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Hepatitis C virus polyprotein vaccine formulations capable of inducing broad antibody and cellular immune responses

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Although approximately 3 % of the world's population is infected with *Hepatitis C virus* (HCV), there is no prophylactic vaccine available. This study reports the design, cloning and purification of a single polyprotein comprising the HCV core protein and non-structural proteins NS3, NS4a, NS4b, NS5a and NS5b. The immunogenicity of this polyprotein, which was formulated in alum, oil-in-water emulsion MF59 or poly(DL-lactide co-glycolide) in the presence or absence of CpG adjuvant, was then determined in a murine model for induction of B- and T-cell responses. The addition of adjuvants or a delivery system to the HCV polyprotein enhanced serum antibody and T-cell proliferative responses, as well as IFN-γ responses, by $CD4^+$ T cells. The antibody responses were mainly against the NS3 and NS5 components of the polyprotein and relatively poor responses were elicited against NS4 and the core components. IFN-γ responses, however, were induced against all of the individual components of the polyprotein. These data suggest that the HCV polyprotein delivered with adjuvants induces broad B- and T-cell responses and could be a vaccine candidate against HCV.

INTRODUCTION

Hepatitis C virus (HCV) is the leading cause of parenterally transmitted non-A, non-B viral hepatitis (Armstrong *et al.*, 2000; Choo *et al.*, 1989). Approximately 3 % of the world's population are infected with HCV (Cohen, 1999) and about 30 000 newly acquired HCV infections occur in the USA annually, mostly as a result of intravenous drug abuse (Alter, 1993). Currently, there is no vaccine available to prevent HCV infection and the only available therapies, IFN-α and ribavirin, are effective in fewer than half of the patients treated (McHutchison *et al.*, 1998; Poynard *et al.*, 1998). Therefore, there is an urgent need for the development of an efficacious vaccine to prevent HCV infection.

We previously developed an experimental vaccine comprising a recombinant gpE1 and gpE2 heterodimer that protects chimpanzees against experimental challenge with both homologous (Choo *et al.*, 1994) and heterologous (Coates *et al.*, 2004) genotype 1a strains, which predominate in the USA and also occur worldwide. Natural immunity to HCV infection has been linked with early and broad Th1-type cellular immune responses to non-structural proteins NS3, NS4 and NS5 and the nucleocapsid (C) protein (Diepolder *et al.*, 1995; Ferrari *et al.*, 1997; Gerlach *et al.*, 1999; Tsai *et al.*, 1997). More recent data have shown the importance of Th1-type $CD4^+$ T-cell responses in protection in chimpanzees (Grakoui *et al.*, 2003) and humans (Lechner *et al.*, 2000).

To enhance the immunogenicity of recombinant protein-based vaccines, adjuvants are required. The most widely used adjuvants are insoluble aluminium salts, generically called alum. However, although alum adjuvants have been used for decades and are generally safe, they induce predominantly a Th2-type cytokine response (Lindblad, 2004; Raz & Spiegelberg, 1999; Valensi *et al.*, 1994). Therefore, alternative adjuvants may be required for the successful development of an HCV vaccine. Among many alternative approaches available, polymeric microparticles have been evaluated as vaccine adjuvants (Eyles *et al.*, 2003; O'Hagan & Singh, 2003; Singh *et al.*, 2004a, b). Moreover, in a non-human primate model, poly(DL-lactide co-glycolide) (PLG) microparticles have already been shown to induce potent T-cell lymphoproliferative responses against an adsorbed

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recombinant antigen from human immunodeficiency virus (HIV) (Otten *et al.*, 2003). Hence, PLG microparticles appear to be an attractive approach for the development of new-generation adjuvants and in these studies we assessed these microparticles for the delivery of the polyprotein from HCV.

Various oil-in-water emulsions have also been developed as alternative adjuvants to alum. The most advanced of these is a squalene oil-in-water emulsion (MF59), which is a potent adjuvant with an acceptable safety profile and has gained approval for licensure in an influenza vaccine product in Europe called Fluad (Ott *et al.*, 1995; Podda & Del Giudice, 2003).

To enhance Th1-type responses to vaccines, immunopotentiating adjuvants may be added to 'delivery'-based adjuvants. CpG is an immune potentiator that has been shown to induce Th1-type responses selectively in small-animal models (Klinman *et al.*, 2004; Schetter & Vollmer, 2004). In addition, the inclusion of CpG into MF59 or PLG formulations for HIV vaccines has been shown to enhance antibody, as well as Gag-specific, cytotoxic T-cell responses (O'Hagan *et al.*, 2002; Singh *et al.*, 2001).

In this study, we designed, cloned, expressed and purified a single polyprotein comprising the HCV core and NS3, NS4a, NS4b, NS5a and NS5b proteins, designated NS345Core. We determined the *in vivo* immunogenicity of the purified polyprotein formulated with alum, MF59 or PLG in the presence or absence of CpG for induction of B- and T-cell responses in a murine model.

METHODS

Construction of HCV-1a ΔNS3–NS5 (aa 1242–3011) with the core protein (aa 1–121 or 1–173). The plasmid pT7-HCV was created in a polylinker-modified pUC18 vector (New England Biolabs) to contain full-length HCV-1a cDNA (Choo *et al.*, 1991) preceded by a synthetic T7 promoter. pT7-HCV also contains the complete 5′ UTR and the poly(A) version of the 3′ UTR followed by an *Xba*I site. The plasmid pCMV-KMΔNS3-5 was generated by ligating a PCR fragment that encodes a Kozak initiator methionine followed by aa 1242 of HCV-1a to a 3′ *Stu*I site. This product was ligated to a *Stu*I–*Xba*I fragment from pT7-HCV along with pCMV-KM2 vector (Murphy *et al.*, 1998).

The termini of the polyprotein-encoding DNA were modified to be compatible with restriction sites in the pBS24.1 yeast shuttle vector, which contains 2μ sequences for autonomous replication in yeast, the α-factor terminator to ensure transcription termination, the yeast genes *leu2-d* and URA3 for selection, the *Col* E1 origin of replication and the β-lactamase gene required for plasmid replication in bacteria (Lin *et al.*, 2005). Thereafter, the hybrid ADH2/GAPDH promoter was cloned 5′ to the polyprotein-coding sequence to generate pd.ΔNS3NS5.PJ. This plasmid was transformed into *Saccharomyces cerevisiae* strain AD3 [*MAT*α, *leu*Δ, *ura3-52*, *prb-1122*, *pep4-3*, *prc1-407* (CIR^0), ::pDM15 (GAP/ADR)]. Clone 5 was analysed for expression and a band with the expected molecular mass of 194 kDa was observed by Coomassie blue staining (Fig. 1). Thereafter, the core was appended on to the C terminus of the ΔNS3NS5 protein. Two core sequences were used: one terminated at aa 121 and the other at aa 173.

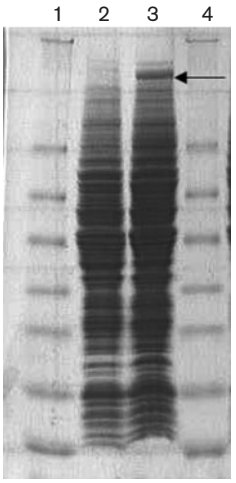

Fig. 1. Expression level of clone pd.ΔNS3NS5.PJ from the plasmid pd.ΔNS3NS5 clone #17 shown by Coomassie blue staining. See Methods for details. A protein band of the expected molecular mass of 194 kDa is shown (arrow). Lanes: 1 and 4, size standards; 2, control yeast plasmid; 3, pd.ΔNS3NS5.PJ colony 1.

The core sequences used the reverse-transcribed version, where aa 9 is Arg rather than Lys and aa 11 is Thr rather than Asn. Strain AD3 was transformed with pd.ΔNS3NS5.PJ.core121RT clone #6 and pd.ΔNS3NS5.PJ.core173RT clone #15 and checked for expression. Protein bands of the expected molecular masses of 206 and 210 kDa, respectively, were observed by Coomassie blue staining. Expression levels of the pd.ΔNS3NS5.PJ.core173RT construct were consistently less (data not shown) than that of the pd.ΔNS3NS5.PJ.core121RT construct (Fig. 2a). Therefore, in all of the immunogenicity studies, the pd.ΔNS3NS5.PJ.core121RT construct was used.

Purification of the HCV NS345Core$_{121}$ polyprotein. Yeast cells expressing insoluble HCV polyprotein intracellularly were lysed by milling with glass beads in a buffer containing 50 mM Tris/HCl, 100 mM sodium chloride, 10 mM dithiothreitol (DTT) and 1 mM PMSF at pH 8·0. The lysate was collected and centrifuged at 39 200 ***g*** (Beckman JA-20 rotor at 18 000 r.p.m.) at 22 °C for 30 min. The pellet was washed by resuspending in 50 mM Tris/HCl, 100 mM sodium chloride, 2 M urea, 0·1 % octyl glucoside and 1 mM PMSF at pH 8·0 and mixing well with an OmniMixer. The centrifugation was repeated and the pellet was washed a second time by resuspending in PBS plus 50 mM DTT and 1 % Tween 20 (Sigma). The pH of the suspension was raised to pH 12 with 6 M sodium hydroxide and the suspension was mixed by stirring at room temperature for 30 min. The pH was lowered to pH 8·0 with 6 M hydrochloric acid, more DTT was added to a final concentration of 0·1 M and the suspension was centrifuged as before.

HCV polyprotein was extracted from the insoluble pellet by suspending in 50 mM Tris/HCl, 50 mM DTT and 2 % SDS at pH 8·0, raising the pH to 12 for 30 min while stirring, followed by neutralization to pH 8·0. The centrifugation was repeated and the supernatant containing soluble HCV polyprotein was purified further by size-exclusion chromatography using a Superdex 200 column equilibrated with PBS containing 0·1 % SDS and 5 mM DTT. Fractions containing the purest HCV polyprotein as determined by SDS-PAGE were pooled and dialysed using Spectrapore dialysis tubing (molecular mass cut-off of 25 kDa) against water for 6 h at room temperature to

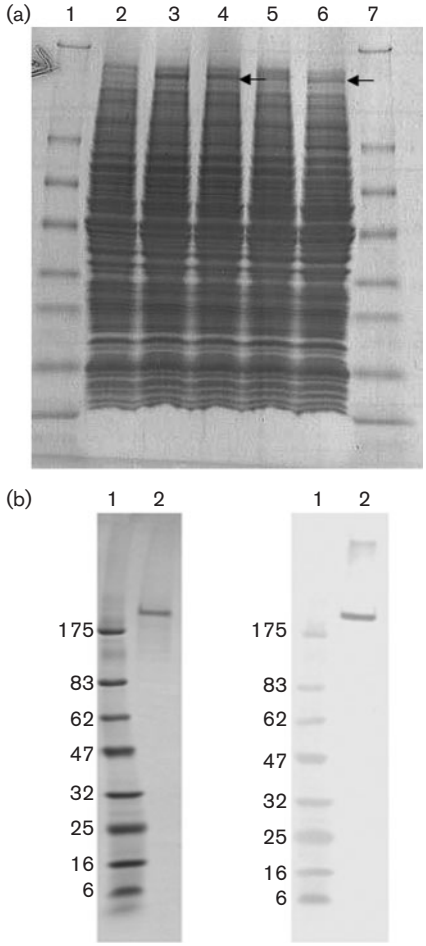

Fig. 2. (a) Expression levels of the pd.ΔNS3NS5.PJ.core173RT and pd.ΔNS3NS5.PJ.core121RT constructs shown by Coomassie blue staining. See Methods for details. Protein bands of the expected molecular masses of 206 and 210 kDa, respectively, are shown (arrows). Expression of the pd.ΔNS3NS5.PJ.core173RT construct was considerably lower than that of the pd.ΔNS3NS5.PJ.core121RT construct. Lanes: 1 and 7, size standards; 2, control yeast plasmid; 3, 4, pd.ΔNS3NS5.PJ.core121RT colonies 1 and 2; 5 and 6, pd.ΔNS3NS5.PJ.core173RT colonies 3 and 4. (b) Coomassie blue staining (left) and Western blot (right) of the purified HCV $NS345Core_{121}$ polyprotein. Lane 1, Pre-stained molecular mass markers (Broad Range; New England Biolabs); lane 2, 0·5 mg Superdex 200 gel filtration pool.

remove salts. The purified HCV polyprotein was concentrated by lyophilizing to dryness and redissolving the protein in a minimal volume of buffer (20 mM Tris/HCl, 8 M urea, 5 mM DTT at pH 8·0) to achieve complete solubilization. Buffer exchange into the final buffer was performed by dialysing into a buffer of PBS plus 6 M urea and 5 mM DTT. The expected molecular mass was confirmed by Coomassie blue staining and the antigenicity was confirmed by Western blotting (Fig. 2b). The Western blot was performed by transferring a Tris/glycine gradient gel to 0·2 mm nitrocellulose (Bio-Rad), blocking for 10 min with TBST (Tris-buffered saline with 0·2 % Tween 20) containing 1 % powdered milk and shaking at room temperature for 1 h. The primary antibody was an anti-HCV-C22 monoclonal antibody (specific for the HCV core protein) (developed at Chiron Corporation), which was diluted 1 : 2000 in TBST with 1 % powdered milk and incubated with shaking at room temperature for 1 h. The membrane was washed three times for 5 min each with TBST. The secondary antibody was an alkaline phosphatase-conjugated goat anti-mouse antibody (Boehringer Mannheim) diluted 1 : 2000 in TBST with 1 % powdered milk and incubated with shaking at room temperature for 1 h. The membrane was washed three times for 5 min each with TBST and developed using Western blue stabilized substrate for alkaline phosphatase (Promega).

Formulation of the HCV polyprotein with adjuvants. RG503 PLG with a 50 : 50 co-polymer composition (intrinsic viscosity of 0·4 from the manufacturer's specifications) was obtained from Boehringer Ingelheim. Dioctylsulfosuccinate (DSS) and urea were from Sigma. $NS345Core_{121}$ and MF59C were obtained from Chiron Corporation. Aluminium phosphate was obtained from Superfos. All other reagents were obtained from Sigma. CpG (1826; Oligos Incorporation) was added at a concentration of 25 μg per dose to the delivery system (alum, PLG or MF59) before immunization.

PLG/$NS345Core_{121}$ formulation. PLG microparticles were prepared by a solvent evaporation method as described previously (Kazzaz *et al.*, 2000; Singh *et al.*, 2004a). Briefly, microparticles were prepared by homogenizing 10 ml 6 % (w/v) polymer solution in methylene chloride with 2·5 ml PBS using a 10 mm probe (Ultra-Turrax T25; IKA-Labortechnik). The water-in-oil emulsion thus formed was then added to 50 ml distilled water containing DSS and the mixture was homogenized at high speed with a 20 mm probe (ES-15 Omni International) for 25 min in an ice bath. This procedure resulted in the formation of water-in-oil-in-water emulsion, which was then stirred at 1000 r.p.m. for 12 h at room temperature and the methylene chloride allowed to evaporate. The size distribution of the resulting microparticles was determined with a particle size analyser (Master Sizer; Malvern Instruments).

Microparticles with adsorbed protein were prepared by incubating a suspension containing 100 mg blank PLG microparticles with 1 mg protein in PBS with 8 M urea at pH 9. The suspension was allowed to mix on a laboratory rocker (Aliquot Mixer; Miles Laboratories) overnight. The suspension was then dialysed against several changes of water and lyophilized in aliquots containing 250 μg protein each, which comprised ten doses.

Alum/$NS345Core_{121}$ formulation. The alum formulation was made by incubating 100 mg aluminium phosphate with 1 mg protein in PBS with 8 M urea at pH 5·5 at a final concentration of 0·5 mg ml^{-1}. The suspension was aliquotted in vials containing 250 μg protein each, which comprised ten doses.

MF59/$NS345Core_{121}$ formulations. MF59 formulations were prepared by adding 250 μl MF59C (citrate) with 50 μl 5 mg $NS345Core_{121}$ ml^{-1} and 200 μl PBS and mixing immediately prior to injection.

Mice and immunizations. Female BALB/c or C57BL/6 mice aged 6–8 weeks at the onset of the study were used. Six (proliferation) or ten (ELISPOT and ELISAs) mice per group were used for each immunization. All animal studies were approved by the Chiron Animal Use and Care Committee. Intramuscular (i.m.) immunizations were performed in the thigh with or without anaesthesia once or twice at 3 week intervals. Mice were sacrificed at 8 days after one immunization or at 2 weeks following the second immunization to collect tissues for the proliferation and ELISPOT assays, respectively.

Sera and tissue collection. Mice were bled through the retro-orbital plexus 2 weeks after each immunization to prepare sera

for the ELISAs. Popliteal lymph nodes and spleen were meshed through a nylon mesh to prepare single-cell suspensions. Spleens were assayed from pools of all mice in each group unless stated otherwise.

Proliferation assay. Popliteal lymph node cells were prepared from six mice per group 8 days after a single immunization to measure proliferative responses against HCV NS345Core$_{121}$ polyprotein (10 μg ml^{-1}). Concanavalin A was used as positive control and cells with no antigen in the medium were used as negative controls. The data are represented as mean c.p.m. ± SD of six individual mice per group. Results are representative of one experiment of three.

ELISPOT assay. Single-cell suspensions from pooled spleen from five mice per group were prepared and adjusted to concentrations of 1×10^7 to 3×10^7 cells ml^{-1} in culture medium (50 % RPMI 1640, 50 % alpha-MEM with 10 % heat-inactivated fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol and 1 % antibiotics). One hundred microlitres from each cell preparation was added in duplicate to the first rows of 96-well PVDF plates (Milipore) pre-coated with rat anti-mouse IFN-γ (BD PharMingen) and 2-fold serial dilutions were performed. Pooled synthetic peptides (20mers overlapping by 10 aa, 1–3 μg per well) encompassing the entire core, NS3, NS4, NS5a and NS5b sequences of the HCV-1a genotype or the NS345Core$_{121}$ protein (10 μg ml^{-1} in 100 μl), synthesized using Fmoc solid-phase methods by Research Genetics or Chiron Mimotopes, were then added to the cells and stimulated overnight for 14–16 h at 37 °C. As a negative control, cells were incubated without the pool of peptides. All peptides had free N and C termini. Following overnight incubation, the plates were washed and biotinylated anti-IFN-γ (BD PharMingen) was added. The plates were incubated at room temperature for 2 h and washed. Avidin–peroxidase (BD PharMingen) was added and the plates were incubated for 30 min at 37 °C and then washed. The plates were developed with aminoethyl carbazole solution (Sigma) for 30 min. The results from two experiments are presented as the mean ± SD of IFN-γ-secreting cells per 1×10^7 mononuclear cells from a minimum of four wells from pools of five mice per group with splenocytes added in duplicate. The background number of spots averaged 20 in the wells with cells without peptide stimulation.

Flow cytometric analysis following intracellular and surface staining. Two weeks after the final immunization, spleens were harvested from two pools of five mice each and single-cell suspensions were prepared. Spleen cells (1×10^6 cells in 200 μl) were cultured at 37 °C in the presence or absence of pools of 20mer overlapping peptides covering the NS3, NS4 and NS5a + b region at a concentration of 10 μg ml^{-1} using 100 μl per well or with monoclonal antibodies directed against CD3 and CD28 (both from BD PharMingen) as a positive control. BD GolgiPlug (BD PharMingen) was added to block cytokine secretion. After 6 h, cells were washed, incubated with BD Fc Block (anti-CD16/32; BD PharMingen) to block Fc-γ receptors and stained for cell-surface antigens CD4 and CD8 using the BD Cytofix/Cytoperm kit (BD PharMingen) following the manufacturer's instructions. Cells were then fixed in 2 % (w/v) paraformaldehyde and stored overnight at 4 °C. The following day, cells were permeabilized and stained for intracellular IFN-γ with a phycoerythrin (BD PharMingen)-conjugated mouse IFN-γ monoclonal antibody in the presence of 0·1 % (w/v) saponin. Cells were washed and analysed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). A 2-fold increase above the background response of 0·01 % was considered to be positive for CD4$^+$ or CD8$^+$ intracellular IFN-γ-expressing cells. For all flow cytometric analyses, the data are presented as means ± SD of two pools of five mice each from two experiments.

ELISAs. Serum IgG titres specific for NS345Core$_{121}$, NS3, NS4, NS5 or core protein were quantified by a standard ELISA. Briefly, ELISA plates (96-well U-bottom plates; Nunc MaxiSorp) were coated with purified NS345Core$_{121}$ protein at a concentration of 3 μg per well in PBS overnight at 4 °C. After washing with PBS plus 0·03 % Tween 20, the wells were blocked with PBS plus 2 % fetal calf serum for 30 min at 37 °C and serum samples were added in an initial dilution of 1 : 5000. Serial dilutions of 1 : 2 were performed in the blocking reagent. A standard serum was included in each assay as a positive control. The samples and standard sera were incubated at 4 °C overnight and washed with PBS plus 0·03 % Tween 20. The plates were washed and horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) diluted 1 : 10 000 in PBS plus 2 % fetal calf serum was added and incubated for 2 h at room temperature. The plates were washed and developed with tetramethylbenzidine (Kirkegaard & Perry Laboratories) for 15 min and then development was stopped with 2 M HCl. The absorbance of each well was measured using a Titertek absorbance reader at 450 nm. The data are presented as mean titres ± SEM of six to ten mice per group.

Statistical analysis. Student's *t*-test on log-transformed values was performed for two geometric means assuming equal variances (*F*-test, $P>0{\cdot}05$), using Microsoft EXCEL.

RESULTS

Enhanced serum anti-HCV responses following immunizations with polyprotein in delivery systems

To determine whether the designed and yeast-expressed HCV polyprotein could induce B-cell responses *in vivo*, we immunized mice i.m. with the HCV polyprotein in various delivery systems. We found that the HCV polyprotein alone did not induce antigen-specific serum antibodies (Fig. 3a). In contrast, mixing the HCV polyprotein with the oil-in-water emulsion MF59 or adsorption to PLG microparticles induced serum antibody responses. Immunization i.m. with the HCV polyprotein adsorbed to PLG induced the highest antibody responses, followed by mixing with MF59, whereas adsorption to alum induced very low antibody titres (Fig. 3a). To determine whether differential specific responses were induced to each component of the polyprotein, we next measured the serum antibody responses against each of the vaccine components, i.e. NS3, NS4, NS5, core and the polyprotein, in the group immunized with alum. We found that serum antibody titres were greatest against the NS3 and NS5 proteins, whilst they were considerably lower against the NS4 protein, three orders of magnitude lower than anti-NS3 and -NS5 responses (Fig. 3b). Moreover, the serum antibody responses were also relatively low against the core antigen, two orders of magnitude lower than anti-NS3 and -NS5 titres. These data showed that, although the expressed and purified HCV core polyprotein was immunogenic in mice when given together with various delivery systems, the response against each of the components was different, with the majority of the antibody response being against the NS3 and NS5 components.

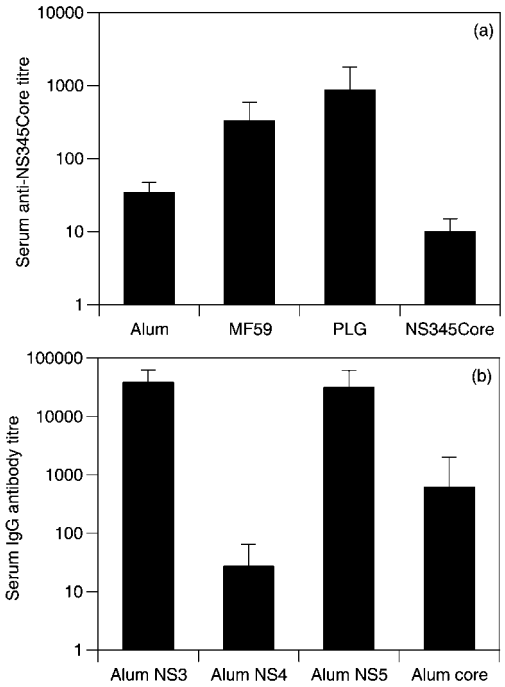

Fig. 3. Serum anti-NS345Core$_{121}$ (a) or anti-NS3, -NS4, -NS5 and -core IgG (b) responses following immunization with NS345Core$_{121}$ in the various delivery systems. Ten mice per group were immunized i.m. twice with a 3 week interval. The mice were bled 2 weeks after the second immunization to collect sera for the ELISA. The data are presented as mean serum IgG titres ± SD.

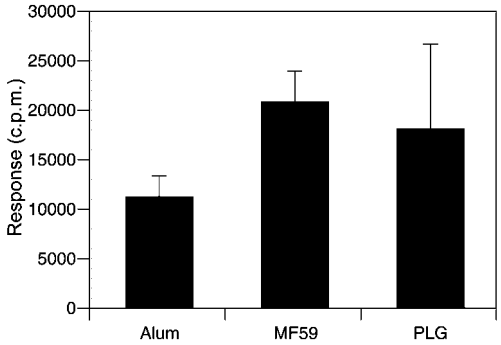

Fig. 4. T-cell proliferative responses in lymph nodes following immunization with NS345Core$_{121}$ in the various delivery systems. Six mice per group were immunized once i.m. in the tibialis anterior muscle. Eight days later, single-cell suspensions from popliteal lymph nodes were prepared for the proliferation assay and incubated over 5 days with the NS345Core$_{121}$ polyprotein. The data are represented as mean c.p.m. ± SD of six individual mice per group and are representative of one experiment of three.

T-cell proliferative responses following restimulation of cells with HCV polyprotein

As both B- and T-cell responses are considered to be important in immunity against HCV, we next determined the T-cell response. Eight days after a single i.m. immunization with HCV polyprotein in the various delivery systems, single-cell suspensions were prepared from popliteal lymph nodes, which drain the tibialis anterior muscle, and restimulated *in vitro* in the presence of the HCV polyprotein for 3 days. We found that the polyproteins alone did not induce antigen-specific proliferative responses (data not shown). However, mixing the HCV polyprotein with MF59 or adsorption to alum or PLG microparticles induced T-cell proliferative responses (Fig. 4). Alum induced the lowest proliferative response, whilst the responses were similar following immunization with PLG or MF59. These data showed that immunization with the expressed and purified HCV polyprotein in the various delivery systems was able to induce T-cell proliferative responses.

T-cell cytokine responses following restimulation of cells with various peptides derived from the HCV polyprotein

As a measure of T-cell responses, we next measured IFN-γ responses in the spleen following immunization with HCV polyprotein. In order to distinguish which proteins within the polyprotein were the most immunogenic, we stimulated the splenocytes with peptides derived from the core, NS3, NS4, NS5a and NS5b proteins and the whole polyprotein, i.e. NS345Core$_{121}$. We found that immunization with PLG or alum induced the highest frequency of IFN-γ-secreting cells following restimulation with the polyprotein or any of the peptides, whilst immunization with MF59 induced the lowest frequency of IFN-γ-secreting cells following restimulation with the polyprotein or any of the peptides (Fig. 5). Restimulation with NS345Core$_{121}$ generally induced the highest response, whilst restimulation with the core peptide induced the lowest response. NS3, NS4, NS5a and NS5b appeared to be equally immunogenic for the induction of IFN-γ responses. Thus, these data showed differential IFN-γ responses to various components of the HCV polyprotein.

Enhanced serum anti-HCV responses following immunization with polyprotein in various delivery systems plus CpG

To enhance the serum anti-HCV polyprotein responses further, we immunized mice i.m. with the HCV polyprotein in the various delivery systems in the presence or absence of the immunopotentiating adjuvant CpG. We found that the HCV polyprotein given as a mixture with MF59 plus CpG induced serum antibody responses that were >20-fold higher compared with protein in MF59 without CpG (Fig. 6a). Immunization i.m. with the HCV polyprotein adsorbed to alum plus CpG also induced >20-fold higher serum antibody responses compared with alum alone. The lowest increase in serum antibody titres (<3-fold) was seen when the polyprotein was adsorbed to PLG in the presence of CpG compared with in the absence of CpG. The serum

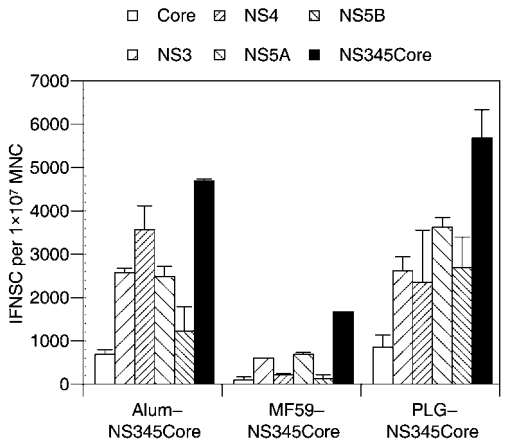

Fig. 5. IFN-γ-secreting cells in response to *ex vivo* restimulation with various HCV non-structural- and core-derived peptide pools. Ten mice per group were immunized i.m. twice with a 3 week interval. The mice were sacrificed 2 weeks after the second immunization to collect spleens for the IFN-γ ELISPOT assay. The data are presented as the mean number of IFN-γ-secreting cells (IFNSC) per 1×10^7 mononuclear cells (MNC) from two pools of five mice each ± SD. The background number of spots (not shown) averaged 20 per 1×10^7 mononuclear cells in the wells with cells without peptide stimulation.

antibody responses against NS4 and core protein were far lower than the responses against NS3 and NS5. The addition of CpG to alum enhanced the serum antibody responses against NS3, NS5 and NS345Core$_{121}$, but not against NS4 and core, in a statistically significant manner ($P<0{\cdot}00019$ for NS3; $P<0{\cdot}0002$ for NS5; $P<0{\cdot}0008$ for NS345Core$_{121}$; Fig. 6b). These data showed that the addition of the immunopotentiating adjuvant CpG to the HCV NS345Core$_{121}$ polyprotein differentially enhanced serum antibody responses depending on the nature of the delivery system.

Enhanced T-cell proliferative anti-HCV response following immunization with polyprotein in various delivery systems plus CpG

As CpG enhanced the serum anti-HCV polyprotein response, we next determined whether the addition of CpG would also enhance the T-cell response. Eight days after i.m. immunization with HCV polyprotein in the various delivery systems plus CpG, single-cell suspensions were prepared from popliteal lymph nodes and restimulated *in vitro* in the presence of the HCV polyprotein for 3 days. We found that the addition of CpG to alum enhanced the proliferative response compared with alum alone (Fig. 7). However, the addition of CpG to MF59 or PLG did not enhance the proliferative response further. These data showed that immunization with the expressed and purified HCV core polyproteins in the presence of the various delivery systems and CpG induced differential enhancement of the T-cell proliferative response depending on the delivery system used.

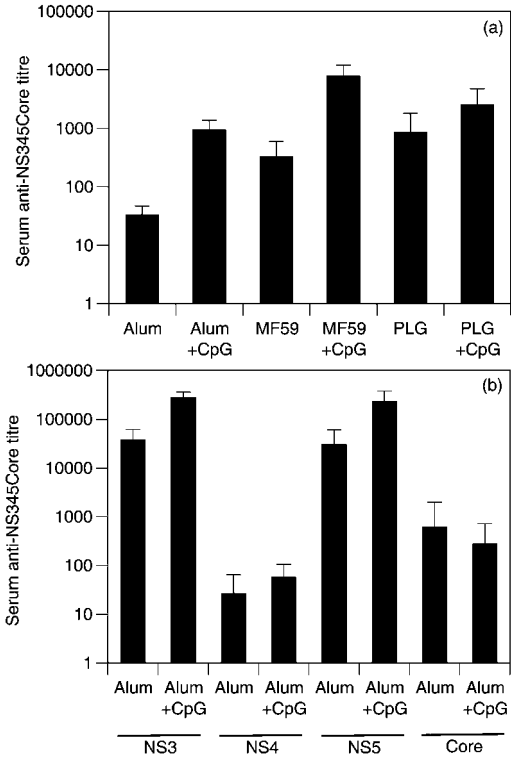

Fig. 6. Serum anti-NS345Core$_{121}$ (a) or anti-NS3, -NS4, -NS5 and -core IgG (b) responses following immunization with NS345Core$_{121}$ in the various delivery systems with or without CpG. Ten mice per group were immunized i.m. twice with a 3 week interval. The mice were bled at 2 weeks after the second immunization to collect sera for the ELISA. The data are presented as mean serum IgG titres ± SD.

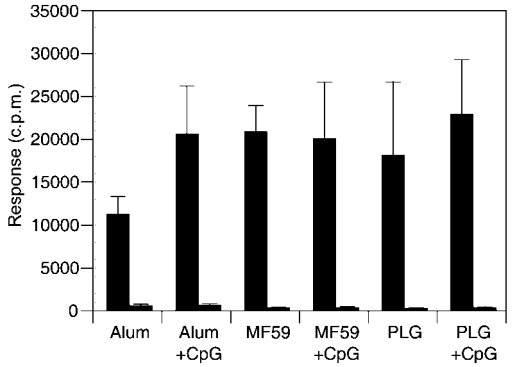

Fig. 7. T-cell proliferative responses in lymph nodes following immunization with NS345Core$_{121}$ in the various delivery systems with or without CpG. Six mice per group were immunized once i.m. Eight days later, single-cell suspensions from popliteal lymph nodes were prepared for the proliferation assay. The data are represented as mean c.p.m. ± SD of six individual mice per group.

Next, to determine whether a $CD4^+$ or $CD8^+$ T-cell response was induced by immunization with and without CpG, we measured the antigen-specific responses to pools of peptides derived independently from each of the NS3, NS4 and NS5 proteins by intracellular and surface staining and by flow cytometric analysis. We found that the majority of the $CD4^+$ T-cell response was directed against NS4, followed by the NS5 component (Fig. 8a). Moreover, CpG appeared to enhance the response against all three antigens, although this was not statistically significant (Fig. 8a). Importantly, $CD8^+$ T-cell responses were not induced against any of the non-structural components in either the presence or absence of CpG (Fig. 8b). These data suggest that the T-cell response was predominantly induced in the $CD4^+$ T-cell population and that this response was enhanced in the presence of CpG.

DISCUSSION

Understanding the correlates of immune response versus protection will result in the design of more effective vaccines against a number of infectious diseases for which vaccines do not currently exist. Although the correlates of protection against HCV infection and disease are not well established, there are growing indications for the role of HCV-specific immune responses in the resolution or amelioration of infection and disease. As induction of antibody and T-cell responses against HCV appears to be increasingly important in designing an effective vaccine, we investigated whether both types of immune response could be induced by immunization with a recombinant polyprotein vaccine. DNA vaccines comprising one or all of the HCV proteins have been designed and evaluated previously (O'Hagan *et al.*, 2004; Xavier *et al.*, 1994). However, it has been difficult to produce a single polyprotein vaccine comprising the entire core and most of the non-structural proteins. Whilst the expression and purification of individual structural and non-structural HCV proteins has been performed (Selby *et al.*, 1993), our study is the first to report the expression, purification and *in vivo* immunogenicity of a protein vaccine that includes most of the non-structural proteins.

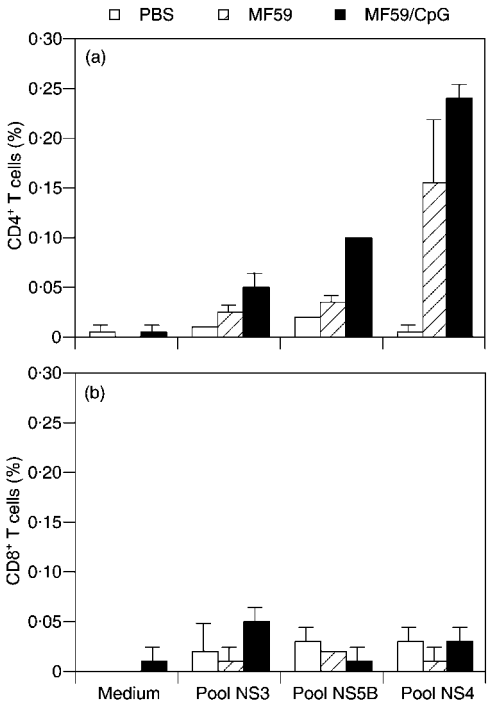

Fig. 8. IFN-γ responses by $CD4^+$ and $CD8^+$ cells against NS3, NS4 and NS5 measured by flow cytometry. Mice were immunized three times with the HCV $NS345Core_{121}$ polyprotein and splenocytes were restimulated with pools of peptides encompassing NS3, NS4 and NS5 proteins. The cells were stained intracellularly for IFN-γ and surface stained for CD4 (a) or CD8 (b). The data are presented as the mean percentage ± SD of IFN-γ-positive cells of total $CD4^+$ or $CD8^+$ cells of two experiments each with pools of spleens from five mice.

In this study, we evaluated three different delivery systems – alum, MF59 emulsion and PLG microparticles – for their ability to induce immune responses against the HCV polyprotein. Although alum is widely used in human vaccines, it was the least effective adjuvant for inducing serum IgG responses in mice. In contrast, MF59, which is also approved for human use, induced stronger responses than alum. This observation is consistent with previous studies using alternative antigens, which also showed enhanced immune responses using MF59 (Cataldo & van Nest, 1997; Heineman *et al.*, 1999; Higgins *et al.*, 1996; O'Hagan *et al.*, 1997; Traquina *et al.*, 1996).

The core protein is the most conserved region among the various HCV strains, making it an ideal vaccine component. However, there have been reports that the HCV core protein can cause cleavage of NS5a by caspases and induce apoptosis in transfected cells *in vitro* (Goh *et al.*, 2001). Nevertheless, a recent report provided evidence that, *in vivo*, co-expression of HCV core and NS5a did not reduce the immune response to either core or NS5a and did not induce measurable adverse effects (Liu *et al.*, 2002). More importantly, it has been suggested that the core protein inhibits tumour necrosis factor α-mediated apoptosis of HCV-infected cells, resulting in chronic infection (Ray *et al.*, 1998). Therefore, in our study, it was important to include not only the core, but also the other viral non-structural proteins, in a protein-based vaccine. In this regard, it was interesting to find that the serum antibody responses to core and NS4 were far lower than the responses to NS3 or NS5. It is also important to note that the inclusion of CpG further enhanced the humoral response, particularly in the MF59- and alum-based vaccines. The immunostimulatory effect of unmethylated CpG dinucleotides (Krieg *et al.*, 1995) is thought to be caused by recognition of the CpG motifs by cells of the innate immune system to allow discrimination of pathogen-derived DNA from self DNA (Bird, 1987).

Several studies have suggested that T-cell immunity to HCV can determine the outcome of HCV infection and disease

(Cooper *et al.*, 1999; Diepolder *et al.*, 1995; Lechner *et al.*, 2000; Missale *et al.*, 1996). One study concluded that individuals displaying predominant Th0/Th1 $CD4^+$ T-helper responses resolved their HCV infections, whilst those with Th2-type responses tended to progress to chronicity (Tsai *et al.*, 1997). In addition, it has been shown that there is an inverse correlation between the frequency of HCV-specific cytotoxic T lymphocytes and virus load (Nelson *et al.*, 1997). More recently, it was shown that the control of HCV in chimpanzees was associated with a Th1-type cellular immune response (Major *et al.*, 2002). Therefore, accumulated evidence suggests an important role for HCV-specific T-cell responses in controlling HCV infection. In this study, we sought to induce lymphoproliferative responses as well as broad cellular IFN-γ responses against various components of the HCV polyprotein vaccine.

The T-cell proliferative response in our study was considerable, bearing in mind that the animals had received only a single immunization with the vaccine candidates. Interestingly, although alum is widely used in human vaccines, it was the least effective in inducing lymph node proliferative responses. In contrast, MF59 and PLG induced similar and stronger responses than the alum vaccine. Although formulation of the polyprotein vaccine in delivery systems enhanced the response compared with no formulation, the inclusion of CpG did not enhance the proliferative response further, except for the alum group. To analyse in more detail the T-cell response with regard to the various components of the vaccine, IFN-γ responses following restimulation of splenocytes with various non-structural proteins or the core protein were evaluated. The response to the whole polyprotein was generally the highest. However, although the response to the core protein was the lowest, it was detectable. This is important in light of the fact that the core antigen is the most conserved region of HCV. The level of response to the other antigens was similar. Of note, whilst the MF59 formulation induced the lowest IFN-γ response, the alum and PLG formulations induced generally similar responses and PLG induced the highest response. These data demonstrate that formulation with PLG or alum induces cellular IFN-γ responses against each of the components of the polyprotein vaccine, including the core antigen.

In this study, one of our aims was to induce broad cellular responses against the various components of the HCV polyprotein. Interestingly, the addition of CpG enhanced both serum IgG and lymph node proliferative responses when the vaccine was administered in alum. In contrast, whilst the addition of CpG with MF59 enhanced serum IgG responses, it had little effect on proliferative responses when the vaccine was formulated in PLG or MF59. CpGs have been shown in small animal models to selectively induce Th1-type responses (Klinman *et al.*, 2004; Schetter & Vollmer, 2004). In our study, we demonstrated that the T-cell response generated was predominantly within the $CD4^+$ T-cell population and little or no $CD8^+$ T-cell IFN-γ response was generated in either the presence or absence of CpG. It is noteworthy that, whilst the antibody responses against NS3 and NS5 were higher than the antibody response against NS4, the IFN-γ response against NS4 was greater than the IFN-γ response against NS3 or NS5. We have made similar observations using DNA or virus-like particles for vaccinations, where a higher IFN-γ response resulted in a lower antibody response (unpublished observations). It has been shown that cellular responses to CpG DNA are dependent on the presence of toll-like receptor 9 (Hemmi *et al.*, 2000). In addition, it has been reported that CpG is taken up by non-specific endocytosis and that endosomal maturation is necessary for cell activation and the release of pro-inflammatory cytokines (Pulendran, 2004; Sparwasser *et al.*, 1998). Our data suggest that formulation of the HCV polyprotein vaccine in PLG induced the same level of serum antibody and antigen-specific proliferative responses as using MF59 or alum together with CpG. Furthermore, the PLG formulation induced higher IFN-γ responses compared with MF59. Therefore, PLG appears to be the optimal delivery system for the HCV polyprotein vaccine.

The mechanism of immune enhancement by the PLG microparticles was not addressed in this study. However, several other studies have shown that the immune enhancement mechanism of PLG involves presentation of multiple copies of antigen to the immune system and promotion of trapping and retention of antigens in local lymph nodes (O'Hagan & Singh, 2003; Singh *et al.*, 2004b). In addition, antigen uptake by antigen-presenting cells is enhanced by association of antigen with particles or by the use of polymers or proteins that self-assemble into particles (Kanke *et al.*, 1983; Tabata & Ikada, 1988, 1990).

In conclusion, our HCV polyprotein vaccine, formulated in various delivery systems with an adjuvant, was able to generate both humoral and cellular responses against the polyprotein antigen as whole or against its individual components. These and other formulations of the polyprotein will be tested in future for prophylactic and immunotherapeutic activity against HCV infection.

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