Humoral and CD4⁺ T helper (Th) cell responses to the hepatitis C virus non-structural 3 (NS3) protein: NS3 primes Th1-like responses more effectively as a DNA-based immunogen than as a recombinant protein

Una Lazdina, Catharina Hultgren, Lars Frelin, Margaret Chen, Karin Lodin, Ola Weiland, Geert Leroux-Roels, Juan A. Quiroga, Darrell L. Peterson, David R. Milich and Matti Sällberg

The non-structural 3 (NS3) protein is one of the most conserved proteins of hepatitis C virus, and T helper 1 (Th1)-like responses to NS3 in humans correlate with clearance of infection. Several studies have proposed that DNA-based immunizations are highly immunogenic and prime Th1-like responses, although few head-to-head comparisons with exogenous protein immunizations have been described. A full-length NS3/NS4A gene was cloned in eukaryotic vectors with expression directed to different subcellular compartments. Inbred mice were immunized twice in regenerating tibialis anterior (TA) muscles with either plasmid DNA or recombinant NS3 (rNS3). After two 100 µg DNA immunizations, specific antibody titres of up to 12960 were detected at week 5, dominated by IgG2a and IgG2b. NS3-specific CD4⁺ T cell responses in DNA-immunized mice peaked at day 13, as measured by proliferation and IL-2 and IFN-γ production. Mice immunized with 1–10 µg rNS3 without adjuvant developed antibody titres comparable to those of the DNA-immunized mice, but dominated instead by IgG1. CD4⁺ T cell responses in these mice showed peaks of IL-2 response at day 3 and IL-6 and IFN-γ responses at day 6. With adjuvant, rNS3 was around 10-fold more immunogenic with respect to speed and magnitude of the immune responses. Thus, immunization with rNS3 in adjuvant is superior to DNA immunization with respect to kinetics and quantity in priming specific antibodies and CD4⁺ T cells. However, as a DNA immunogen, NS3 elicits stronger Th1-like immune responses, whereas rNS3 primes a mixed Th1/Th2-like response regardless of the route, dose or adjuvant.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. HCV can be divided into different genotypes, of which genotypes 1, 2 and 3 are the most common in Europe. Today’s therapies based on IFN-α and ribavirin can induce a sustained virological and biochemical response in 20–60% of treated patients, depending on the infecting genotype, the virus load and the age of the patient (Reichard et al., 1998; Schvarcz et al., 1995). In patients infected by HCV of genotype 1b, response rates as low as 10% are not uncommon (Schalm et al., 1996). Thus, additional compounds or treatment strategies are urgently needed to improve the treatment of chronic HCV infections.

Several studies have stressed the importance of HCV-specific CD4⁺ T cell responses in clearance of HCV infections (Diepolder et al., 1995; Missale et al., 1996). It has been suggested that T helper 1 (Th1) CD4⁺ T cell responses to the non-structural 3 (NS3) protein are present in patients who clear...
undertook a study to determine how these immune responses shown to correlate with clearance of HCV infections, we

**Methods**

- **Mice.** Inbred mouse strains C57/BL6 (H-2b), BALB/c (H-2d) and CBA (H-2k) were obtained from the breeding facility at Møllegård Denmark, Charles River Uppsala, Sweden, or B&K Sollentuna Sweden. All mice were female and were used at 4–8 weeks of age.

- **Recombinant NS3 ATPase/helicase domain protein.** The production in *E. coli* of an rNS3 protein corresponding to the NTPase/helicase domain has been described in detail previously (Jin & Peterson, 1995). Prior to use, the rNS3 protein was dialysed overnight against PBS and sterile-filtered.

**Construction of eukaryotic vectors expressing NS3/NS4A.**

A full-length NS3/NS4A gene fragment was amplified from a patient infected with HCV genotype 1b using primers flanking the start of NS3 and the end of NS4A (Zhang et al., 2000). The NS3/4A gene was cloned into two different vector backbones. The vector pcDNA3.1 (Invitrogen) produces a form of NS3 that is retained within the cell, in which subcellular localization is determined by the NS3 protein itself. The vector pSecTag2 (Invitrogen) has a multiple cloning site for in-frame cloning of NS3 with an IgG leader sequence to translocate the NS3/NS4A protein to secretory compartments such as the Golgi apparatus.

A 2.1 kb DNA fragment of HCV encoding amino acids 1007–1711, covering NS3 and NS4A, was amplified with a high-fidelity polymerase (Expand High Fidelity PCR, Roche Applied Science). The amplicon was inserted into BamHI/XbaI-digested pcDNA3 (Invitrogen) to produce the NS3–pcDNA plasmid. The NS3/4A gene was ligated with the pSecTag2 plasmid through BamHI/XhoI digestion to give the NS3–pSec plasmid. All expression constructs were sequenced to ensure the correct reading frame. Plasmids were grown in competent *E. coli* BL21 cells. The plasmid DNA used for *in vivo* injection was purified by using Qiagen DNA purification columns according to the manufacturer's instructions. The concentration of the resulting plasmid DNA was determined spectrophotometrically (Dynaquant, Pharmacia Biotech). Purified DNA was dissolved in sterile PBS at 1 mg/ml.

**In vitro translation assay and transient transfections.** In order to characterize *in vitro* translation of the NS3 plasmids, the T7-coupled reticulocyte lysate system (TntT, Promega) was used as described previously (Zhang et al., 2000). *In vitro* translation of the NS3 plasmids was carried out at 30°C with [35S]methionine (Amersham). Labelled proteins were separated on a 12% SDS–polyacylamide gel and visualized by exposure to X-ray film (Hyper film-MP, Amersham) for 6–18 h.

BHK cells were transiently transfected by standard protocols (Zhang et al., 2000) with the two plasmids and NS3 protein expression was analysed by immunofluorescence with NS3-specific hyperimmune sera.

**Protein and DNA immunization of mice.** rNS3, NS3–pcDNA and NS3–pSec were used to immunize groups of four to 18 mice. Both rNS3 (without adjuvant) and plasmids were injected directly into regenerating tibial anterior (TA) muscles as described previously (Davis et al., 1993). In brief, mice were injected intramuscularly with 50 µl of 0.01 mM cardiotxin (Latoxan) in 0.9% sterile NaCl per TA muscle. Five days later, each TA muscle was injected with 50 µl PBS containing either rNS3 or DNA. In control experiments, groups of mice pre-treated with cardiotxin were injected with rNS3 either intraperitoneally or subcutaneously at the base of the tail with the antigen emulsified in Freund's complete adjuvant (CFA).

For T cell studies, the mice were immunized once only. For monitoring of humoral responses, all DNA-immunized mice got a booster injection of 50 µg of the same plasmid DNA per TA muscle every fourth week. The mice receiving rNS3 were immunized twice at most. All mice were bled twice a month.

**Enzyme immunoassays (EIAs) for the detection of antibodies to NS3.** All EIAs for the detection of murine anti-NS3 antibodies were performed essentially as described previously (Chen et al., 1998; Sällberg et al., 1996). In brief, rNS3 was adsorbed passively overnight at 4°C to 96-well microtitre plates (Nunc) at 1 µg/ml in 50 mM sodium carbonate buffer (pH 9.6). The plates were then blocked by incubation with dilution buffer containing PBS, 2% goat serum and 1% BSA for 1 h at 37°C. Serial 5-fold dilutions of mouse sera starting at

acutec HCV infections, whereas those who progress to chronic infection lack these responses (Diepolder et al., 1995; Missale et al., 1996; Tsai et al., 1997). In established chronic HCV infections, CD4+ T cell responses to the NS3 protein are almost totally absent, whereas antiviral therapy appears to activate these responses (Cramp et al., 2000; Leroux-Roels et al., 1996; Zhang et al., 1997b). In general, HCV proteins produced during HCV infection do not appear to be highly immunogenic in the infected host (Chen et al., 1999). The importance of HCV-specific cytotoxic T lymphocyte (CTL) responses in clearance is not clear, because the differences between acute and chronic HCV infections are smaller (Erickson et al., 1993). Furthermore, activated HCV-specific CTL can persist in the liver of patients with chronic HCV infections and CTL escape mutations within the NS3 protein have been described (He et al., 1999; Weiner et al., 1995).

The N terminus of NS3 contains a protease domain that forms a tight non-covalent complex with the cofactor NS4A (Bartenschlager et al., 1995; Failla et al., 1994, 1995; Grakoui et al., 1993). The C-terminal two-thirds of the NS3 protein contain helicase and NTPase activities (Jin & Peterson, 1995). These vital enzymatic functions may explain the limited sequence variation within the NS3 region and suggest that the NS3 protein may constitute a good therapeutic vaccine candidate.

One approach to enhance the endogenous CD4+ T cell response during chronic infection is through therapeutic vaccination using conserved HCV proteins. This may be achieved by several different approaches. For example, genetic immunization offers the possibility of priming antiviral CD4+ and CD8+ T cells. In addition, genetic immunization, in particular DNA immunization, has been suggested to activate Th1-like immune responses preferentially. The reason for this is not clear, but it may be important when considering therapeutic vaccination. We have shown previously with a recombinant NS3 protein (rNS3) that NS3-specific immune responses are cross-reactive between different genotypes of HCV (Sällberg et al., 1996). However, despite there being less evidence for the importance of CTL in clearance of HCV infections, most murine vaccine studies have focused on the induction of CTL responses (Encke et al., 1998; Gordon et al., 2000). Because Th1-like CD4+ T cell responses have been shown to correlate with clearance of HCV infections, we undertook a study to determine how these immune responses can be most effectively primed.
1:60 were then incubated on the plates for 1 h. Bound murine serum antibodies were detected by an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Cellprods) followed by addition of the substrate p-nitrophenyl phosphate (one tablet in 5 ml of 1 M diethanolamine with 0.5 mM MgCl2). The reaction was stopped by addition of 1 M NaOH. The A405 was then read.

ELAs for determination of IgG subclasses to NS3 were performed as described above with the exception that IgG subclass-specific antibodies were used when detecting the bound IgG. The protocol has been described in detail previously (Chen et al., 1998; Sällberg et al., 1996).

T cell proliferation and cytokine assays. All mice received cardiotoxin pre-treatment, regardless of the immunization schedule they were to receive, in order to exclude any influence on the general immune responses that this treatment might induce. Five days later, groups of mice received either an intramuscular injection of 50 μg NS3–pcDNA or NS3–pSec per TA muscle or 20 μg rNS3 emulsified in CFA subcutaneously at the base of the tail. For kinetic studies, two or three mice from each group were sacrificed at days 1, 3, 6, 9, 13 and 21 and their spleens and draining lymph nodes were harvested. Single-cell suspensions were prepared in either Click’s medium or RPMI 1640 and were plated in microplates at 6 × 10^4 cells per well, together with dilutions of rNS3 starting at 20 μg/ml for proliferation assays and cytokine assays (Sällberg et al., 1996, 1997). Phytohaemagglutinin was used as a positive control in each experiment and was added at a final concentration of 1 μg/ml. Supernatants were removed at 24 and 48 h for determination of IL-2, IL-4, IL-6 and IFN-γ. To measure T cell proliferation, the plates were incubated for 72–96 h with addition of 1 µCi [3H]thymidine (Tdr; Amersham) for the last 16 h. Labelled cells were harvested on cellulose filters and quenched and the level of [3H]Tdr incorporation was determined in a beta-counter.

The presence of cytokines in culture supernatants was determined as described previously (Hultgren et al., 1998). In brief, culture supernatants were analysed for the presence of IL-2, IL-4, IL-6 and IFN-γ by commercial ELAs (Endogen). All commercial ELAs were performed according to the manufacturer’s instructions. Results of representative experiments are presented in the figures.

Results

Characterization of NS3-expression plasmids

Expression of NS3/NS4A from the two plasmids was analysed by an in vitro transcription/translation assay in which transcription is initiated through the prokaryotic T7 promoter. In vitro translation resulted in full-length NS3/NS4A proteins from both plasmids (Fig. 1a).

Both plasmids were used for transient transfection of BHK cells. Immunofluorescence revealed that both plasmids expressed NS3/NS4A, but in different subcellular locations. The NS3–pcDNA vector expresses NS3/NS4A in both the cytoplasm and nucleus (Fig. 1d), whereas NS3–pSec expresses NS3 in compartments consistent with secretory pathways, e.g. the Golgi (Fig. 1e).

Humoral responses following immunization with NS3

Groups of four to six mice of three different haplotypes, CBA (H-2k), BALB/c (H-2b) and C57/B16 (H-2d), were immunized with NS3 in the form of the two different expression plasmids. The mice were boosted 4 weeks later. Since the responder hierarchies to rNS3 and rNS4A have been determined previously, only H-2k mice were immunized as a control with 20 μg rNS3 in CFA at day 0 and were boosted 4 weeks later with 5 μg rNS3 in Freund’s incomplete adjuvant. As additional controls, groups of five H-2b mice were immunized at weeks 0 and 4 with 0.1, 1.0 and 10.0 μg rNS3 in PBS in regenerating TA muscles.

In a previous study, the responder hierarchy to rNS3 in adjuvant was H-2k followed by H-2d and H-2b mice (Sällberg et al., 1996). With respect to HCV NS4A genotype 1 in adjuvant, the H-2k haplotype was a non-responder, whereas H-2d mice were low responders (Zhang et al., 1997a). Thus, the influence from NS4A-derived T cell site should be limited in these two strains. The responder hierarchies to the two DNA plasmids were similar (Fig. 2), with H-2b and H-2d mice as the best responders, in contrast to rNS3 in adjuvant. Also, the NS3–pcDNA plasmid consistently gave rise to higher humoral responses than the NS3–pSec plasmid. Both plasmids induced humoral responses that were at least 10-fold lower in magnitude compared with rNS3 immunization in adjuvant. Interestingly, by comparing the DNA-primed immune responses in H-2d mice with those of rNS3 in PBS, we found that 100 μg NS3 DNA was equivalent to immunization with 1–10 μg rNS3 in PBS (Fig. 2). Thus, the DNA-mediated protein expression during in vivo immunization corresponds to 1–10 μg exogenous rNS3.

In order to analyse the humoral responses to NS3 further, specific IgG subclass levels were determined in H-2b and H-2d mice. rNS3 immunization in adjuvant induced all IgG subclasses and the response was dominated by IgG1 (Fig. 3; Sällberg et al., 1996). In contrast, in H-2d and H-2k mice, NS3–pcDNA induced predominantly IgG2a and IgG2b and no IgG1. To clarify further whether the route of DNA immunization or the DNA plasmids themselves was responsible for the skewing of the IgG subclass distribution, mice were immunized with rNS3 without adjuvant in regenerating TA muscles. The H-2d mice immunized with rNS3 without adjuvant showed antibody levels similar to those of the DNA immunization groups, whereas the IgG subclass distribution was similar to that of rNS3/CFA-immunized mice (Figs 2 and 3). Therefore, the DNA plasmids themselves rather than the route of immunization determined the IgG subclass distribution of NS3-specific antibodies. DNA-based immunization with NS3 thus induces a different IgG subclass distribution compared with rNS3 in adjuvant.

Kinetics of the cell-mediated immune response: proliferative responses in peripheral lymph nodes

When analysing in vivo-primed rNS3-specific T cells in CBA mice (H-2k) by in vitro recall on peripheral lymph nodes, a marked difference was seen in T cell proliferation between mice given recombinant protein and those given DNA (Fig. 4).
Mice immunized with rNS3/CFA had clearly detectable proliferation at day 3, with peak proliferation between days 3 and 13. In DNA-immunized mice, T cell proliferation started after day 3 and peaked at day 13. The lowest in vitro concentrations of rNS3 able to recall a detectable response at day 13 were 0.00128 µg/ml for rNS3/CFA-immunized mice.
and 0.8 μg/ml for the DNA-immunized mice, indicating a 625-fold difference in T cell priming. Regardless of the immunogen, proliferation had declined by day 21. Thus, with respect to proliferative CD4+ T cell responses in local draining lymph nodes, rNS3 in CFA primes the most rapid and vigorous response.
Kinetics of the cell-mediated immune response: proliferative and cytokine responses in the spleen

The CD4⁺ T cell responses were evaluated further in the spleen. Proliferative CD4⁺ T cell responses were analysed in the spleen following immunization in regenerating TA muscles with 100 µg NS3/4A-pSec or 40 µg rNS3 in PBS. In the splenic compartment, the proliferative responses to immunization with DNA and rNS3/PBS were of similar magnitudes (Fig. 4). Both immunizations primed proliferative responses that could be recalled at in vitro rNS3 concentrations of 4 µg/ml, much less efficient than the proliferative responses in draining lymph nodes primed by rNS3 in CFA. Interestingly, both DNA and rNS3/PBS primed splenic proliferative responses of similar kinetics that peaked around days 6–9. Thus, 40 µg rNS3 in PBS primed splenic proliferative responses of the same order of magnitude as 100 µg NS3–pSec-based DNA immunization.

The splenic cytokine responses were determined following different modes of NS3 immunization. A first comparison was made between immunization with 20 µg rNS3 in CFA given intraperitoneally and the two DNA plasmids given in regenerating TA muscles. Mice immunized with rNS3/CFA displayed peak IL-2 production at day 3 and peak IL-6 and IFN-γ production at day 6 (Fig. 5). In contrast, mice immunized with either NS3–pcDNA or NS3–pSec displayed peak IL-2 and
IFN-γ levels at day 13. The levels of CD4+ T cell priming were consistently much higher for NS3/CFA-immunized mice, as determined by the lowest recall antigen dose that induced a detectable response (Fig. 5). Thus, NS3 in CFA primes more vigorous proliferative and cytokine responses compared with NS3 as a DNA immunogen. The enhanced overall cytokine response, and that of IL-6 in particular, may explain the presence of all IgG subclasses following rNS3/CFA immunization.

Next, the splenic cytokine responses to immunization with
40 µg rNS3/PBS and 100 µg NS3–pSec DNA were compared, both given in regenerating TA muscles. The two routes induced comparable kinetics, cytokine patterns and levels of cytokine production (Table 1). This emphasizes the importance of the adjuvant for rNS3 immunization. The differences in cytokine responses between the two modes of immunization were consistent with the observed differences in IgG subclass distribution. T cells from DNA-immunized mice were more prone to produce IL-2 and IFN-γ than those from the rNS3-immunized mice, since cytokine production was induced in vitro at lower recall concentrations of NS3 at days 13–20 (Table 1). Also, it is likely that the IFN-γ production following DNA immunization, peaking at days 9–20, influenced the IgG subclass distribution (Stevens et al., 1988).

### Discussion

There are several reasons why the HCV NS3 protein may be a suitable candidate for a therapeutic vaccine. Firstly, the NS3 protein is a genetically stable protein sequence, whereby the likelihood of sequence differences between the infecting virus and the vaccine is reduced. NS3 is also a large protein, which may reduce the likelihood of immunological non-responder status. Furthermore, many studies have shown that CD4+ T cell responses to HCV NS3 are related to clearance of acute HCV infections (Diepolder et al., 1995; Missale et al., 1996). CD4+ T cell responses to HCV NS3 are absent or extremely weak in untreated chronic HCV infections (Leroux-Roels et al., 1996; Zhang et al., 1997b) and antiviral therapy seems to induce these cell-mediated responses in patients with chronic HCV infection (Cramp et al., 2000; Lohr et al., 1998; Zhang et al., 1997b). Finally, rNS3 has been shown to be fairly immunogenic in mice (Sallberg et al., 1996). Thus, vaccine studies of HCV NS3 may be important.

We compared different vehicles for priming NS3-specific CD4+ T cells. rNS3 in adjuvant was clearly superior to other modes of immunization with respect to the kinetics and magnitude of both humoral and CD4+ T cell responses. We have found previously that this is also true for retroviral vectors (Sallberg et al., 1997, 1998; Townsend et al., 1997). This suggests that combined immunization with DNA and recombinant proteins may be of interest and should be explored further. Although DNA immunization primed responses that were weaker, some other properties may be of great importance. In line with previous reports, we observed that CD4+ T cell responses following DNA immunization seemed to be dominated by IFN-γ-producing Th1-like cells, as evidenced by both the IgG subclass distribution and the cytokine profile (Encke et al., 1998). Because IFN-γ-producing CD4+ T cells have been correlated with clearance of HCV infections, a therapeutic vaccine based on NS3 DNA may be suitable. However, the route of immunization with DNA has to be optimized further.

An important observation was that, when comparing DNA immunization and rNS3/PBS immunization in regenerating TA muscles, we noted that DNA immunization primed immune responses comparable to 1–10 µg rNS3. Thus, after in vivo transfection with 100 µg DNA, the immunogenicity of endogenously expressed NS3 is equal to administration of 1–10 µg rNS3. Also, the differences in IgG subclass distribution are most likely due to the nature of DNA immunization and not to the route or adjuvant used. Thus, the Th1-like immune responses primed by DNA-based immunization seem to be intrinsic to this mode of immunization. However, by enhancing in vivo transfection efficiencies and expression levels, it is possible that a more mixed Th1/Th2 population may appear.

NS3 seems to localize naturally to the cytoplasmic and nuclear compartments (Errington et al., 1999; Muramatsu et al., 1997; Wolk et al., 2000). We noted that DNA-based immunization with expression vectors with products targeted to different subcellular compartments had some effect on
immune responses. Artificial targeting of NS3/4A expression to secretory compartments generally primed lower humoral responses, with an IgG subclass distribution restricted to IgG2a in H-2^d mice. This might possibly be explained either by the unnatural localization of NS3/4A or by some particular properties of the pSec plasmid itself.

When comparing our data with existing data on DNA-based immunization with NS3, we noted that our antibody levels were 10- to 100-fold better (Gordon et al., 2000). This may be explained either by the modes of immunization or by the fact that we included NS4A in our construct in order to obtain the complete functional protease complex. The presence of NS4A in eukaryotic expression vectors was shown recently to increase stability and prolong the intracellular half-life (Wolk et al., 2000). Thus, NS3/4A fusion genes should be explored further as the basis for a DNA-immunization construct.

In conclusion, although DNA-based immunizations are much less potent than rNS3 in adjuvant in priming CD4^+ T cell responses, some factors favour DNA immunization. In particular, the priming of a more Th1-like CD4^+ T cell population may be desired in therapeutic vaccination of chronic HCV infections. A major difficulty will be maintaining the observed immunogenicity of the NS3/4A gene in higher animals, such as humans. Thus, the administration and formulation of the DNA-based immunization will be of vast importance in further studies.

Mailin Weiland is gratefully acknowledged for excellent technical assistance. The study was supported by grants from the Medical Research Council of Sweden (K2000-06X-12617-03A) and the European Community (QLK2-1999-00588).

References


Received 22 November 2000; Accepted 15 February 2001