Intranasal immunization of mice with a formalin-inactivated bovine respiratory syncytial virus vaccine co-formulated with CpG oligodeoxynucleotides and polyphosphazenes results in enhanced protection

John W. Mapletoft, Mustapha Oumouna, Jennifer Kovacs-Nolan, Laura Latimer, George Mutwiri, Lorne A. Babiuk and Sylvia van Drunen Littel-van den Hurk

Vaccine and Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road, Saskatoon, SK S7N 5E3, Canada

As respiratory syncytial virus (RSV) targets the mucosal surfaces of the respiratory tract, induction of both systemic and mucosal immunity will be critical for optimal protection. In this study, the ability of an intranasally delivered, formalin-inactivated bovine RSV (FI-BRSV) vaccine co-formulated with CpG oligodeoxynucleotides (ODN) and polyphosphazenes (PP) to induce systemic and mucosal immunity, as well as protection from BRSV challenge, was evaluated. Intranasal immunization of mice with FI-BRSV formulated with CpG ODN and PP resulted in both humoral and cell-mediated immunity, characterized by enhanced production of BRSV-specific serum IgG, as well as increased gamma interferon and decreased interleukin-5 production by in vitro-restimulated splenocytes. These mice also developed mucosal immune responses, as was evident from increased production of BRSV-specific IgG and IgA in lung-fragment cultures. Indeed, the increases in serum and mucosal IgG, and in particular mucosal IgA and virus-neutralizing antibodies, were the most critical differences observed between FI-BRSV formulated with both CpG ODN and PP in comparison to formulations with CpG ODN, non-CpG ODN or PP individually. Finally, FI-BRSV/CpG/PP was the only formulation that resulted in a significant reduction in viral replication upon BRSV challenge. Co-formulation of CpG ODN and PP is a promising new vaccine platform technology that may have applications in mucosal immunization in humans.

INTRODUCTION

Human respiratory syncytial virus (HRSV) is a leading cause of respiratory disease in infants and young children worldwide (Heilman, 1990) and is responsible for significant economic loss; in the year 2000 alone, there were 86,000 HRSV infection-related hospitalizations in the USA, costing a total of $394,000,000 (Paramore et al., 2004). Nearly 98% of these hospitalizations occurred in children under 5 years old. Between 1997 and 2000, HRSV bronchiolitis was the leading cause of infant hospitalization and, in 1999, an estimated 360 HRSV-associated post-neonatal deaths (i.e. in children aged between 28 days and 1 year) occurred in the USA (Leader & Kohlhase, 2003).

Like HRSV, bovine respiratory syncytial virus (BRSV) is an enveloped, non-segmented, single-stranded RNA pneumonia virus of the family Paramyxoviridae and order Mononegavirales. BRSV is responsible for significant economic loss to the cattle industry (Stott & Taylor, 1985) and is one of the four known viral components of bovine shipping fever. HRSV and BRSV have similar clinical outcomes in their respective host species, ranging from asymptomatic infection to bronchiolitis and pneumonia, and sometimes death (Philippou et al., 2000). The distribution of both viruses is worldwide.

Whilst there are several commercial BRSV vaccines currently available for immunizing cattle, better vaccines that are more efficacious in the face of maternal antibodies and that induce longer-lasting protection would be desirable. Also, there is currently no safe and effective vaccine against HRSV available for use in humans. Several
Formulation of BRSV with CpG ODN and polyphosphazenes

studies using parenterally delivered, formalin-inactivated (FI)-HRSV vaccines were carried out in children in the 1960s (Chin et al., 1969; Fulginiti et al., 1969; Kim et al., 1969; Weibel et al., 1966). Not only did FI-HRSV vaccines fail to protect upon natural infection but, in most cases, disease was enhanced. In one study, 80% of vaccinated children were hospitalized and two of them died (Kim et al., 1969).

It has been reasonably well established that the failure of FI-RSV vaccines and other vaccines, including recombinant vaccinia virus (rVV) encoding the RSV G protein (Openshaw et al., 1992), in mice is due to a Th2-biased immune response (Connors et al., 1992, 1994; Waris et al., 1996, 1997). An adjuvant that induces Th1-type or balanced immune responses would therefore be a candidate component of a successful vaccine formulation. CpG oligodeoxynucleotides (ODN) are short pieces of DNA that contain unmethylated CG dinucleotides flanked by two 5’ purines and two 3’ pyrimidines. CpG ODN bind Toll-like receptor 9 (TLR9), inducing production of interleukin-1 (IL-1), IL-6, IL-12 and tumour necrosis factor alpha by dendritic cells and macrophages, as well as production of gamma interferon (IFN-γ); IL-6 and IL-10 by natural killer cells (Ballas et al., 1996; Hartmann & Krieg, 2000; Hartmann et al., 2000). CpG ODN generally induce an overall Th1-type immune response characterized by production of IFN-γ and antigen-specific IgG2a in mice (Chu et al., 1997; Davis et al., 1998; Ioannou et al., 2002; Jakob et al., 1998). We have previously demonstrated the ability of CpG ODN to shift the immune response induced by parenteral immunization with FI-BRSV from a Th2-biased response to a more balanced response in mice (Oumouna et al., 2005) and calves (Mapletoft et al., 2006).

An alternative or complementary approach to avoid enhanced disease while maintaining or increasing protection is to change the route of immunization. Several intranasal-immunization strategies have been employed to protect rodents from RSV challenge, including recombinant F protein adjuvanted with CpG ODN (Prince et al., 2003), cholaer toxin (Tebbey et al., 2000; Walsh, 1993) or caprylic/capric glycerides and polyoxyethylene-20-sorbitan monolaurate (Tebbey et al., 1999), as well as live viral (Kahn et al., 2001; Matsuoka et al., 2002; Stott et al., 1987) or bacterial (Cano et al., 2000; Falcone et al., 2006) vectors expressing whole RSV proteins or peptides. One of the challenges of intranasal immunization is delivering the vaccine components in such a manner that they are not degraded or flushed out prior to the initiation of the immune response. Polyphosphazenes (PP) are synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms and organic side groups attached at each phosphorus atom (Payne & Andrianov, 1998). PP form non-covalent complexes when mixed with compounds of interest, i.e. antigens and other adjuvants, increasing their stability and allowing for multimeric presentation. Delivery of antigens formulated with PP has been successful in enhancing antibody responses to influenza (Mutwiri et al., 2007; Payne et al., 1995, 1998), rotavirus (McNeal et al., 1999) and cholera (Wu et al., 2001). Protection was also enhanced in the latter two models.

We have previously shown the adjuvant effects of CpG ODN on parenteral FI-BRSV vaccines in mice (Oumouna et al., 2005) and calves (Mapletoft et al., 2006). Here, we report on the adjuvant effects of CpG ODN and PP, individually and as co-adjuvants, on an intranasal FI-BRSV vaccine in mice.

METHODS

Cells and virus. The 375 strain of BRSV (ATCC) was propagated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco/Invitrogen) supplemented with 1% heat-inactivated fetal bovine serum of New Zealand origin (FBS; Gibco/Invitrogen), 25 mM HEPES (EMD Biosciences Inc.), 48 mM sodium bicarbonate (EMD Biosciences Inc.) and 50 μg gentamicin ml⁻¹ (Gibco/Invitrogen). Infected cells were incubated at 37 °C. Four to seven days after infection, infected cells were collected by scraping and were either frozen at −70 °C until vaccine or challenge virus preparation, or used at a dilution of 1:10 to infect more cells for up to two infection cycles. Virus titres were determined by plaque assay on BT cells. Briefly, tenfold serial dilutions in DMEM with 1% FBS were added to 70–80% confluent BT monolayers in 96-well tissue-culture plates (Corning Inc.). Cells were incubated for 7 days at 37 °C, and BRSV plaques were visualized by immunostaining. Cells were fixed with 80% acetone in 0.1 M PBS (38 mM NaH₂PO₄, 6.1 mM Na₂HPO₄, 7H₂O, 0.15 M NaCl, pH 7.2). Polyclonal goat anti-BRSV IgG (VMRD Inc.) at a dilution of 1:5000, followed by biotinylated rabbit anti-goat IgG (Vector Laboratories Inc.) at a dilution of 1:750, were used to detect BRSV plaques. Vectastain avidin–biotin complex horseradish peroxidase (Vector Laboratories Inc.), followed by diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories Inc.), were used to visualize BRSV plaques. Viral titres were expressed in p.f.u. ml⁻¹.

FI-BRSV was prepared as described by Kim et al. (1969). Briefly, infected-cell lysate was centrifuged at 450 g until vaccine or challenge virus preparation, or used at a dilution of 1:10 to infect more cells for up to two infection cycles. Virus titres were determined as indicated above.

Challenge virus was prepared as follows. Infected-cell lysate was clarified by centrifugation for 15 min at 550 g. The resulting pellet was visibly homogenized. Challenge virus titres were determined as indicated above.

Challenge virus was prepared as follows. Infected-cell lysate was centrifuged at 450 g for 30 min at 4 °C. The resulting pellet was resuspended in 1/10 of the original volume in serum-free DMEM (Gibco/Invitrogen) and assayed for protein concentration. The final vaccine protein concentration was 150 μg ml⁻¹, and 1,875 μg was given per immunization.

Immunization and challenge. Six- to eight-week-old female BALB/c mice (Charles River) were allocated randomly into seven groups of 10 animals and immunized intranasally three times with a total volume of 25 μl (12.5 μl in each nostril) as indicated in Table 1. CpG ODN 1826 (TCCATGACTTCTCGAGCTT) was provided by Merial. Non-CpG ODN (TCATGACTTCTCGAGCTT) was synthesized by Operon Biotechnologies Inc. The underlined dinucleotides indicate the unmethylated cytosine–guanosine dinucleotides. Two mice died during the course of the study and were...
Table 1. Immunization protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunizations (days 0, 21, 42)</th>
<th>Challenge (day 56)</th>
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<tr>
<td>1</td>
<td>FI-BRSV</td>
<td>BRSV</td>
</tr>
<tr>
<td>2</td>
<td>FI-BRSV/CpG</td>
<td>BRSV</td>
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<tr>
<td>3</td>
<td>FI-BRSV/non-CpG</td>
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<td>4</td>
<td>FI-BRSV/PP</td>
<td>BRSV</td>
</tr>
<tr>
<td>5</td>
<td>FI-BRSV/CpG/PP</td>
<td>BRSV</td>
</tr>
<tr>
<td>6</td>
<td>Saline</td>
<td>BRSV</td>
</tr>
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<td>7</td>
<td>Saline</td>
<td>Saline</td>
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excluded from all analyses. All ODNs were phosphorothioate-modified during synthesis to enhance nuclease resistance and were given at 20 μg per immunization. Polyphosphazene polymer 6 was synthesized by John Klaehn (Idaho National Laboratory) according to a previously published method (Andrianov et al., 2004) and was given at 25 μg per immunization. The components of each vaccine were mixed prior to immunization and were given in a total volume of 25 μl as a single administration. Two weeks after the third immunization, all groups were challenged. Following sedation with ketamine and xylazine (sera) and continuing in fourfold dilutions. Alkaline phosphatase was visualized as described above. Virus-neutralizing titres are expressed as the highest titre of sample that resulted in <50% of cells displaying cytopathic effects.

**IFN-γ and IL-5 ELISPOT assays.** Splenocytes were isolated as described previously (Baca-Estrada et al., 1996) with a few modifications. Six days after challenge, mice were euthanized and spleens were removed into tubes containing minimal essential medium (MEM; Gibco/Invitrogen) supplemented with 50 μg gentamicin ml⁻¹ and 10 mM HEPES buffer (Gibco/Invitrogen), on ice. Following removal of excess fat, spleens were cut into pieces and passed gently through sterile 100 μm cell strainers (BD Biosciences) into Petri dishes containing MEM. Splenocytes were centrifuged for 10 min at 310 g at 4 °C with serially diluted samples, or 1 : 20 (pooled sera) and continuing in twofold dilutions. Lung-fragment culture supernatants were heat-inactivated at 56 °C for 30 min prior to dilution. BRSV strain 375 (500 p.f.u. per well) was added to each dilution plate and incubated for 1 h at 37 °C. Sample–virus mixtures were then added to duplicate BT cell cultures and incubated at 37 °C for 6 days. BRSV plaques were visualized by immunostaining as described above. Virus-neutralizing titres are expressed as the highest titre of sample that resulted in <50% of cells displaying cytopathic effects.

**Lung-fragment culture supernatants.** Lung-fragment cultures were prepared as described by Etchart et al. (2006) and Logan et al. (1991), with a few modifications. Six days after challenge, mice were euthanized and lungs were lavaged and removed into tubes containing RPMI 1640 medium (Gibco/Invitrogen) supplemented with 10% FBS (Gibco/Invitrogen), 10 mM HEPES buffer, 0.1 mM non-essential amino acids (Gibco/Invitrogen), 1 mM sodium pyruvate (Gibco/Invitrogen), 50 μg gentamicin ml⁻¹ (Gibco/Invitrogen) and 1× antibiotic/antimyotic (Gibco/Invitrogen), on ice. Under sterile conditions, lungs were cut into four pieces of roughly equal size and deposited into 48-well plates (one piece of lung per well) containing 500 μl RPMI 1640 medium supplemented as indicated above per well. Following 5 days incubation at 37 °C, supernatants were collected, pooled for each individual mouse, clarified by centrifugation at 10 000 g for 1 min and stored at −80 °C until they could be assayed for IgG and IgA, as described below.

**ELISA.** Sera and lung-fragment culture supernatants were assayed for BRSV-specific IgG and IgA. Ninety-six-well polystyrene Immulon 2 microtitre plates (Thermo Electron) were coated overnight at 37 °C with BRSV antigen composed of Nonidet-P40 (Sigma-Aldrich)-treated BT cells previously infected with the 375 strain of BRSV, as described above, and were frozen at −20 °C until use. Mock-infected BT cells were used as negative-control antigen. Plates were washed, then incubated overnight at 4 °C with serially diluted samples, beginning at 1 : 10 (lung-fragment culture supernatants) or 1 : 40 (sera) and continuing in fourfold dilutions. Alkaline phosphatase (AP)-labelled goat anti-mouse IgG or IgA (Kirkegaard & Perry Laboratories) at dilutions of 1 : 5000 and 1 : 2500 were used to detect bound IgG and IgA, respectively. Reactions were visualized with p-nitrophenylphosphate (Sigma-Aldrich).

**Virus-neutralization assay.** BT cell cultures were challenged overnight in 96-well tissue-culture plates (Corning Inc.) to achieve 70–80% confluent monolayers. Samples were diluted in 96-well round-bottom culture plates (Corning Inc.), beginning at 1 : 2 (lung-fragment culture supernatants) or 1 : 20 (pooled sera) and continuing in twofold dilutions. Lung-fragment culture supernatants were heat-inactivated at 56 °C for 30 min prior to dilution. BRSV strain 375 (500 p.f.u. per well) was added to each sample dilution and plates were incubated for 1 h at 37 °C. Sample–virus mixtures were then added to duplicate BT cell cultures and incubated at 37 °C for 6 days. BRSV plaques were visualized by immunostaining as described above. Virus-neutralizing titres are expressed as the highest titre of sample that resulted in <50% of cells displaying cytopathic effects.
Detection of viral RNA. Four days after challenge, mice were euthanized and lungs were removed into 2 ml screw-cap tubes (VWR International) containing 2.4 mm zirconia microbeads (BioSpec Products Inc.) and 1 ml Trizol reagent (Invitrogen), and were homogenized in a mini-beadbeater (BioSpec Products Inc.) for 10 s. RNA was isolated from lung homogenates by using the Trizol reagent method according to the manufacturer’s instructions. DNA removal and cDNA synthesis were performed by using a QuantiTect reverse transcription kit (Qiagen) according to the manufacturer’s instructions. Real-time quantitative PCRs (qPCRs) were prepared by using Platinum SYBR green qPCR Supermix-UDG (Invitrogen) according to the manufacturer’s instructions, in iCycler iQ PCR plates (Bio-Rad) sealed with iCycler iQ optical tape (Bio-Rad). Primers to the BRSV F gene (primer A, 5′-AACCGGCCTCTTTCAGTAGA-3′; primer B, 5′-TGACACTGCTACCCACCTT-3′) were designed by using primer-design software for personal computers (Clone Manager version 6.00; Sci-Ed) from a consensus sequence generated from 27 different BRSV F gene sequences by using the MultAlin multiple sequence alignment tool (available online at http://bioinfo.genopole-toulouse.prd.fr/multalin/; Corpet, 1988). qPCR was performed on an iCycler iQ Multicolor Real-Time PCR detection system (Bio-Rad) for 45 cycles using the following parameters: denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C and extension for 30 s at 72 °C. Serial dilutions of a plasmid that contains a truncated version of the BRSV F gene used in qPCRs carried out under the conditions outlined above allowed construction of a standard curve that enabled the determination of gene copy number. Results are expressed as viral RNA copies (ml lung homogenate)−1.

Statistical analysis. All data were analysed by using statistical software for personal computers (GraphPad Prism version 3.00; GraphPad Software). As outcome variables were found to be not distributed normally, differences among all groups were examined by using a Kruskal–Wallis test. If a significant difference was found among the groups, medians between pairs of groups were compared by using a Mann–Whitney U test. Differences were considered significant if P<0.05.

RESULTS

BRSV-specific humoral immune responses

Humoral immune responses induced by the various vaccine formulations were examined by measuring the BRSV-specific IgG titres in the serum after each immunization and after challenge. After the final immunization, significantly increased IgG production (P=0.0002) was observed by addition of PP to the FI-BRSV vaccine (Fig. 1a). This was increased further by addition of CpG ODN to the FI-BRSV/PP vaccine. Indeed, the levels of IgG produced by the FI-BRSV/CpG/PP group were significantly higher than those in all other groups (P=0.0003 compared with the FI-BRSV/PP group; P=0.0001 compared with all other groups). Examination of the kinetics of the BRSV-specific serum IgG response (Fig. 1b) revealed that only two intranasal immunizations of the FI-BRSV/CpG/PP vaccine were required to induce a robust humoral immune response.

The additive or synergistic effect due to co-formulation with CpG ODN and PP in terms of humoral immunity against BRSV was confirmed upon quantification of virus-neutralizing antibodies in pooled sera: immunization with FI-BRSV/CpG/PP resulted in the highest virus-neutralizing titres before and after challenge (Fig. 1c).

In addition, BRSV-specific IgA was measured after challenge (Fig. 1d). All adjuvanted vaccine groups performed significantly better than FI-BRSV alone (P=0.006 compared with FI-BRSV/CpG; P<0.0001 compared with FI-BRSV/non-CpG; P=0.0002 compared with FI-BRSV/PP; P=0.0006 compared with FI-BRSV/CpG/PP). The only significant difference observed between adjuvanted vaccine groups, however, was that between FI-BRSV/CpG and FI-BRSV/PP (P=0.02).

BRSV-specific cell-mediated immune responses

To evaluate further the type of immune response induced by the various vaccine formulations, BRSV-induced secretion of IFN-γ and IL-5 by splenocytes was measured 6 days after challenge. IFN-γ-secreting cells were induced in the groups that received FI-BRSV or FI-BRSV/PP (Fig. 2a). Addition of CpG ODN to these vaccines increased IFN-γ secretion significantly in the FI-BRSV/CpG and FI-BRSV/CpG/PP groups (P=0.008 and 0.02, respectively). Although there was an increase in IFN-γ production in the FI-BRSV/non-CpG group in comparison with the FI-BRSV group (P=0.008), suggesting a phosphorothioate backbone effect, there was a further increase in the FI-BRSV/CpG group in comparison with the FI-BRSV/non-CpG group (P=0.008). In contrast to the CpG ODN effects, addition of PP to the FI-BRSV vaccine decreased IFN-γ secretion significantly (P=0.008).

High numbers of IL-5-secreting cells were induced in the groups that received FI-BRSV or FI-BRSV/PP (Fig. 2b). Addition of CpG ODN to these vaccines decreased IL-5 secretion significantly in the FI-BRSV/CpG and FI-BRSV/CpG/PP groups (P=0.008 and 0.02, respectively). Non-CpG ODN also decreased the amount of IL-5 secretion compared with FI-BRSV alone (P=0.008). There were no significant differences between the FI-BRSV/CpG and FI-BRSV/CpG/PP groups in terms of IFN-γ or IL-5 secretion by splenocytes. These results indicate that the addition of CpG ODN and, to an extent, non-CpG ODN to the vaccine formulations shifted the cell-mediated immune response from a Th2-type response, characterized by high levels of IL-5 secretion, to a Th1-type response, characterized by high levels of IFN-γ secretion, but exactly what effect PP had on the cell-mediated immune response was less clear.

BRSV-specific mucosal immune responses

To evaluate the mucosal immune responses induced by the various vaccine formulations, the secretion of IgG and IgA in lung-fragment culture supernatants was measured. Low levels of IgG were produced in the group that received FI-BRSV (Fig. 3a). Addition of CpG or PP to the vaccine increased IgG production significantly (P=0.008 and 0.03, respectively), whereas there was no significant effect of non-CpG. The group that received FI-BRSV/CpG/PP
produced significantly higher levels of IgG than any other group \((P=0.03\) compared with FI-BRSV/PP; \(P=0.02\) compared with all other groups). Low levels of IgA were produced in the group that received FI-BRSV. Addition of CpG, but not non-CpG ODN or PP, to the vaccine increased production of IgA significantly \((P=0.008)\). The group that received FI-BRSV/CpG/PP produced significantly higher levels of IgA than any other group \((P=0.02)\). These results indicate that the addition of CpG ODN and PP to FI-BRSV enhanced the mucosal immune response.

To evaluate further the biological effectiveness of the antibodies produced in the lungs, virus-neutralizing titres were determined. Neutralizing-antibody titres were found in all immunized mice, but the group that received CpG ODN and PP performed significantly better than all other groups \((P=0.03\) compared with FI-BRSV/CpG; \(P=0.02\) compared with all other groups).
compared with all other groups) (Fig. 3c). There were no significant differences among the other immunized groups.

**IFN-γ and IL-5 production in the lungs**

The effects of the various vaccine formulations on the type of immune response induced were examined further by measuring the amounts of IFN-γ and IL-5 produced in the lungs. Mice that received FI-BRSV/PP or FI-BRSV/CpG/PP produced significantly lower amounts of IFN-γ than those that received FI-BRSV (P=0.008) or FI-BRSV/CpG.
(P=0.008) (Fig. 4a), indicating the possibility for PP to reduce lung IFN-γ production. This agrees somewhat with our observations with in vitro-restimulated splenocytes, in which addition of PP to the FI-BRSV vaccine decreased the number of IFN-γ-secreting cells significantly. Addition of CpG ODN to the FI-BRSV/PP did, however, increase lung IFN-γ production significantly (P=0.05). Mice that received FI-BRSV/CpG, FI-BRSV/non-CpG or FI-BRSV/CpG/PP produced significantly lower amounts of IL-5 than those that received FI-BRSV (P=0.03, 0.008 or 0.008, respectively) (Fig. 4b), indicating a role for CpG and non-CpG ODN in the reduction of lung IL-5 production. Incidentally, there was no significant difference between the FI-BRSV/CpG and FI-BRSV/CpG/PP groups in terms of lung IL-5 production, demonstrating that PP does not interfere with the ability of CpG ODN to reduce IL-5 production.

Detection of viral RNA in the lung tissue

To assess the ability of CpG ODN and PP to enhance protection from infection, the level of virus replication in the lungs was determined by detection of viral RNA. The unvaccinated group (saline/BRSV) displayed the highest level of viral replication (Fig. 5). Compared with the saline/BRSV group, all groups that received a vaccine displayed some level of reduction in viral replication, but the largest significant reduction in viral replication was observed in the group that received FI-BRSV/CpG/PP (P=0.008). The FI-BRSV/CpG/PP group also displayed significantly less viral replication than all other vaccine groups (P=0.008). The small number of viral RNA copies observed in the uninfected mice was due to the formation of non-specific PCR products synthesized in the absence of viral RNA. These results indicated a synergy between CpG ODN and PP in terms of enhancing protection against BRSV.

DISCUSSION

Like many pathogens, RSV targets the mucosal surfaces of the respiratory tract, so the induction of both systemic and mucosal immune responses is expected to be critical for optimal disease protection. In order to achieve effective mucosal immunity by intranasal vaccination, non-replicating vaccines need to be formulated with effective adjuvants and/or delivery vehicles. In this study, we demonstrated the ability of an intranasally delivered FI-BRSV vaccine co-formulated with CpG ODN and PP to induce systemic and mucosal immunity, as well as protection from BRSV challenge in mice.

Humoral immunity, although not the only component of a successful immune response, especially in the case of a BRSV infection, is a traditional measure of the success of an immunization protocol. Here, we found that co-formulation of FI-BRSV with CpG ODN and PP resulted in significantly higher levels of BRSV-specific IgG in the serum compared with all other vaccine formulations, indicating a synergy between CpG ODN and PP. It is well known, however, that humoral immunity, in the absence of cell-mediated immunity, is not sufficient to combat RSV infections, whereas cell-mediated immunity is universally recognized to be an important factor in the resolution of RSV infections. Addition of CpG ODN to the FI-BRSV and FI-BRSV/PP vaccines resulted in significant increases in IFN-γ secretion. This agrees with an earlier report in which high levels of IFN-γ were induced by parental immunization of mice with FI-BRSV formulated with CpG ODN (Oumouna et al., 2005). In contrast, IL-5 secretion was reduced significantly to virtually zero when CpG ODN was added to the FI-BRSV and FI-BRSV/PP vaccines. Although non-CpG ODN also enhanced IFN-γ and reduced IL-5

Fig. 4. IFN-γ (a) and IL-5 (b) production in lung-homogenate supernatants. Mice were immunized intranasally with FI-BRSV, FI-BRSV/CpG, FI-BRSV/non-CpG, FI-BRSV/PP, FI-BRSV/CpG/PP or saline. CpG and non-CpG ODN were given at 20 μg per immunization, and PP was given at 25 μg per immunization. All animals were challenged with BRSV 2 weeks after the final immunization (except for the saline/saline group) and euthanized 6 days later. Each data point represents an individual animal, and median values are indicated by horizontal bars. *P<0.05; **P<0.01.
levels, suggesting some phosphorothioate backbone effect, the increase in IFN-γ induction by CpG ODN was greater in magnitude than that induced by non-CpG ODN. It was less clear, however, what the exact effects of PP were on the type of immune response induced in in vitro-restimulated splenocytes or in the lungs, but this is not surprising, given an earlier study that concluded that parenteral immunization of mice with influenza virus antigens formulated with a similar PP results in mixed Th1/Th2 immune responses (Mutwiri et al., 2007).

Whilst humoral and cell-mediated immunity are traditionally considered to be important correlates of protection, in BRSV disease, the first encounter between the host and pathogen takes place in the lung mucosa. Addition of CpG ODN to the FI-BRSV and FI-BRSV/PP vaccines resulted in significant increases in production of both IgG and IgA, whereas there was no effect of non-CpG ODN. Furthermore, the group co-formulated with CpG ODN and PP produced significantly higher levels of IgG and IgA than all other groups. These findings correspond to those in a previous study in which intranasal immunization of mice with hepatitis B surface antigen with CpG ODN primes mice for enhanced lung immunopathology (Openshaw et al., 1992), intranasal and intraperitoneal immunization with rVV-G results in protection with no lung lesions (Stott et al., 1986). Another study, featuring rVV expressing the RSV F protein, demonstrated that, whilst intranasal immunization induced some pathological lung inflammation upon challenge with live BRSV, it was milder than that induced following intradermal immunization (Matsuoka et al., 2002).

The most important quality offered by a successful BRSV vaccine formulation is protection upon viral challenge. Because of the labile nature of BRSV, as well as its limited growth in experimental animals and cell culture, RT-PCR assays have been developed to detect viral RNA in the lungs of mice as an alternative to the isolation of live virus (Almeida et al., 2004). We have increased the sensitivity of these assays via the use of RT-qPCR reagents and detection systems. Despite reductions in the median viral RNA copy number in all vaccinated groups, the only group that experienced a significant reduction in viral replication was the group that received FI-BRSV/CpG/PP. This group also demonstrated the largest reduction in median viral RNA copy number. This agrees with an earlier report in which intranasal immunization of cotton rats with RSV F protein plus CpG ODN reduced viral production upon challenge (Prince et al., 2003). They, however, required amounts of purified antigen and/or CpG ODN higher than those used in
the present study to achieve this level of protection. Furthermore, intranasal delivery of CpG co-formulated vaccines consisting of human immunodeficiency virus (HIV) immunogen (Dumais et al., 2002) or herpes simplex virus (HSV) recombinant glycoprotein B (Gallichan et al., 2001) induced protection of mice from intravaginal challenge with rVV expressing HIV-1 gag or HSV-2, respectively.

Because of their ability to form non-covalent complexes with antigens and other adjuvants, PP are attractive for use in mucosal immunization, a situation in which constant secretion of mucous membrane fluids and high turnover of epithelial cells threaten the stability and uptake of vaccine components. We demonstrated that intranasal immunization of mice with FI-BRSV co-formulated with CpG ODN and PP induced both humoral and cell-mediated immunity. In addition, mice immunized intranasally with FI-BRSV/CpG/PP developed enhanced mucosal immunity, characterized by increased production of BRSV-specific IgG and IgA in lung-fragment cultures. Indeed, the production of enhanced serum and lung IgG and, in particular, lung IgA and virus-neutralizing antibodies is probably the most important characteristic of this formulation in comparison with the individual components. Finally, the FI-BRSV/CpG/PP formulation induced a reduction in viral replication upon BRSV challenge, which correlates with the enhanced mucosal IgG and IgA. Based on these results, we conclude that FI-BRSV co-formulated with CpG ODN and PP, delivered intranasally, is a good candidate vaccine formulation for protection against BRSV. As BRSV and HRSV cause similar clinical disease in their respective host species, an intranasally delivered RSV vaccine co-formulated with CpG ODN and PP might also be efficacious in humans.

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Formulation of BRSV with CpG ODN and polyphosphazenes


