Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3

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Cell-mediated immune responses to hepatitis C virus (HCV) proteins play a key role in recovery from infection. The NS3 protein of HCV is of special interest, since it is one of the most conserved proteins and NS3-specific immune responses are stronger and more frequently observed in patients resolving the infection than in chronically infected patients. Since these characteristics make NS3 an attractive vaccine candidate, the objective of this study was to optimize NS3-specific immune responses. Results from this group first demonstrated that a plasmid enriched with 24 CpG motifs (pBISIA24-NS3) tends to induce the strongest and most consistent Th1-biased immune response. Subsequently, it was shown that NS3 formulated with CpG oligodeoxynucleotide and Quil A (rNS3+CpG+Quil A) adjuvants induces a balanced immune response in mice, whereas rNS3 combined with either CpG or Quil A elicits a Th2-biased response. To further enhance NS3-specific cell-mediated immune responses, a vaccination regime consisting of priming with pBISIA24-NS3, followed by boosting with rNS3+CpG+Quil A, was explored in mice and pigs. When compared to immunization with rNS3+CpG+Quil A, this regime shifted the immune response to a Th1-type response and, accordingly, enhanced MHC I-restricted killing by cytotoxic T lymphocytes in mice. Although immunization with pBISIA24-NS3 also induced a Th1-biased response, including cytotoxicity in the mice, the humoral response was significantly lower than that induced by the DNA prime–protein boost regime. These results demonstrate the advantage of a DNA prime–protein boost approach in inducing a strong NS3-specific cell-mediated, as well as humoral, immune response, in both inbred laboratory and outbred large animal species.

INTRODUCTION

About 170 million people are infected with hepatitis C virus (HCV) worldwide, and 55–85 % of acute HCV infections result in chronic hepatitis, which may lead to cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997; Saito et al., 1990). Treatment with IFN-α and ribavirin is the most common therapy against HCV infection, but the overall response rate is less than 40 % (Poynard et al., 1998). Thus, developing strategies for vaccination as well as for treatment of HCV infection is of great importance.

HCV is an enveloped, plus-stranded RNA virus of the family Flaviviridae. Its genome is 9–5 kb in length with one open reading frame encoding a polyprotein comprising structural (core and envelope glycoproteins E1 and E2) and non-structural (NS2, NS3, NS4a/b and NS5a/b) proteins (Grakoui et al., 1993; Hijiakata et al., 1991). Since HCV exists as numerous genotypes and within an infected host as numerous quasispecies, it is a notoriously difficult target for immunization (Inchauspe & Feinstone, 2003). There is growing evidence that Th1 and cytotoxic T-lymphocyte (CTL) responses to HCV proteins play a major role in recovery (Thimme et al., 2001; Cooper et al., 1999). The NS3 protein, which has serine protease and helicase activity (Grakoui et al., 1993) and is one of the most conserved proteins of HCV, seems to play a key role in virus clearance (Jiao et al., 2003). NS3 contains an immunodominant CD4+ T helper epitope and several CTL epitopes. Since these epitopes have been associated with control of HCV in patients with self-limiting infection (Diepolder et al., 1997; Kurokohchi et al., 1996; Battegay et al., 1995), NS3 represents a potential vaccine candidate expected to induce both CD4+ and CD8+ lymphocyte-mediated protective immune responses.
DNA vaccines preferentially induce Th1 immunity and CTL responses (Boyer et al., 1996; Raz et al., 1996; Ulmer et al., 1993) and elicit protective immunity to a variety of pathogens (Tedeschi et al., 1997; Donnelly et al., 1995; Fynan et al., 1993). However, DNA immunization has also been demonstrated to generate weaker antibody and CTL responses than protein and live attenuated vaccines (Manickan et al., 1997). Several approaches have been explored to enhance immune responses induced by DNA vaccines, including co-administration of cytokine-expressing plasmids (Kim et al., 2001; Geissler et al., 1997), delivery in a Salmonella vector (Wademeyer et al., 2001), targeting of DNA to dendritic cells (You et al., 2001), incorporation of immunostimulatory DNA sequences (Klinman, 2003; Pontarollo et al., 2002) and heterologous prime and boost vaccination regimes (Pancholi et al., 2003; Song et al., 2000).

In general, recombinant protein vaccines stimulate primarily Th2 cells and thus elicit strong humoral responses, but weak cell-mediated immune responses. However, if proteins are formulated with appropriate adjuvants, it is also possible to induce Th1-type responses. Oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG ODNs) are novel adjuvants known to promote Th1-biased immune responses (Chu et al., 1997). Indeed, out of 19 different adjuvants tested, CpG ODN was most effective at eliciting a Th1-type immune response to a tumour antigen (Davis, 2000). CpG ODNs are capable of augmenting antigen-specific humoral and cellular immune responses against peptides, viral and bacterial proteins and tumour antigens in a number of different species (Klinman, 2003; Ioannou et al., 2002a; Davis et al., 2000). Furthermore, CpG ODNs cause minor adverse reactions in comparison to most immunostimulatory agents used to date (Ioannou et al., 2003).

Unlike many other vaccine adjuvants, the saponin-derived adjuvant Quil A promotes a broad immune response, by simultaneously inducing strong antibody and T-cell responses including enhanced cytokine secretion and activation of CTL responses (Cox & Coulter, 1997; Barr & Mitchell, 1996). One of our previous studies showed that Quil A promotes a balanced immune response to a truncated form of bovine herpesvirus-1 glycoprotein D (BHv-1 gD), while causing minimal tissue damage. When Quil A was co-administered with CpG ODN, the immune response was further enhanced and shifted to a Th1-type response (Ioannou et al., 2002b). Since the purity of Quil A-based adjuvants has significantly improved, virtually non-reactogenic vaccines can be made with these highly purified Quil A components (Rimmelzwaan & Osterhaus, 1995). This makes it one of the few adjuvants safe enough to be licensed for human use.

There is increasing evidence that the cellular immune response is of major importance for the control of HCV infection. Therefore, our goal was to enhance cell-mediated immunity to HCV NS3 by testing different vaccine formulations and strategies in both in-bred laboratory and outbred large animal species. A comparison of various vaccination regimes demonstrated that priming with plasmid encoding NS3, followed by boosting with rNS3 formulated with CpG ODN and Quil A results in optimal NS3-specific immune responses.

**METHODS**

**Cloning, expression and purification of rNS3.** The NS3 gene derived from pHCVrep1bl, a plasmid containing the NS3, NS4ab and NS5ab genes of the HCV-1b strain, was cloned in-frame into the prokaryotic expression vector pRSETA (Invitrogen). A recombinant clone, pRSET-NS3, was selected and transformed into Escherichia coli BL21(DE3). NS3 protein consisting of amino acids 1027–1657 of the polyprotein plus an amino-terminal extension of six histidine residues from the vector sequence was induced by IPTG and expressed as inclusion bodies. To purify rNS3, bacteria were lysed, and the insoluble inclusion body proteins were pelleted and resuspended in 6 M guanidine.HCl, 0·1 M NaH2PO4, 0·01 M Tris/HCl, pH 8·0. The lysate supernatant was loaded onto a Protein resin (Invitrogen) column. Unbound protein was removed with 8 M urea, 0·1 M NaH2PO4, 0·01 M Tris/HCl, pH 6·5, and bound rNS3 was eluted with 8 M urea, 0·1 M NaH2PO4, 0·01 M Tris/HCl, pH 4·5. The NS3-containing fractions were pooled and renatured by reduction of the urea concentration by dialysis (Jin & Peterson, 1995). The concentration of the purified NS3 was determined by a Microplate Reader 4.0 (Bio-Rad) protein assay. The endotoxin level, determined using the QCL-1000 Chromogenic Limulus amoebocyte lystate test (BioWhittaker), was 51 ng mg⁻¹.

**Construction of NS3 expression vectors.** Plasmid pMASIA and plasmids enriched with different numbers of CpG motif TCGTGT, pBSIA24, pBSIA40, pBSIA88 and pBSIA160, were constructed as described previously (Pontarollo et al., 2002; Krieg et al., 1998). To create plasmids encoding NS3, the NS3 gene was cut from pRSET-NS3 with BamHI and HindIII restriction enzymes and inserted into pMASIA, pBSIA24, pBSIA40, pBSIA88 and pBSIA160 digested with the same enzymes. The recombinant plasmids were transformed into E. coli DH5α, purified using an EndoFree Plasmid Giga kit (Qiagen), and stored at −20 °C. In vitro expression of NS3 protein in COS-7 cells transfected by these plasmids was confirmed by immunohistochemistry, as described previously by Bryan et al. (1988).

**CpG ODN and Quil A.** The adjuvants used in this study were Quil A (Accurate Chemical and Scientific) and two synthetic ODNs containing unmethylated CpG dinucleotides (Qiagen). Although generally conserved, there is a certain degree of species specificity in the recognition of CpG motifs (Hartmann et al., 2000). ODN 1826 (5'-TCCATGAGCCTCCTGACGTT-3'), which is a strong B cell mitogen known to stimulate mouse spleenocytes in vitro (Rankin et al., 2001; Davis et al., 1998), was used for mice. ODN 2007 (5'-TGGCTGT- TGGCTGTTTGGTGGCTGTT-3'), which stimulates porcine and human peripheral blood mononuclear cells (PBMCs) in vitro (Rankin et al., 2001; Hartmann et al., 2000), was used for pigs. These two CpG ODNs were phosphorothioate modified to increase resistance to nuclease degradation (Samani et al., 2001).

**Immunization of mice and piglets.** Eight-week-old female BALB/c (H-2b) mice were used in all mouse trials. In the first trial, groups of six mice were immunized three times intradermally (i.d.) with saline or 50 μg plasmid in the back as follows: (1) saline; (2) pMASIA-NS3; (3) pBSIA24-NS3; (4) pBSIA40-NS3; (5) pBSIA88-NS3; or (6) pBSIA160-NS3. In the second trial, four groups of five mice were immunized three times subcutaneously (s.c.) in the back with one of the following formulations: (1) saline; (2) 20 μg rNS3 with 10 μg ODN 1826 (rNS3+CpG); (3) 20 μg rNS3 with 10 μg

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Quil A (rNS3 + Quil A); or (4) 20 µg rNS3 with 10 µg ODN 1826 and 10 µg Quil A (rNS3 + CpG + Quil A). In the third trial, six groups of six mice were immunized in the back as follows: (1) three times with saline; (2) three times with 50 µg pBISIA24-NS3 (3) twice with 50 µg pBISIA24-NS3 followed by 5 µg rNS3 with 10 µg ODN 1826 and 10 µg Quil A; (4) three times with 5 µg rNS3, 10 µg ODN 1826 and 10 µg Quil A; (5) twice with 50 µg pBISIA24-NS3 followed by 20 µg rNS3 with 10 µg ODN 1826 and 10 µg Quil A; or (6) three times with 20 µg rNS3, 10 µg ODN 1826 and 10 µg Quil A. The protein formulations were injected s.c., whereas the plasmids were delivered i.d.

In the pig trial, 5/5-week-old cross-bred piglets were randomly allocated to four groups of six piglets each, and immunized with pBISIA24-NS3 and/or rNS3 with CpG ODN and Quil A. The vaccination regimes were the same as treatments 1–4 in the second mouse trial. However, CpG ODN 2007 instead of CpG ODN 1826 was used. Secondly, i.d. immunizations were performed in the ears of the piglets. Thirdly, the doses of plasmid (500 µg), protein (50 µg), CpG ODN (100 µg) and Quil A (100 µg) in the pig trial were 10-fold higher than the doses in the mouse trial.

In all animal trials, vaccinations were given on days 0, 28 and 49. All mice were bled at regular intervals for ELISAs and they were sacrificed on day 63 to isolate splenocytes for ELISPOT and CTL assays. The piglets were also bled at regular intervals for ELISA, ELISPOT and lymphocyte proliferation assays. All experiments were carried out according to the guidelines provided by the Canadian Council for Animal Care.

ELISA. In order to determine antibody responses in mice and pigs, 96-well polystyrene microtitre plates (Immulon 2; Dynatech) were coated overnight at 4°C with 5 µg ODN 1826 and 10 µg Quil A; (5) twice with 50 µg pBISIA24-NS3 followed by 20 µg rNS3 with 10 µg ODN 1826 and 10 µg Quil A; or (6) three times with 20 µg rNS3, 10 µg ODN 1826 and 10 µg Quil A. The protein formulations were injected s.c., whereas the plasmids were delivered i.d.

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ELISA. In order to determine antibody responses in mice and pigs, 96-well polystyrene microtitre plates (Immulon 2; Dynatech) were coated with 0.1 µg per well of purified rNS3 and incubated with serially diluted murine or porcine sera. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG or AP-conjugated goat anti-porcine IgG (Kirkegaard & Perry Laboratories) was used at a dilution of 1:5000 to detect bound murine and porcine antibodies, respectively. The reactions were visualized with p-nitrophenyl phosphate (PNPP) (Sigma). The NS3-specific antibody titres are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the value of negative control sera.

Immunoglobulin isotyping ELISA. To determine the NS3-specific IgG subtypes in mice and pigs, serially diluted murine and porcine sera were incubated in rNS3-coated 96-well plates. Murine antibodies were detected with biotinylated goat anti-murine IgG1 and IgG2a (Caltag Laboratories) at a dilution of 1:5000 to detect bound murine and porcine antibodies, respectively. The reactions were visualized with streptavidin–AP (Gibco) at a 1:1000 dilution. The porcine IgG1 and IgG2 (Serotec) diluted 1:200 and 1:100, respectively, followed by AP-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) diluted 1:5000. The reaction was visualized with PNPP (Sigma). The results are expressed as murine IgG1 : IgG2a ratio of titres and porcine IgG1 : IgG2 ratio of titres.

Cytokine ELISPOT. A cytokine-specific enzyme-linked immunospot (ELISPOT) assay was performed as described previously (Ioannou et al., 2002a; Lewis et al., 1999). Briefly, 96-well MultiScreen-HA filtration plates (Millipore) were coated overnight at 4°C with 0.1 µg per well of murine IFN-γ or IL-4 specific monoclonal antibodies (Pharmingen) or porcine IFN-γ specific monoclonal antibodies (BioSource International). Splenocytes isolated from mice (Baca-Estrada et al., 1996) or PBMCs isolated from piglets (Rankin et al., 2002) in AIM-V medium were added to the coated plates at 10^5 cells per well in the absence and presence of rNS3 at a final concentration of 1 µg ml^-1 for mice and 0.1 µg ml^-1 for piglets. After 20 h incubation at 37°C and 5% CO2, the plates were washed extensively, and incubated with biotinylated anti-murine IFN-γ or IL-4 monoclonal antibodies (Pharmingen) or biotinylated anti-porcine IFN-γ monoclonal antibodies (BioSource International) at 2 µg ml^-1. This was followed by incubation with streptavidin–AP (Gibco) at a 1:1000 dilution. The spots were visualized with a substrate consisting of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma). The number of cytokine-secreting cells is expressed as the difference between the number of spots per 10^6 cells in rNS3-stimulated wells and the number of spots per 10^6 cells in non-stimulated wells.

CTL assay. To prepare effector cells, splenocytes were isolated from each group of mice and pooled. Syngeneic splenocyte stimulators were prepared by infection for 1 h at 37°C with a recombinant vaccinia virus VP1461, which encodes NS3/NS4/NS5 from HCV-1b strain BK (kindly provided by Aventis), at an m.o.i. of 10. After infection, the stimulators at a concentration of 10^6 cells ml^-1 were irradiated with 3000 rads. The pooled splenocytes from each group were cultured with the stimulators at 37°C and 5% CO2 for 5 days in AIM-V medium. Mouse IL-2 (Boehringer Mannheim) was added to a final concentration of 10 U ml^-1. To generate target cells, we stably transformed P815 cells (H-2d) (ATCC) with NS3. NS3-transformed and control P815 cells were labelled for 1 h with 100 µCi of Na24CrO4 per 10^6 cells. Cells were washed four times and used as targets at 5 × 10^3 cells ml^-1. One hundred microlitres of labelled target cells was added to each well of a U-bottom 96-well plate and 100 µl of effector cells were added to the target cells in triplicate wells at various effector to target (E : T) ratios. Plates were incubated for 4 h at 37°C and 5% CO2. The supernatant from each well was counted in a 1470 Wizard gamma counter (Perkin Elmer). The percentage specific cytotoxicity was calculated as [(experimental 51Cr release–spontaneous release)/(total 51Cr release–spontaneous release)] × 100.

Lymphocyte proliferation assay. PBMCs from piglets were dispensed at 3.5 × 10^5 cells ml^-1 in AIM-V medium and cultured in 96-well tissue culture plates at 3.5 × 10^5 cells per well in the absence and presence of 0.1 µg rNS3 ml^-1. After 72 h in culture, the cells were pulsed with 0.4 µCi (14.8 kBq) of [methyl-3H]thymidine (Amersham) per well. The cells were harvested 18 h later and radioactivity was determined by scintillation counting. Proliferative responses were calculated as the means of triplicate wells and are expressed as a stimulation index (SI) where SI represents the counts per minute (c.p.m.) in the antigen-stimulated wells divided by the c.p.m. in wells with medium alone.

Statistical analysis. All data were analysed with the aid of a software program (GraphPad Prism 3.0). Differences between the means of experimental groups were analysed using an independent, two-tailed t-test at the level of P < 0.05.

RESULTS

Production of NS3 and construction of plasmids encoding NS3

Purified NS3 protein was analysed by SDS-PAGE and shown to be 90% pure (Fig. 1a). The NS3 gene derived from an HCV-1b strain was cloned into CpG-enriched vectors (Fig. 1b). To determine whether the recombinant plasmids express the NS3 protein in vitro, COS-7 cells were transfected. As shown for pBISIA24-NS3 transfected cells in Fig. 1c-1, a strong and very similar signal was observed for all of the plasmids encoding NS3. In contrast, no signal was found in the pBISIA24 transfected cells (Fig. 1c-2). These results indicate that all plasmids efficiently expressed NS3 in transfected cells in vitro.
Immune responses induced by CpG-enriched plasmids encoding NS3

Since ODNs containing a GTCGTT motif are strong and consistent stimulators of lymphocyte proliferation and IFN-γ secretion in several species including mice, pigs and humans (Rankin et al., 2001; Hartmann et al., 2000), we constructed plasmids encoding NS3 with different numbers of this motif as a means of optimizing the induction of cellular immunity. Immunization of mice with pBISIA24-NS3 tended to induce more IFN-γ-secreting cells than vaccination with any of the other plasmids (Fig. 2a). The difference in IFN-γ production between the pBISIA24-NS3 and pBISIA160-NS3 mice was significant (P<0.05). Furthermore, pBISIA24-NS3 induced higher levels of IgG2a (Fig. 2b) and therefore was selected for further experiments.

Immune responses of mice immunized with rNS3 formulated with CpG ODN and/or Quil A

Since we observed previously that BHV-1 tgD formulated with Quil A and CpG ODN induces a Th1-type response, BALB/c mice were immunized with rNS3 formulated with Quil A, ODN 1826, or both Quil A and ODN 1826. All mice developed high NS3-specific antibody titres and there was no significant difference between the three vaccinated groups (Fig. 3a). In contrast, there was a difference in isotype profiles. Although all three groups had similar IgG1 titres, the rNS3 + CpG group developed a significantly lower IgG2a titre (P<0.05) when compared to the rNS3 + CpG + Quil A and rNS3 + Quil A groups. Although the rNS3 + Quil A group tended to have a lower IgG2a titre than the rNS3 + CpG + Quil A group, there was no significant difference (Fig. 3a).

Antigen-specific IFN-γ and IL-4 production by splenocytes further reflects the bias of the immune response. The rNS3 + CpG + Quil A group had a significantly higher number of IL-4-secreting cells (P<0.05) than the rNS3 + CpG group. However, there was no difference between the numbers of IL-4-secreting cells in the rNS3 + CpG + Quil A and rNS3 + Quil A groups. Interestingly, the numbers of IFN-γ-secreting cells in the rNS3 + CpG and rNS3 + Quil A groups were very low; however, when rNS3 was formulated with both CpG ODN and Quil A, IFN-γ production increased dramatically (P<0.01) (Fig. 3b). The IFN-γ : IL-4 ratios are consistent with the IgG2a : IgG1 ratios observed for the three vaccinated groups. These results demonstrate that immunization with rNS3 generally facilitates strong immune responses. However, rNS3 formulated with CpG ODN and Quil A induced a balanced immune response, whereas rNS3 combined with either CpG ODN or Quil A stimulated a Th2-type response.

Humoral immune responses of mice immunized with pBISIA24-NS3 and/or rNS3 formulated with CpG ODN and Quil A

To further enhance the cell-mediated immune response to NS3, a regime of priming with pBISIA24-NS3 followed by boosting with rNS3 + CpG + Quil A was explored. A second plasmid immunization was given prior to immunization with protein, because previous reports have demonstrated that this greatly enhances the immune response (Song et al., 2000). After the first, second and third immunization,
the groups immunized with 5 μg rNS3 + CpG + Quil A or 20 μg rNS3 + CpG + Quil A had significantly higher total IgG titres (P<0.01) than the other groups. Furthermore, the mice primed with pBISIA24-NS3 and boosted with rNS3 + CpG + Quil A developed stronger antibody responses compared to the mice immunized with plasmid alone (P<0.05) (Fig. 4a). The NS3-specific IgG1 : IgG2a
ratios were determined to evaluate the type of responses induced. Immunization with pBISIA24-NS3 resulted in the lowest IgG1 : IgG2a ratio, whereas the IgG1 : IgG2a ratio was highest in mice immunized with 20 μg rNS3 + CpG + Quil A. Furthermore, the mice primed with pBISIA24-NS3 and boosted with rNS3 + CpG + Quil A developed a significantly (P<0.05) lower IgG1 : IgG2a ratio compared to the mice immunized with rNS3 + CpG + Quil A. Noticeably, immunization with higher doses of rNS3 did not increase the total IgG titre, but tended to increase the IgG1 : IgG2a ratio in the protein groups and significantly (P<0.05) increased the IgG1 : IgG2a ratio in the groups primed with plasmid and boosted with protein (Fig. 4b).

Cellular immune responses of mice immunized with pBISIA24-NS3 and/or rNS3 formulated with CpG ODN and Quil A

The mice immunized with rNS3 + CpG + Quil A developed high numbers of both IFN-γ- and IL-4-secreting cells, indicating that a balanced immune response was induced. In contrast, the mice immunized with pBISIA24-NS3 followed by rNS3 + CpG + Quil A had equivalent numbers of IFN-γ-producing cells, but much lower numbers of IL-4-producing cells (P<0.01) compared to the protein vaccinated animals. The high IFN-γ : IL-4 ratio in these groups is suggestive of a Th1-biased immune response. The group immunized with pBISIA24-NS3 produced similar numbers of IFN-γ-secreting cells compared to the DNA prime–protein boost group, but no IL-4-secreting cells were detected, which again is indicative of a Th1-biased immune response (Fig. 5a). These results are consistent with the IgG1 : IgG2a ratios in the serum.

Because CTL responses are essential to eliminate virus from infected cells, we studied the ability of splenocytes derived from mice treated with different vaccination regimes to lyse P815 target cells stably expressing NS3 protein. The CTLs of mice immunized with pBISIA24-NS3 followed by 5 μg rNS3 + CpG + Quil A showed 32 % specific lysis at an E:T ratio of 90 : 1, while the CTLs of mice immunized with pBISIA24-NS3 or 5 μg rNS3 + CpG + Quil A showed 25 % and 17 % specific lysis, respectively. When the dose of protein increased from 5 μg to 20 μg, the specific lysis at the 90 : 1 ratio decreased from 32 % to 22 % in the DNA prime–protein boost groups, and from 17 % to 13 % in the protein groups (Fig. 5b). These results were confirmed in a second trial and demonstrate the induction of an NS3-specific CTL response in mice immunized with plasmid, protein or plasmid followed by protein.

Humoral immune responses of piglets immunized with pBISIA24-NS3 and/or rNS3 formulated with CpG ODN and Quil A

Since DNA vaccines tend to be less effective in large, outbred species, it was critical to confirm the efficacy of the NS3 DNA vaccine in another model species. Furthermore, the rNS3 vaccine formulation needed to be validated with a pan-activating CpG ODN. Because of the strong similarity with humans in size, physiology and immunology, we used pigs to validate these vaccination strategies. In contrast to the responses in mice, even after three immunizations with pBISIA24-NS3, only low NS3-specific antibody levels were detected in the sera of the piglets (Fig. 6a). However,
antibody responses to NS3 increased dramatically \((P<0.01)\) in piglets immunized with pBISIA24-NS3 followed by rNS3+CpG+Quil A. In the piglets immunized with rNS3+CpG+Quil A, there was a significant increase in antibody titre after the second immunization, but little change after the third immunization. There was no difference in antibody titres between the protein-vaccinated piglets and the piglets immunized with plasmid followed by protein.

To characterize the type of immune response generated, the NS3-specific IgG1 and IgG2 titres in the porcine sera were determined (Fig. 6b). Since the plasmid-vaccinated group
had low antibody titres, the IgG1 : IgG2 ratios showed more variance. However, there appeared to be a slight predo-
minance of IgG2 over IgG1. Furthermore, priming with pBISIA24-NS3 followed by boosting with rNS3 + CpG +
Quil A resulted in very similar IgG1 and IgG2 levels, whereas vaccination with rNS3 + CpG + Quil A tended to induce
higher IgG1 than IgG2. However, the differences between
these three groups were not significant. These data suggest
that all vaccination regimes result in a rather balanced
immune response in piglets.

Cellular immune responses of piglets
immunized with pBISIA24-NS3 and/or rNS3
formulated with CpG ODN and Quil A

To assess NS3-specific cell-mediated immune responses in
the piglets, lymphocyte proliferation and IFN-γ ELISPOT
assays were performed after in vitro stimulation of PBMCs
with rNS3. All vaccinated groups showed significantly
($P<0.05$) stronger NS3-specific lymphocyte proliferation
than the saline group on days 63 and 84 (Fig. 7a). Further-
more, both the plasmid group and the plasmid prime–protein
boost group had a significantly stronger ($P<0.05$)
lymphocyte proliferative response than the protein group
on day 84.

In the plasmid prime–protein boost group, the number
of IFN-γ-producing cells dramatically increased after the
protein boost and, consequently, was significantly higher
($P<0.05$) in this group than in the plasmid or protein
group on days 63 and 84 (Fig. 7b). The number of IFN-γ-
producing cells remained the same on days 63 and 84 in
both the plasmid and the plasmid prime–protein boost
groups, but decreased significantly ($P<0.05$) in the pro-
tein group. Consequently, the number of IFN-γ-producing
cells in the plasmid group was similar to that in the protein
group on day 63, but was significantly higher ($P<0.05$) on
day 84. These results suggest that the NS3-specific cellular
immune responses elicited by vaccination with pBISIA24-
NS3 or by priming with pBISIA24-NS3 followed by boosting
with rNS3 + CpG + Quil A were stronger and maintained
longer than the responses induced by immunization with
rNS3 + CpG + Quil A.

**DISCUSSION**

Since cell-mediated immunity to HCV proteins plays a
major role in virus resolution, the objective of this study
was to optimize cellular immune responses induced by
NS3, while retaining the ability to induce humoral immune
responses. Amongst the three vaccination regimes evalu-
ated, priming with plasmid encoding NS3, followed by
boosting with rNS3 formulated with CpG ODN and Quil A,
resulted in optimal NS3-specific immune responses.

A previous study has demonstrated that plasmid encod-
ing NS3 induces strong CTL responses in BALB/c mice,
whereas NS3 formulated with complete Freund’s adjuvant
(CFA) elicited antibody, but no CTL responses (Encke et al.,
1998). In another report, NS3 in PBS or CFA was shown to
induce primarily IgG1 in BALB/c mice, whereas plasmid
coding NS3 elicited predominantly IgG2a. Furthermore,
T-cells from DNA-vaccinated mice tended to produce more IFN-γ and IL-2 than those from rNS3-immunized mice (Ladzina et al., 2001). In the current study, rNS3 was formulated for the first time with CpG ODN and/or Quil A. Generally, either CpG ODN or Quil A can enhance both cellular and humoral immune responses (Klinman, 2003; Stittelaar et al., 2000). However, we showed that only when rNS3 was formulated with both CpG ODN and Quil A was a balanced immune response induced with production of high numbers of IFN-γ- and IL-4-secreting cells, while rNS3 combined with either CpG ODN or Quil A produced high levels of IL-4, but low IFN-γ. The dramatic increase of IFN-γ production in the rNS3 + CpG + Quil A group may be related to either the dose or the properties of the protein. NS3 is a non-structural protein, which tends to be less immunogenic compared to structural proteins. Therefore, the dose of NS3 was higher than that of, for example, BHV-1 gD, which was formulated with 10 μg of ODN 1826 at 0-2 μg per dose, and induced a Th1-type response (Ioannou et al., 2002b). In support of this contention, a reduction of the rNS3 dose from 20 to 5 μg in the second mouse trial tended to shift the response to be more Th1-biased. Formulation of NS3 with CpG ODN and Quil A might be an excellent approach to induce strong and balanced immune responses in humans, since both CpG ODN and purified fractions of Quil A cause low side-effects (Ioannou et al., 2003; Rimmelzwaan & Osterhaus, 1995). Furthermore, this adjuvant combination will be useful in general for proteins that elicit low or Th2-biased immune responses.

As an approach to induce stronger cell-mediated immune responses to HCV NS3, we evaluated two additional vaccination regimes consisting of either vaccination with pBISIA24-NS3 or priming with pBISIA24-NS3 followed by boosting with rNS3 + CpG + Quil A. Previous studies have demonstrated varying efficacy of heterologous prime–boost strategies for NS3 compared to a single DNA vaccination regime. A DNA prime–canarypox virus boost regime elicited stronger cellular immune responses to HCV structural and non-structural proteins when compared to vaccination with HCV DNA alone (Pancholi et al., 2003). In contrast, no difference was observed between the immune responses induced by a DNA-based NS3 vaccine, recombinant Semliki Forest virus (SFV) particles expressing NS3 (rSFV), or a DNA prime–rSFV boost regime (Brinster et al., 2002). In our study, the DNA-based NS3 vaccine and the DNA prime–protein boost regime elicited very similar cell-mediated responses, both inducing a Th1-type response characterized by a predominance of IFN-γ and high cytotoxicity in mice.

Interestingly, compared to previous reports (Ladzina et al., 2001; Pancholi et al., 2003; Encke et al., 1998), pBISIA24-NS3 induced relatively high NS3-specific antibody titres, which may be due to the selection of the intradermal route for immunization. Furthermore, the overall antibody responses induced by the DNA prime–protein boost regime were significantly stronger than the responses elicited by the DNA vaccine alone. Several studies suggest a role for virus neutralizing antibodies in resolution of HCV infection (Shimizu et al., 1996; Farci et al., 1994; Choo et al., 1994), whereas two other studies rule out the role of such antibodies in recovery (Takaki et al., 2000; Cooper et al., 1999). This suggests that humoral immunity might have an impact on prevention of infection. Although NS3-specific antibody production is not expected to neutralize the virus, the DNA prime–protein boost regime might be the appropriate approach for induction of both humoral and cellular immunity to the surface proteins E1 and E2.

Because the natural host and the closely related primates are not readily available, only a few HCV vaccine studies have been carried out in large animal species. In addition to non-human primate and monkey trials, most of which have tested the envelope proteins of HCV (Bukh et al., 2001; Dueñas-Carrera et al., 2004), sheep have been used in a vaccine study on HCV core (Acosta-Rivero et al., 2002). Furthermore, plasmids encoding other human products such as human growth hormone have been tested in both dogs and pigs (Anwer et al., 1999). We chose pigs as our model because they are more closely related to humans and are used for xenografts. The NS3-specific cellular immune responses produced in pigs after vaccination with pBISIA24-NS3 followed by rNS3 + CpG + Quil A were very strong and appeared to be at least as good as those in mice vaccinated with the same regime. The mean number of IFN-γ-secreting cells in the ELISPOT assay was more than 200 per 10^6 cells and this number was maintained on day 35 after the last immunization, suggesting that, as in the mice, excellent and long-lasting immunity to NS3 was produced in this group. Interestingly, the humoral immune responses induced to NS3 in mice and pigs were quite different. As expected from previous large animal studies, the mice produced much higher serum IgG titres than the pigs after vaccination with pBISIA24-NS3. However, after vaccination with pBISIA24-NS3 followed by rNS3 + CpG + Quil A, the NS3-specific IgG titre increased about fivefold in mice and 100-fold in pigs compared to immunization with plasmid alone. These data suggest that the piglets were very well primed by the DNA vaccine, resulting in significantly enhanced NS3-specific antibody responses following the protein boost. Thus, immune responses developed in the mouse and pig model may not entirely correlate, which supports the need for testing vaccine strategies in an outbred large animal. Based on the excellent immune responses induced in the pig model, it will be of interest to test the DNA prime–protein boost vaccination strategy in primates.

In conclusion, our study demonstrated that a vaccination regime of priming with pBISIA24-NS3 followed by boosting with rNS3 formulated with CpG ODN and Quil A elicited a strong cell-mediated, as well as humoral, immune response, both in mice and in piglets. This strategy might be of great value in general for vaccine development against HCV as well as many other pathogens.
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