following primers and probe: 5′-AGCAGAGCTCTTGCGGAACACATTCA-3′ (forward primer), 5′-GTCGCCGGCGTGACAGGTTGATAGTGCA-3′ (reverse primer), and 6-FAM-AACCCAC-dabcyl-dT-GCTT ACTGCCTTAAGATTAACGTA-3′-phosphate (probe). Oligonucleotides were synthesized by Scandinavian Gene Synthesis. The qPCR was optimized using standard protocols and had a sensitivity of nine plasmid copies per milligram of tissue. Using a standard dilution of the plasmid, the number of plasmid copies per milligram of tissue could be calculated.

The whole body pharmacokinetics and biodistribution of the coNS3/4A DNA plasmid delivered by in vivo EP was determined in a total of 30 New Zealand White rabbits divided into five groups of three males and three females in each group. All animals received 70 μg of coNS3/4A DNA in 0.3 ml of saline administered by i.m. injection into the right TA muscle. Immediately after the injection, in vivo EP with two 60-ms pulses of 246 V/cm were applied with a four-needle array using the MedPulser DNA delivery system. At days 1, 3, 7, 14, and 28 after the vaccination, 2 ml of blood was obtained from the ear artery, after which the animals were euthanized. Samples from the liver, kidney (left and right), spleen, heart, ovaries/testicles, ileum, mesenteric lymph nodes, brain, and right and left TA muscles were dissected and divided into two parts. 0.5–1 g of tissue in each part. The tissue samples were immediately frozen on dry ice. Blood and tissue samples were stored at ~70°C until analysis. The presence of the coNS3/4A plasmid DNA was quantified by qPCR in blood, serum and all organ tissues, which were collected as described above.

Right and left mouse TA muscles were analyzed for the infiltration of CD3+ T cells and NS3 protein expression by immunohistochemistry. TA muscles were fixed in a buffered 4% formaldehyde solution, dehydrated, and embedded in paraffin. The embedded tissues were sectioned in 4- to 6-μm sections. The sections were mounted onto glass slides and stained with H&E, an Ab to CD3 (clone A0452; DakoCytomation), or sera from a coNS3/4A DNA-immunized rabbit as previously described (24).

Expression of NS3 protein in the right and left TA muscles for rabbit was detected using an immunoperoxidase-Western blot procedure described previously (24). The presence of NS3 protein was detected as visible bands after staining with an insoluble substrate. Blots were photographed with a GeneGenius gel documentation camera (Syngene) and band intensities were determined by densitometry using Gene Tools. Each band was normalized according to the band intensity of the positive control. Intensities of <20% of the positive control represented by baby hamster kidney (BHK) cells transiently transfected by coNS3/4A plasmid were considered as negative for NS3 protein.

Acute and repeated toxicological studies in rabbits

In the acute toxicological study 24 rabbits (New Zealand White) were used consisting of 12 females and 12 males divided into three groups of four females and four males per group. coNS3/4A DNA (70 or 700 μg) in saline or saline alone was administered by a single i.m. injection (0.3 ml) as described above. Also, the body temperature was measured in all animals on two occasions before the study and at 0, 4, and 24 h after vaccination followed by repeated measurements every 24 h for 7 days. Seven days after the vaccination, blood was obtained from the ear artery and collected in potassium EDTA tubes. The serum tubes before euthanasia were collected for analysis of white blood cells, hemoglobin, hematocrit, and platelets (Quintiles). The serum samples were tested for aspartate aminotransferase, alanine aminotransferase, bilirubin, albumin, creatinine, lactate dehydrogenase, γ-glutamyl transferase, potassium, sodium, and glucose (Quintiles). Also, the liver, kidney, heart, brain, spleen, ovaries/testicles, ileum, mesenteric lymph nodes, brain, and right and left TA muscles were collected for histological evaluation. The organs were fixed in a buffered 4% formaldehyde solution, dehydrated, and embedded in paraffin. The embedded tissues were sectioned in 4- to 6-μm sections. The sections were
mounted onto glass slides and stained with H&E. All sections were evaluated for histopathological changes that could be related to the treatment by an experienced pathologist.

In the repeated toxicological study, groups of eight male or female rabbits were immunized as indicated above at a total of five times at monthly intervals. One month after the last vaccination, arterial blood was obtained before euthanizing. The blood samples, serum samples, and organs were analyzed the same way as in the acute toxicological study.

**Determination of in vivo clearance of HCV NS3/4A expressing liver cells**

Four groups of mice were vaccinated once in the right TA muscle on wk 0 as described with 0.5, 5, or 50 μg of DNA. In one group the mice were immunized with NS3/4A cT lymphocyte peptide, produced as described previously (25), and in one group the mice received an i.m. injection of normal saline only. Also, one group of mice that received 50 μg of coNS3/4A-DNA i.m. did not undergo in vivo EP. Two weeks after the vaccination all mice were given a hydrodynamic injection of the coNS3/4A plasmid, exactly as described previously (24). By this treatment the liver cells (mainly hepatocytes) are permeabilized to take up the plasmid and to express the NS3/4A complex (24). The presence of NS3/4A expression was determined 24 h later by immunoprecipitation-Western blot as described previously (24). Blots were photographed with a GeneGenius gel documentation camera (SynGene) and band intensities were determined by densitometry using GeneTools (SynGene). Each band was normalized according to the band intensity of the positive control (100%; lysate from NS3/4A-transfected BHK cells). Intensities of <20% of the positive control were considered as negative for NS3 protein. The number of NS3/4A-positive livers was then compared between the groups.

**Detection of Abs to NS3**

Detection of mouse and rabbit Abs to NS3 by enzyme immunoassay was performed essentially as described (25, 26). Ab titers were determined as the last serum dilution giving an OD at 405 nm of three times the OD at the same dilution of a nonimmunized animal serum.

**Detection of lytic CTLs and γ-IFN-producing CTLs and Th cells to NS3**

Lytic CTLs in immunized mice were measured in individual mouse spleens using a standard 4-h 31P-uptake assay using NS3 peptide-loaded RMA-S target cells as described previously (25). The presence of γ-IFN-producing CTLs and Th cells to NS3 were detected in pooled splenocyte or lymph node cultures by a commercially available ELISPOT assay as described (8). In brief, the spleens and lymph nodes from each group were pooled and immediately tested for the presence of HCV NS3-specific T cells. The ability of NS3-specific Th and CTLs to produce γ-IFN recalled by a recombinant NS3 protein (nNS3; kindly provided by Dr. D. L. Peterson, Commonwealth University, Richmond, VA) and the H-2Kk-restricted NS3-derived CTL peptide produced as described previously (33), was determined by using a commercially available ELISPOT assay. The number of γ-IFN-producing cells or spots was determined at each concentration of protein or peptide and the results have been given as the number of γ-IFN-producing cells per 106 cells. A mean number of γ-IFN-producing cells of <50 per 106 cells were considered as negative.

Proliferative responses to NS3 were determined in rabbit whole blood. A total of 4 ml of whole blood was obtained from the ear vein of each rabbit immediately before the first vaccination and 2 wk after each vaccination and collected in heparin tubes, and plasma and PBMCs were isolated by gradient centrifugation. Plasma was stored at −80°C until the analysis of NS3-specific Abs by enzyme immunoassay. PBMCs were immediately assayed for in vitro proliferative recall responses using a standard 96-h proliferation assay (34). In brief, microplates were seeded with 200,000 cells per well and the cells were incubated with medium alone (sample to negative (S/N) ratio). Groups were compared by the mean S/N ratios at each time point.

**Statistical analysis**

Statistical comparisons were performed using the StatView 5.0 (3/20/98; Power PC version; SAS Institute) and Excel-Mac (version 11.2.5; Microsoft) software packages for Macintosh. Parametrical data were compared using Student’s t test (StatView and Excel), and nonparametrical data were compared by using the Mann-Whitney U test (StatView). Frequencies were compared by using Fisher’s exact test (StatView).

**Results**

**Biodistribution of plasmid and gene expression after i.m. injection in the mouse TA muscle**

Distribution of the plasmid DNA in muscle tissues was determined in mice injected i.m. with 50 μg of a firefly luciferase reporter plasmid without in vivo EP or coNS3/4A DNA with or without in vivo EP. Using the luciferase reporter plasmid, expression was monitored using in vivo imaging and the distribution of the coNS3/4A-DNA was monitored by qPCR in tissues harvested at 0.5, 24, 48, and 72 h after injection. Expression of luciferase reporter was analyzed from 3 h to 20 days, and expression was only detected at the site of injection (Fig. 1). Consistent with this were the highest plasmid levels detected at the site of injection with or without in vivo EP, but with in vivo EP the levels were 1 or 2 log10 higher (Fig. 1). Surprisingly high levels of plasmid were recovered in both thigh muscles and in the contralateral TA muscle at 30 min after injection, regardless of whether in vivo EP was administered (Fig. 1). The DNA levels in the noninjected leg were comparable regardless of the use of in vivo EP (Fig. 1). The DNA levels in the quadriceps muscles seemed to decrease at a more rapid pace than the TA muscles, and some were almost negative for DNA already at 72 h after injection (Fig. 1). Again, as determined by the in vivo imaging, none of the other tissues had detectable expression of the luciferase reporter at any time point (Fig. 1). Thus, it seems that DNA levels at the site of injection decrease by up to 1 log per day, whereas outside the injection site the plasmid can be cleared by up to 3 logs per day. This very rapid elimination of the plasmid in the other muscle tissues suggests that the plasmid is rapidly degraded when not expressed in the nucleus. Also, we could only detect moderate levels of DNA (~5 × 104 copies/ml) in the serum of a single mouse at 30 min after immunization (data not shown), suggesting that plasma clearance is extremely rapid. Interestingly, the only tested muscle that had levels close to the site of injection was the thigh muscle of the injected leg (Fig. 1). This suggests that, at least in the mouse, the in vivo EP may extend beyond the site of injection.

To test whether in vivo EP promoted long-term persistence of plasmid DNA at the site of injection, groups of six mice were given two monthly injections with 0, 0.5, 5, or 50 μg of coNS3/4A in the TA followed by in vivo EP or 50 μg of coNS3/4A DNA without in vivo EP. Sixty days after the last injection, all of the mice were sacrificed and the treated TA muscles were harvested and analyzed for the presence of plasmid DNA by qPCR. Plasmid DNA was only recovered in up to half of the muscles receiving the highest dose of DNA, independent of in vivo EP. In the in vivo EP-treated group the plasmid copy numbers were 52, 55, and 72 plasmid copies/μg total DNA, and in the group not receiving in vivo EP the corresponding figures were 47 and 378 plasmid copies/μg total DNA. Thus, there is no evidence that in vivo EP promotes persistence of the coNS3/4A plasmid.

We next studied the effects of in vivo EP in mice on NS3/4A expression and the local inflammatory response at the site of injection and in the contralateral TA muscle. An inflammatory response or NS3 expression was never detected in the noninjected contralateral TA muscle (data not shown). At the site of injection, the needle and physiological saline alone caused minimal tissue damage detectable at day 3 (Fig. 2). The injection of 50 μg of coNS3/4A DNA caused local inflammation, regeneration, and fibrosis at days 3 and 7 that extended to <50% of the section area (Fig. 2). NS3 expression, as detected by immunohistochemistry in the adjoining sections, peaked at day 3 and was almost gone at day
The expression of NS3 had disappeared, and the local inflammatory response had almost completely resolved at day 14 (Fig. 2). The combination of injecting coNS3/4A-DNA with in vivo EP enhanced the local inflammation, regeneration, and fibrosis, as well as the number of NS3-expressing muscle fibers (Fig. 2). The protein expression peaked at days 3–7, was much decreased by day 14, and the local damage was resolving by day 14 (Fig. 2). Importantly, the inflammatory infiltrate in the injected muscle contained a large proportion of CD3⁷ T cells (Fig. 2). The extent of the infiltration CD3⁷ T cells at the site of injection was compared between the three groups on days 7 and 14 (Fig. 2). This revealed a statistically significant increase in infiltrating CD3⁷ T cells on both occasions when using in vivo EP. Thus, in vivo EP increases and prolongs Ag expression as well as local inflammation and the infiltration of CD3⁷ T cells and, subsequently, also the tissue damage. These factors may additively assist in the priming of immune responses using in vivo EP.

The local tissue damage was also examined in the groups of mice that were studied for the long-term persistence of plasmid DNA at 60 days after a second monthly injection. At the highest
dose, with or without in vivo EP, the histology revealed none to moderate degeneration/necrosis and inflammation, none to mild fibrosis, and a generally moderate regeneration. Consistent with the complete or almost complete clearance of the injected plasmid, this represented the overall histological picture at the site of injection, with ongoing regeneration at a late but not fully mature stage. Thus, repeated treatments with high doses of coNS3/4A DNA and in vivo EP did not result in maintained tissue damage.

Biodistribution and gene expression after in vivo EP-enhanced i.m. injection of coNS3/4A DNA in rabbits

A detailed analysis of the biodistribution and clearance after a single injection of 70 μg of coNS3/4A DNA was performed in rabbits. As expected, the highest plasmid copy numbers were detected at the site of injection within 24 h, after which the levels rapidly decreased during the first week (Fig. 3 and data not shown). Consistent with the mouse experiments, we found that DNA could also be recovered from the contralateral TA muscles within the first 24 h, but at much lower levels (data not shown). After 28 days, low levels of DNA were found only in TA muscles, suggesting an ongoing clearance (data not shown). One TA muscle had >10^5 DNA copies/mg tissue at day 28 (Fig. 3 and data not shown). Very low plasmid copies numbers of <10^2 copies/mg were occasionally found in the right kidney, contralateral TA muscle, ileum, and a single brain (data not shown). Thus, most of the injected plasmid was recovered from the site of injection and at lower levels in the contralateral TA muscle. Plasmid DNA was only rarely, and at random time points, recovered from other organs. The vast majority (>99%) of the injected DNA seems to...
Pharmacokinetics of NS3/4A DNA and protein at the injection site

![Graph showing the distribution of NS3 expression](image)

<table>
<thead>
<tr>
<th>Vaccine Dose</th>
<th>Degeneration/necrosis</th>
<th>Inflammation</th>
<th>Fibrosis</th>
<th>Regeneration</th>
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<td>Four weeks/five injections</td>
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<td>700 µg</td>
<td>12/16 (++++)</td>
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<td>13/16 (+)</td>
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</tr>
</tbody>
</table>

^ Intensity of changes: +++, Very severe and extensive; ++, severe; +, moderate; +, mild.
^ p < 0.01, Fisher’s exact test and χ² test.
^ p < 0.05, χ² test only.

have been cleared during the first week after injection and the rest appears to have been cleared sometime between 14 and 28 days.

Again consistent with the findings from mice, we found that NS3 protein expression was detectable only at the site of injection (Fig. 3 and data not shown). NS3 protein expression levels peaked after 3 days and became undetectable by day 28 (Fig. 3). As in the mice, the contralateral TA was consistently negative for protein expression (data not shown). Thus, in both mouse and rabbit the injected DNA distributes in skeletal muscles outside the site of injection, whereas protein expression is consistently restricted to the injection site.

Acute and repeat toxicological studies in rabbits

In the acute toxicological study the rabbits were given a single dose of the coNS3/4A plasmid or normal saline alone administered using in vivo EP, and all animals were sacrificed 7 days later. Body temperatures lower than the lower 95% confidence interval of untreated animals were observed at 4 h after injections; this may be explained by the fact that the animals were anesthetized during injection, which normally results in a loss of temperature control (data not shown). Hematology showed no statistically significant differences between the treatment groups (data not shown). Clinical chemistry showed, except for glucose levels in females (p = 0.0097; saline vs 70 µg of DNA) and lactate dehydrogenase levels in males (p = 0.0376; saline vs 70 µg of DNA), no statistically significant differences between the treatment groups.

Histopathology showed no lesions consistent with an effect of the vaccine in tissues other than the injection site when all animals were sacrificed 1 wk after the injection (Table I). Animals from all three treatment groups showed, with a few exceptions, some degree of degeneration/necrosis, inflammation, fibrosis, or regeneration at the injection site, clearly showing that the in vivo EP alone causes a local inflammatory response and tissue damage (Table I). This is fully consistent with the findings from the murine experiments. The most pronounced changes were observed in the muscles receiving DNA, suggesting that the plasmid DNA, the NS3 protein, or both add to the inflammation (Table I). Importantly, we have shown that after 2 wk the plasmid has been almost completely eliminated and NS3 protein expression levels are low (Fig. 3 and data not shown). As we observed in mice, these changes are most likely triggered by the treatment, the Ag, and the Ag-specific immune responses within the first week after immunization. Thus, the presence of the DNA and the NS3/4A protein together with the in vivo EP enhances the local inflammation, most likely a factor helping to explain the immunogenicity of the combination.

### Table I. Grading of histological changes observed at the injection site at 1 wk after a single injection and 4 wk after five injections of 0, 70, or 700 µg of coNS3/4A followed by in vivo EP

<table>
<thead>
<tr>
<th>Vaccine Dose</th>
<th>Degeneration/necrosis</th>
<th>Inflammation</th>
<th>Fibrosis</th>
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</tr>
</tbody>
</table>

^ Intensity of changes: +++, Very severe and extensive; ++, severe; +, moderate; +, mild.
^ p < 0.01, Fisher’s exact test and χ² test.
^ p < 0.05, χ² test only.
In the repeated toxicological study, body weight changes were similar between groups within gender throughout the study, which is an indicator that most animals were in good health during the experimental period. The hematology data showed no statistically significant differences between treatment groups. Clinical chemistry showed statistically significant differences only in glucose, lactate dehydrogenase, and potassium among females (data not shown). However, the actual differences within each of these parameters were small and most likely of no biological significance.

No lesions consistent with toxicity were observed in the organs subjected to histopathological examination, except from local effects observed at the site of injection. In the injected muscle some degree of degeneration/necrosis, inflammation, fibrosis, or regeneration was observed in all groups of animals (Table I). Again, it was evident that in vivo EP per se caused a histological reaction that was aggravated by the vaccine, because the most pronounced changes were found in animals treated with the 700-μg DNA dose and EP. Importantly, significantly fewer of the animals that only received buffer and in vivo EP displayed signs of inflammation at the site of injection as compared with the groups receiving the coNS3/4A-DNA (Table I). Thus, the in vivo EP itself causes minor tissue damage, but the presence of the DNA and/or the expressed NS3/4A protein causes an increased inflammatory response at the site of injection. This is fully consistent with the observations from the immunized mice and the previous observations using in vivo EP (35) and is to be expected with respect to vaccination. Finally, it is important to note that the local damage at the site of injection was more pronounced at 1 wk after a single injection as compared with a month after the fifth injection at the same site (Table I). This suggests that repeated treatment of the same site does not aggravate the local tissue damage.

Detection of Abs to NS3 after coNS3/4A-DNA vaccination in mice

With respect to NS3 Ab levels, a clear dose-response relationship was seen after vaccination with different doses of coNS3/4A-DNA administered with or without in vivo EP (Fig. 4). The major boost effect was seen after the second immunization, whereas after the third injection the boost effect was less pronounced (Fig. 4). No differences in mean Ab levels were seen between the 50- and 5-μg doses when using in vivo EP (Fig. 4). The 0.5-μg dose given with in vivo EP induced the same mean NS3-specific Ab levels as the 50-μg dose delivered without in vivo EP. However, both of these were consistently lower than the 50- and 5-μg dose groups receiving in vivo EP (Fig. 4). In conclusion, in vivo EP makes the coNS3/4A DNA-based immunization ~100-fold more effective.
with respect to Ab responses, supporting the benefits of the adjuvant effects mediated by in vivo EP.

Detection of lytic CTLs to NS3 in mice after coNS3/4A-DNA vaccination

The priming of NS3-specific lytic CTLs in mice was determined by using peptide pulsed RMA-S cells in a standard 51Cr-release assay. The two high-dose groups of 50 and 5 μg of coNS3/4A-DNA delivered by in vivo EP were consistently equally immunogenic (Fig. 5). Two weeks after the first priming of the CTL responses there were no differences between any of the dose groups (Fig. 5). However, after the second dose all of the dose groups receiving in vivo EP had higher CTL activity as compared with the group not receiving in vivo EP (Fig. 5). After the third injection, the CTL responses in the 0.5-μg group receiving EP and the 50-μg group not receiving EP seemed to weaken (Fig. 5). Thus, the coNS3/4A-DNA delivered by in vivo EP clearly primed sustained lytic CTL responses. Interestingly, these data suggest that repeated suboptimal delivery or dosing did not prime sustained lytic CTL responses.

Detection of γ-IFN-producing CTLs and Th cells to NS3 after coNS3/4A-DNA vaccination

The priming of γ-IFN-producing NS3-specific Th cells and CTLs was determined by ELISPOT. With respect to the γ-IFN-producing Th and CTLs, the results were consistent with observations from both NS3 Abs and lytic CTLs. After the first dose, the number of γ-IFN-producing splenic Th and CTLs were comparable in all groups, whereas a weak response in lymph nodes was only seen in the highest dose group receiving in vivo EP (Fig. 6). After the second dose, the three groups receiving in vivo EP had comparable numbers of γ-IFN-producing Th cells and CTLs in the spleen and lymph nodes (Fig. 6). Importantly, in the group not receiving in vivo EP the splenic responses were lower and there were still no...
responses detectable in the lymph nodes (Fig. 6). After the third dose the two highest dose groups had comparable responses in the spleen with >1000 spot forming cells (SFCs) per 10⁶ cells recalled at the highest dilutions of the CTL peptide or rNS3. In the lymph nodes, both groups had >500 SFCs per 10⁶ cells recalled at the highest dilutions of the CTL peptide or rNS3 (Fig. 6). In contrast, the group receiving the 50-μg dose without in vivo EP had generally lower CTLs and rNS3-recalled SFCs in the spleen and very low T cell responses in the lymph nodes (Fig. 6). Even a 100-fold lower DNA dose primed γ-IFN-producing Th cells and CTLs more efficiently when delivered with in vivo EP (Fig. 6). Thus, it is clear that the adjuvant effects of in vivo EP improved the priming of γ-IFN-producing NS3-specific Th cells and CTLs in both spleen and lymph nodes. Importantly, these differences are maintained even after a third dose, suggesting that an increased number of doses cannot substitute for the adjuvant effect provided by in vivo EP. Interestingly, suboptimal dosing resulted in slowly increasing numbers of γ-IFN-producing T cells but waning lytic activity.

Vaccine-mediated clearance of NS3 protein expressing murine liver cells in vivo

With respect to hepatotropic infections, it is of importance to determine whether the vaccine-primed immune responses can enter the liver and clear “infected” or Ag-expressing hepatocytes. Clearance of HCV NS3/4A-expressing hepatocytes was therefore tested in vaccinated mice using the hydrodynamic injection technology (36). In brief, if a functional CTL response has been primed by vaccination, these cells will enter the liver and clear HCV NS3/4A-expressing liver cells (24). We could, by this approach, show that among the unvaccinated mice all livers were positive for NS3/4A-expression at 72 h after the hydrodynamic injection as determined by immunohistochemistry and immunoprecipitation-Western blot (Fig. 7). In the groups receiving 50, 5, or 0.5 μg of coNS3/4A DNA with or without in vivo EP, all livers were negative for NS3/4A-expression at 72 h. Also, in a group receiving 2 μg of coNS3/4A-DNA transdermally by use of the gene gun, all except one was negative for hepatic NS3/4A expression (Fig. 7). All vaccinated groups had significantly lower frequencies of NS3/4A-expressing livers as compared with the group not receiving vaccination (Fig. 7; p < 0.05, Fisher’s exact test). Thus, a single vaccination with coNS3/4A DNA primes detectable NS3/4A-specific CTLs that are functional in vivo in that the CTLs enter the liver and clear HCV Ag-expressing liver cells. This is a mechanism of action that is desired in a therapeutic vaccine against HCV.

Immunogenicity of in vivo EP assisted coNS3/4A DNA vaccination in rabbits

In the rabbits receiving five vaccinations with the coNS3/4A DNA we also determined the NS3-specific Ab levels and proliferative
obtained in rabbits receiving an absolute dose of 70 μg of DNA, immediately followed by in vivo EP. Each symbol indicates a dose of coNS3/4A-DNA administered with in vivo EP activated NS3-specific, γ-IFN-producing Th cells and CTLs that enter the infected liver and help the host gain control over the infection. Thus, from a vaccine point of view the activation of NS3-specific Abs is most likely not essential, albeit as shown herein the activation of NS3 Abs may be a good readout of how immunogenic a vaccine is in an naive host. So how should the vaccine be delivered to obtain these responses? First, the vaccine should induce endogenous expression of immunologically relevant components, because this ensures a priming of both Th cells and CTLs (27). Next, the immunization should be as immunogenic as possible because it should prime or enhance an immune response already existing in an infected individual. Thus, the vaccination schedule most likely should include some type of adjuvant that can prime these responses in the setting of a chronic infection. We therefore evaluated the overall effects of delivering the HCV coNS3/4A DNA by using in vivo electroporation, a delivery system that indeed acts as a strong adjuvant in several ways.

We have recently shown that both the inclusion of the NS4A cofactor and codon optimization improved the intrinsic immunogenicity of the NS3 gene by increasing protein persistence and/or gene expression (25, 26). When using this gene we immediately noted that the immunogenicity of the coNS3/4A-DNA vaccine was greatly enhanced when delivered by in vivo EP. Although a 100-fold higher concentration was used without in vivo EP, the 0.5-μg dose of coNS3/4A-DNA administered with in vivo EP activated fully comparable and sometimes even better immune responses. This supports the notion that the efficacy of coNS3/4A-DNA is greatly enhanced when combined with in vivo EP, which is consistent with observations using other Ags (29, 48, 49).

So which mechanisms could we identify as participating in the adjuvant effect of in vivo EP? Two factors play an obvious role: the increased uptake of plasmid DNA and the increased protein expression levels that increase the overall amount of Ag available for the
priming of the immune response. However, two additional factors are most likely of an equal importance: the local tissue damage and the recruitment of muscle-infiltrating CD3\(^+\) cells. Thus, mechanistically the highly localized tissue damage and protein expression results in a potent inflammatory response that most likely assists in the priming and recruitment of CD3\(^+\) cells. It cannot be excluded that some of the priming may even take place in the muscle tissue if the appropriate costimulatory molecules are present, either directly or through cross-presentation. This should be further studied in detail.

In vivo EP improved all tested branches of the NS3-specific immune response. Importantly, the priming of persisting lytic and γ-IFN-producing Th cells and CTLs was clearly improved by in vivo EP, again fully consistent with previous reports (19, 48). It was recently shown that the number of γ-IFN SFCs found in the spleen when using a HCV NS3-NS5 DNA vaccine was greatly enhanced by in vivo EP (19). However, we could now show that a major effect of in vivo EP-supported vaccination was the presence of HCV-specific SFCs in both the spleen and the lymph nodes. For example, three 50-μg doses of coNS3/4A DNA primed γ-IFN-producing Th cells CTLs in both the spleen and the lymph nodes, reaching precursor frequencies of >1000 SFCs per million cells in both compartments. The same dose given without in vivo EP only primed such responses, albeit weaker, in the spleen. Consistent with this result, we found that the lytic CTL responses in the spleen were stronger and were maintained longer after in vivo EP. In the absence of in vivo EP, the lytic responses waned with time even if the same dose was administered up to three times. Taken together, this suggests that to ensure the maintenance of CTL activity a sufficient level of immune stimulation needs to be repeated, whereas repeated insufficient priming leads to waning responses. This observation needs to be explored further because this may have implications for the correct priming of memory T cells.

It is also of importance to address the in vivo functionality of the primed immune responses. We therefore designed experiments to address whether the primed immune responses could enter the liver and clear transiently transgenic hepatocytes in vivo (24). Mice were immunized once and 2 wk later they received a hydrodynamic injection of coNS3/4A DNA to generate NS3/4A-expressing hepatocytes available for immune-mediated clearance. This revealed that a single vaccination with coNS3/4A-DNA, at doses ranging from 0.5 to 50 μg, given i.m. in combination with in vivo EP primed NS3/4A-specific CTLs that were functional in vivo. Thus, the vaccine-primed CTLs entered the liver and cleared liver cells expressing HCV AGs.

The coNS3/4A DNA delivered by in vivo EP was found to be highly immunogenic, and because the vaccine is intended for human use we therefore determined the safety profile of the combined vaccine. These studies included the pharmacokinetics and biodistribution of the plasmid and the expressed protein, as well as toxicity studies in mice and rabbits. A general aim is that a DNA vaccine should be taken up and expressed at the desired site and that the plasmid DNA levels should gradually decrease. Several studies have found that this is indeed the case (50). In addition, although integration of the plasmid into the host genome may occur, this is a very rare event; probably even less common than naturally occurring gene-inactivating mutations (51).

We could show that an i.m. injection of plasmid results in highly localized uptake and expression of the injected DNA regardless of whether in vivo EP was administered or not. However, in vivo EP greatly enhanced DNA uptake, protein expression levels, the degree of local inflammation, and, as already mentioned, the strength of the primed immune responses. In both mice and rabbits the peak copy numbers of the plasmid were consistently detected at the site of injection immediately after injection. In both mice and rabbits most plasmid was cleared within the first 3–7 days, paralleling findings using other plasmids (52, 53). This is consistent with previous reports suggesting a very short half-life for free plasmid DNA, which most likely is cleared through the first passage in the liver (50). A minor surprise was that significant amounts of plasmid could also be detected in the noninjected contralateral TA muscle and other surrounding muscles in mice or rabbits. However, by using a luciferase reporter gene or detection of the NS3 protein by Western blotting we were only able to detect expression at the site of injection. Thus, the lower DNA levels at the contralateral site or surrounding muscles did not seem to be sufficient to be taken up and produce detectable protein expression. Consistent with the targeting of the plasmid and protein expression to the site of injection, we did not find evidence of the distribution of plasmid throughout the body. Only low levels of plasmid were recovered from a few organs at random time points. Thus, most plasmid seems to be cleared from the injection site during the first weak after injection and the rest appears to be almost completely cleared form the body within 14–28 days. As we could show, when plasmid was administered to mice by two monthly injections at doses of 0.02 or 0.2 mg per kilogram of body weight using in vivo EP, the plasmid was cleared at 60 days after the last injection as determined by qPCR. Also, at doses of 2 mg per kilogram of body weight at most 50% of the mice had very low levels of persisting plasmid at the site of injection regardless of whether in vivo EP had been used. This supports the safety of the combination and is consistent with other reports showing that most of the plasmid has generally disappeared within 60 days from injection (53). In addition, these data are also supported by a recent study showed that in vivo EP even reduced the persistence of the plasmid and was not associated with high m.w. DNA (54). This may be explained by the supposition that integration is most probable when high plasmid copy numbers are present in a cell. However, at the same time this cell will have a high expression of the gene of interest, which makes the cell a perfect target for the primed immune response. Thus, the risk for a surviving cell with integrated plasmid DNA is most likely very low when in vivo EP is used.

The results from a toxicological examination was fully consistent with the biodistribution of the plasmid and the protein, because the only pathology detected was located at the site of injection and protein expression. In addition, in a newly developed transgenic mouse with hepatic expression of NS3/4A we could not detect any spontaneously appearing histological changes in the liver or other signs of liver disease (55). Importantly, it is clear that the in vivo EP itself did cause local tissue damage, suggesting that the treatment has two effects: increasing the uptake of the plasmid DNA and protein expression and acting as an adjuvant at the site of injection. This most likely helps to explain the efficiency of in vivo EP. Again, the plasmid copies detected in the contralateral control muscles neither caused protein expression nor resulted in a local inflammation or histological changes. Thus, even in the cases when the plasmid is detected outside the site of injection this does not cause these cells to become detectable targets for immune-mediated attack. This supports the notion that the plasmid present outside the site of injection is not effectively taken up and expressed.

In conclusion, we have shown that a coNS3/4A gene can be effectively delivered using in vivo EP, which results in the activation of a broad immune response. It is hoped that the combination is able to activate strong enough immune responses in any native, “slumbering,” or inactivated CD4\(^+\) and CD8\(^+\) T cells to the conserved NS3 protein and assist the host in gaining control of the infection.

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Disclosures

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References