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Formulation with CpG Oligodeoxynucleotides Prevents Induction of Pulmonary Immunopathology following Priming with Formalin-Inactivated or Commercial Killed Bovine Respiratory Syncytial Virus Vaccine

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Commercial killed bovine respiratory syncytial virus (K-BRSV) and formalin-inactivated BRSV (FI-BRSV) tend to induce Th2-type immune responses, which may not be protective and may even be detrimental during subsequent exposure to the virus. In this study we assessed the ability of CpG oligodeoxynucleotides (ODNs) to aid in the generation of effective and protective BRSV-specific immune responses. Mice were immunized subcutaneously with FI-BRSV formulated with CpG ODN, Emulsigen (Em), CpG ODN and Em, or non-CpG ODN and Em. Two additional groups were immunized with K-BRSV or K-BRSV and CpG ODN. After two vaccinations, the mice were challenged with BRSV. FI-BRSV induced Th2-biased immune responses characterized by production of serum immunoglobulin G1 (IgG1) and IgE, as well as interleukin-4 (IL-4), by in vitro-restimulated splenocytes. Formulation of FI-BRSV with CpG ODN, but not with non-CpG ODN, enhanced serum IgG2a and IFN-γ production by splenocytes, whereas serum IgE was reduced. Although the immune response induced by K-BRSV was not as strongly Th2 biased, the addition of CpG ODN to this commercial vaccine also resulted in a more Th1-type response. Furthermore, the addition of CpG ODN to the BRSV vaccine formulations resulted in enhanced neutralizing antibody responses. Significant production of IL-5, eotaxin, and eosinophilia was observed in the lungs of FI-BRSV- and K-BRSV-immunized mice. However, IL-5 and eotaxin levels, as well as the number of eosinophils, were decreased in the mice vaccinated with the CpG ODN-formulated vaccines. Finally, when formulated with CpG ODN, both FI-BRSV and K-BRSV significantly reduced virus production after challenge with BRSV.

Respiratory syncytial virus (RSV) is an important infectious agent and the leading cause of viral lower respiratory tract infection in infants and young children worldwide (4). Clinical manifestations range from asymptomatic infection to bronchopneumonia, bronchiolitis, and pneumonia. RSV infection is also associated with development of asthma later in life (50, 51). Like human RSV (HRSV), bovine RSV (BRSV) is a negative-stranded RNA virus classified in the *Pneumovirus* genus of the *Paramyxoviridae* family. It has been recognized as an important pathogen for cattle for 30 years, and it affects primarily young calves (2, 38, 58). Infection is characterized by pyrexia, coughing, trachypnea, and dyspnea (7), pneumonia (56), and sometimes death (57). The pathological lesions caused by HRSV and BRSV are very similar. Both viruses induce lymphocyte bronchitis, bronchiolar epithelial necrosis, bronchiolar occlusion, parenchyma inflammation, and alveolar exudation (53).

Despite the importance of HRSV as a respiratory pathogen, there is no safe and effective vaccine available. The first vaccine trial, in the 1960s, in which young children were given formalin-inactivated HRSV (FI-HRSV), had serious consequences upon subsequent natural infection with RSV. The vaccine recipients developed enhanced pulmonary disease leading to hospitalization and even to the deaths of two children (9, 17, 35, 61). It was reported that in mice the failure of the FI-HRSV vaccine was caused by an imbalance in the immune responses to the RSV attachment (G) and fusion (F) proteins, because of poor preservation of the F protein during formalin inactivation (12, 19, 43). Other studies with cotton rats showed an immune response against F after immunization with formalin-inactivated virus (11), although disease complications still occurred. It has now been reasonably well established at least for the mouse model that the immunopathology is caused by polarized type 2 T-helper cell (Th2) responses (13), characterized by high levels of RSV-specific immunoglobulin G1 (IgG1) and IgE, as well as an increase in the amount of Th2 cytokines such as interleukin-4 (IL-4) and IL-5, which result in enhanced pulmonary eosinophilia. A similar exacerbation of respiratory disease occurred in calves given a live or FI-inactivated BRSV vaccine (34, 37) and in laboratory animals immunized with recombinant virus vaccine (54). Furthermore, in cattle activation of complement has been observed (36), and in monkeys a deposition of immune complexes has been observed (46). However, there are commercial BRSV vaccines, which have shown limited success in preventing the disease or stopping the virus from spreading between herds.

Synthetic oligodeoxynucleotides (ODNs) containing un-

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methylated CpG dinucleotides flanked by two 5′ purines and two 3′ pyrimidines (CpG motif) have been reported to have immunomodulatory activity (16). These ODNs require chemical modifications such as the replacement of the nonbridging oxygen atom on the phosphate group with a sulfur atom (phosphorothioate modification) (40) to increase resistance to nuclease degradation and thus activity (48). By binding Toll-like receptors, CpG can activate dendritic cells and macrophages to trigger the production of IL-1, IL-6, IL-12, and tumor necrosis factor alpha, as well as natural killer (NK) cells and lymphocytes, to produce gamma interferon (IFN-γ), IL-6, IL-10 and immunoglobulin, respectively (3, 26, 27). Overall, CpG DNA stimulates Th1-type responses, characterized by IL-12 and IFN-γ secretion with very little secretion of Th2 cytokines and a predominance of IgG2a over IgG1 in the mouse (10, 14, 30, 33). ODNs do not bind major histocompatibility complex molecules and are not recognized as antigens by T cells, and therefore CpG-based adjuvants do not trigger severe granulomatous reactions (52) and are not associated with adverse injection site reactions (32). Although for cattle the Th1/Th2 paradigm has not been as firmly established as it has been for mice, we have observed increased IgG2 levels and IFN-γ production in the blood of calves vaccinated with CpG-formulated bovine herpesvirus 1 gD (31).

In the present study we evaluated the ability of CpG ODNs to shift the immune response bias induced by vaccination with FI-BRSV or commercial killed BRSV (K-BRSV), as well as to enhance protection after BRSV challenge. We chose to use the BRSV challenge model because commercial vaccines are available for BRSV. This allowed us to not only evaluate an experimental FI-BRSV vaccine but also to assess the potential for improvement of a commercial K-BRSV vaccine.

MATERIALS AND METHODS

Cells and virus. BRSV strain 375 was propagated in Madin-Darby bovine kidney (MDBK) cells maintained in Dulbecco's modified Eagle's medium (MEM) (GIBCO-BRL, Grand Island, N.Y.) supplemented with 1% heat-inactivated (56°C) fetal bovine serum (GIBCO-BRL). The virus was propagated until there was a detectable cytopathic effect. Infected cells were collected and frozen at −70°C. The virus titer was determined by plaque assay on MDBK cells.

Formalin-inactivated BRSV vaccine was prepared as described previously (35). Briefly, the supernatant from BRSV-infected MDBK cells was centrifuged for 15 min at 1,600 rpm (Legend RT; Sorval) to remove cellular debris. One part of 37% formalin (Sigma, St. Louis, Mo.) was incubated with 4,000 parts of clarified BSRV lysate at 2×10^6 PFU per ml for 3 days at 37°C and pelleted by ultracentrifugation for 1 h at 17,000 rpm in an SW28 rotor (Optima L-100 XP; Beckman Coulter). The virus pellet was resuspended in 1/25 of the original volume in serum-free MEM (GIBCO-BRL).

Vaccination and challenge. Six- to 8-week-old female BALB/c mice were purchased from Charles River (Montreal, Quebec, Canada) and kept in an in-house animal care facility. Nine groups of six mice each were immunized subcutaneously twice with a 3-week interval with 100 μl of FI-BRSV or K-BRSV. FI-BRSV strain 375 was formulated with either ODN 1826 (10 μg), Emulsigen (Em) (30%, vol/vol) (MVP Laboratories, Ralston, Nebr.), both ODN 1826 and Em, or ODN 1982 (10 μg) and Em. Emulsigen was chosen because of its efficacy as a coadjuvant with CpG ODNs (30). Because the commercially available killed virus vaccine (Triangle 4; Fort Dodge Laboratories Inc., Fort Dodge, Iowa) already contains an undisclosed adjuvant, it was formulated with ODN 1826 alone (10 μg) (Table 1). Both ODN 1826 (TCCATGA**CG**TTCCTGA**CG**TT) and non-CpG ODN 1982 (TCCAGGACTTCTCTCAGGTT) were phosphorothioate modified and synthesized by Qiagen GmbH (Hilden, Germany). ODNs were diluted in endotoxin-free water (GIBCO-BRL). All mice were challenged 3 weeks after the secondary vaccination. Following sedation with ketamine and xylazine (60 mg/kg; Butler Co., Dublin, Ohio), 10^7 PFU of BRSV strain 375 in a final volume of 50 μl was applied in the nostrils. Four days after challenge, all mice were euthanized. The experiment was performed twice to validate the results, and results from a representative trial are shown. All procedures involving the animals were performed in accordance with the recommendations of the Canada Council for Animal Care.

TABLE 1. Vaccination protocol

Group	Vaccination		Challenge (day 42)
	First (day 0)	Second (day 21)	
1	Saline	Saline	Saline
2	FI-BRSV	FI-BRSV	BRSV
3	FI-BRSV/CpG	FI-BRSV/CpG	BRSV
4	FI-BRSV/Em	FI-BRSV/Em	BRSV
5	FI-BRSV/CpG/Em	FI-BRSV/CpG/Em	BRSV
6	FI-BRSV/non-CpG/Em	FI-BRSV/non-CpG/Em	BRSV
7	K-BRSV	K-BRSV	BRSV
8	K-BRSV/CpG	K-BRSV/CpG	BRSV
9	Saline	Saline	BRSV

Enzyme-linked immunosorbent assay (ELISA). To determine BRSV-specific IgG titers, 96-well polystyrene Immulon 2 microtiter plates (Dynatech, Gaithersburg, Md.) were coated overnight with BRSV antigen, which was composed of detergent-treated MDBK cells infected with strain 375 of BRSV. Mock-infected MDBK cells were used as the control antigen as previously described (55). Plates were incubated for 2 h at room temperature (RT) with serially diluted mouse sera. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) at a dilution of 1:5,000 was used to detect bound IgG. The reaction was visualized with *p*-nitrophenyl phosphate (PNPP) (Sigma Chemical Co., Oakville, Ontario, Canada).

To determine BRSV-specific IgG1 and IgG2a titers, 96-well polystyrene microtiter plates were coated overnight with BRSV-infected and mock-infected cell lysates as described above (55). Plates were incubated for 2 h at RT with serially diluted murine sera. Bound antibodies were detected with biotinylated goat anti-murine IgG1 and IgG2a (Caltag Laboratories, San Francisco, Calif.) at 0.05 and 0.5 μg of IgG per ml, respectively, followed by streptavidin-AP (GIBCO-BRL) at a dilution of 1:2,000. The reaction was visualized with PNPP (Sigma Chemical Co.).

To determine BRSV-specific IgE titers, 96-well polystyrene microtiter plates were coated overnight with BRSV-infected and mock-infected cell lysates as described above (55). Murine sera were serially diluted and incubated overnight at 4°C. Bound antibodies were detected with biotinylated rat anti-mouse IgE (Serotec, Oxford, United Kingdom) at a 1:5,000 dilution, followed by streptavidin-AP (GIBCO-BRL) at a dilution of 1:2,500. The reaction was visualized with PNPP (Sigma Chemical Co.).

The titers were determined based on the titer differences between the BRSV-infected and mock-infected cell lysates as previously described (55).

Virus neutralization assay. MDBK cells were cultured overnight in 96-well tissue culture plates (Becton Dickinson, Mountain View, Calif.) to obtain a confluent monolayer. Murine sera were pooled for each group and serially diluted twofold, and known BRSV-high-positive, -low-positive, and -negative sera were used as controls. The 375 strain of BRSV was diluted 1:1 with 100 μl of each serum sample and incubated at 37°C for 1 h. The serum-virus mixture was then added to duplicate MDBK cell cultures and incubated for 48 h. The plates were stained with BRSV-specific goat serum (VMRD, Inc., Pullman, Wash.) and biotinylated goat anti-rabbit serum (Vector Laboratories, Inc., Burlingame, Calif.), followed by detection with the Vectastain ABC horseradish peroxidase kit and diaminobenzidine substrate as recommended by the manufacturer (Vector Laboratories Inc.) to visualize viral plaques before counting. Virus neutralization titers were expressed as the reciprocal of the highest dilution of serum that caused a 100% reduction in plaques relative to the virus control.

IFN-γ and IL-4 enzyme-linked immunospot assays. Splenocytes were isolated as described previously (30). Following the removal of excess fat, spleens were cut into pieces and gently pushed through a sterile strainer into a petri dish containing MEM supplemented with 100 U of penicillin and 50 μg of streptomycin (GIBCO-BRL) per ml and 5 mM HEPES (GIBCO-BRL). The strainer was washed, and the splenocytes were transferred into 15-ml tubes. After 5 min on ice, the cells were centrifuged for 10 min at 4°C and resuspended in 1.5 ml of ammonium chloride lysis buffer. One minute later, 10 ml of MEM was added and the cells were centrifuged again. After the cells were counted with an automatic Coulter counter (Z1; Beckman Coulter, Hialeah, Fla.), they were resuspended in Aim-V medium (GIBCO-BRL) supplemented with 2 mM glutamine (GIBCO-

BRL), 100 U of penicillin and 50 μg of streptomycin (GIBCO-BRL) per ml, 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.), 10 mM HEPES (GIBCO-BRL), and 10% (vol/vol) fetal bovine serum (Sigma Chemical Co.) to 10^7 cells per ml.

Nitrocellulose plates (Whatman, Inc., Clifton, N.J.) were coated overnight at 4°C with mouse IFN-γ- and IL-4-specific monoclonal antibodies (PharMingen, San Diego, Calif.) diluted to 2 μg/ml. Unbound antibody was removed by washing with 0.05% (vol/vol) phosphate-buffered saline (PBS)–Tween 20, with a final wash with PBS alone. Wells were blocked with 100 μl of complete AIM V medium for 1 to 2 h at 37°C. Mouse splenocytes were cultured in triplicate wells at 10^6 cells per well in the presence of BRSV-infected or mock-infected cell lysate. Following 24 h of incubation at 37°C, the plates were washed with double-distilled water (ddH_2O) for 1 min to lyse the cells. Cells were then removed by washing with ddH_2O followed by PBS-Tween 20. The plates were subsequently incubated with biotinylated anti-mouse IFN-γ and IL-4 monoclonal antibodies (PharMingen) at 2 μg/ml for 1 h at RT, followed by streptavidin-AP (GIBCO-BRL) at a 1:1,000 dilution. Bound IFN-γ- and IL-4-specific antibodies were visualized with 5-bromo-4-chloro-3-indolylphospohate and nitroblue tetrazolium substrate tablets (Sigma Chemical Co.). Plates were washed in ddH_2O and air dried. Spots were counted with the aid of an inverted microscope. The numbers of BRSV-specific IFN-γ- and IL-4-secreting cells are expressed as the difference between the number of spots per 10^6 cells in BRSV-stimulated cultures and the number of spots per 10^6 cells in control cultures.

IL-5 and eotaxin ELISA. IL-5 secretion into the lungs was evaluated by ELISA as previously described (22). Murine recombinant IL-5 and anti-IL-5 monoclonal antibodies (TRFK.5 and TRFK.4) were purchased from PharMingen. Bound anti-IL-5 antibodies were visualized with PNPP (Sigma Chemical Co.).

The level of eotaxin, a member of the CC chemokine family of inflammatory and immunoregulatory cytokines, in the lungs was measured by ELISA with a Quantikine M kit (R&D Systems, Minneapolis, Minn.) and was expressed in picograms per milliliter.

Bronchoalveaolar lavage fluids were collected, and cytospin preparations were prepared and stained with Diff-Quick (Dade International, Miami, Fla.). The numbers of eosinophils, lymphocytes, macrophages, and neutrophils were determined for each group of mice by examination of at least 400 leukocytes.

Virus titration. Production of BRSV in the lungs after challenge was assessed by plaque titration (22, 24). The lungs were removed and homogenized four times for 45 s each in a tube containing microbeads (1.0-mm-diameter glass beads; Biospec Products, Inc.) and MEM. To prevent enzymatic degradation of virus, 10 μg of aprotinin and leupeptin per ml was added to the medium. Before each homogenization the tubes containing the lungs were kept in alcohol chilled by dry ice. Tenfold serial dilutions of the lung homogenates were added to 70% confluent MDBK cell monolayers. Cells were incubated for 6 to 8 days at 37°C, and the BRSV plaques were visualized with 2% crystal violet. The BRSV titers were expressed as PFU per milliliter.

Statistical analysis. The differences between the mean values for the vaccine groups were determined by a two-tailed Student *t* test with assumed equal variance. Differences were considered significant if the *P* value was ≤ 0.05.

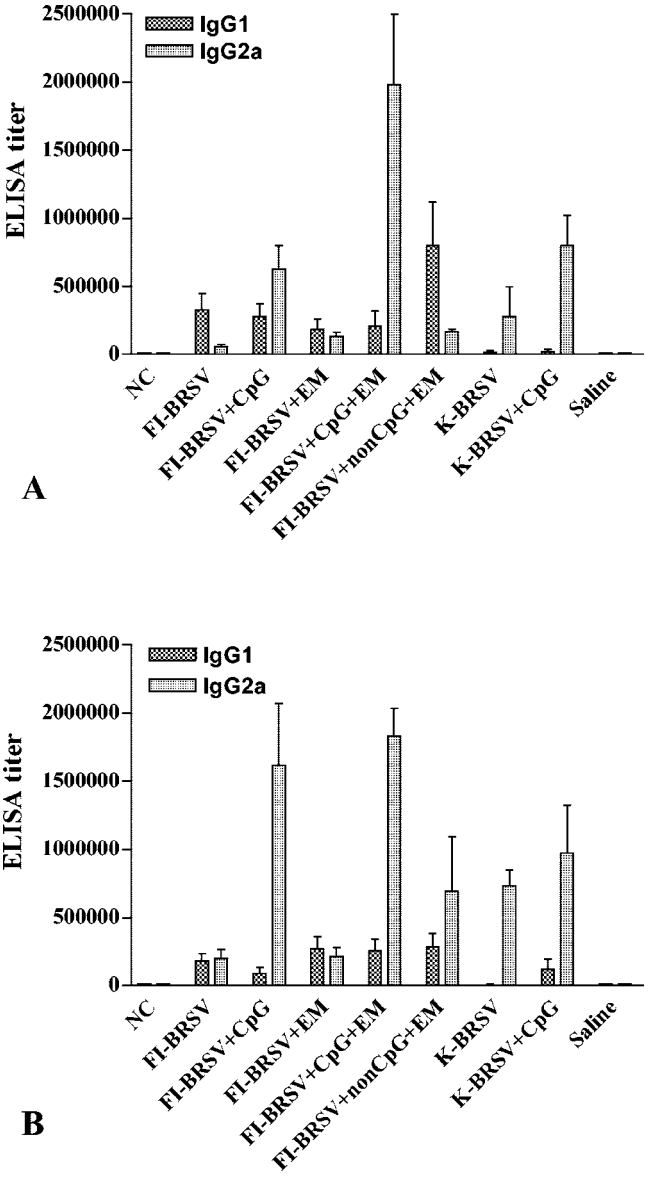

FIG. 1. BRSV-specific IgG1 and IgG2a in mouse sera after two immunizations (A) and challenge (B). Mice were vaccinated twice with either FI-BRSV, FI-BRSV/CpG, FI-BRSV/EM, FI-BRSV/CpG/EM, FI-BRSV/nonCpG/EM, K-BRSV, K-BRSV/CpG or saline. CpG ODN was given at 10 μg/dose, and the amount of Emulsigen (EM) was 30% (vol/vol). IgG1 and IgG2a titers specific for BRSV are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the value of a negative control serum. Results are expressed as the mean ± standard error of the mean. NC, mice injected with saline but not challenged; all other groups were challenged with BRSV.

RESULTS

Humoral immune response to FI-BRSV and K-BRSV. The ability of CpG ODNs to modulate the humoral immune responses induced by FI-BRSV and K-BRSV was investigated by measuring BRSV-specific IgG1 and IgG2a titers in the serum. All vaccinated groups produced higher antibody titers ($P < 0.05$) than the saline group, both before (Fig. 1A) and after (Fig. 1B) viral challenge. However, there were no significant differences in IgG1 levels between any of the vaccinated groups. In contrast, before challenge the groups immunized with FI-BRSV/CpG or FI-BRSV/CpG/Em had significantly higher IgG2a titers than the groups that received FI-BRSV ($P < 0.01$) or FI-BRSV/Em ($P < 0.005$), respectively. The role of CpG ODN in inducing IgG2a was confirmed by the fact that the mice vaccinated with FI-BRSV/nonCpG/Em had significantly ($P < 0.005$) lower IgG2a titers than animals immunized with FI-BRSV/CpG/Em. After challenge, the IgG2a titers were still significantly higher in the mice vaccinated with FI-BRSV/CpG or FI-BRSV/Em/CpG than in the animals immunized with FI-BRSV ($P < 0.01$) or FI-BRSV/Em ($P < 0.0001$). These results suggest that the groups that received FI-BRSV and CpG ODN developed a Th1 response, whereas the groups vaccinated with FI-BRSV had a Th2-biased response. Before challenge, the mice vaccinated with K-BRSV did not produce any IgG1 and produced little IgG2a, but when K-BRSV was formulated with CpG ODN, higher IgG2a titers were observed, al-

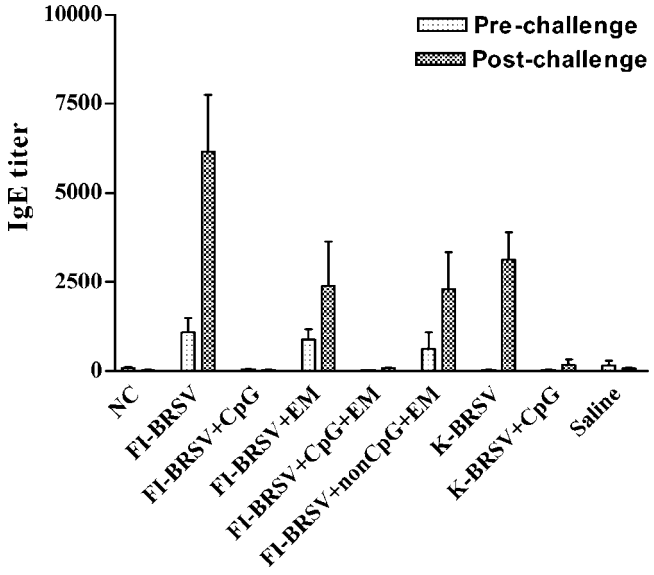

FIG. 2. BRSV-specific IgE in mouse sera after two immunizations and after challenge. Mice were vaccinated twice with FI-BRSV, FI-BRSV/CpG, FI-BRSV/EM, FI-BRSV/CpG/EM, FI-BRSV/nonCpG/EM, K-BRSV, K-BRSV/CpG or saline. IgE titers specific for BRSV are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the value of a negative control serum. Results are expressed as the mean ± standard error of the mean. NC, mice injected with saline but not challenged; all other groups were challenged with BRSV.

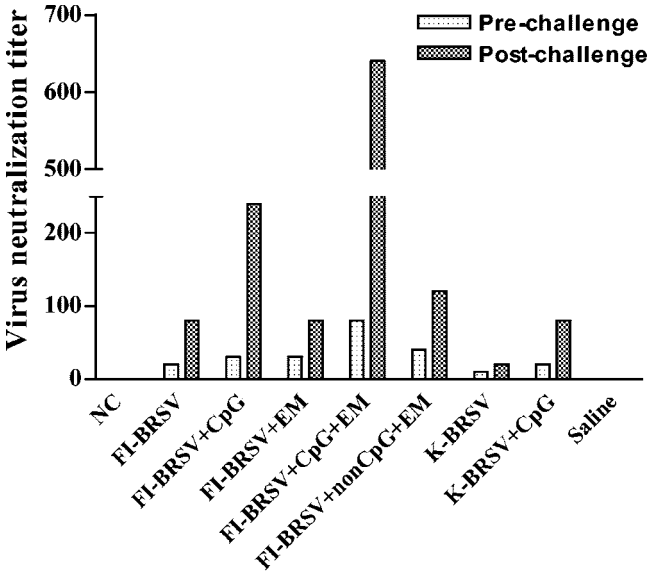

FIG. 3. Virus neutralization titers in sera after two immunizations and after challenge. Mice were vaccinated twice with FI-BRSV, FI-BRSV/CpG, FI-BRSV/EM, FI-BRSV/CpG/EM, FI-BRSV/nonCpG/EM, K-BRSV, K-BRSV/CpG or saline. The neutralizing antibody titers are expressed as the reciprocal of the highest dilution of serum that caused a 100% reduction in viral plaques relative to the virus control.

though there were no statistically significant differences between the two groups. Similarly, no significant difference was observed between K-BRSV- and K-BRSV/CpG-vaccinated mice after challenge. These results were confirmed in a second trial, with the serum IgG2a/IgG1 ratio increasing upon addition of CpG ODN to either FI-BRSV, FI-BRSV/Em, or K-BRSV.

The presence of IgE is an additional indicator of the bias of the immune response (Fig. 2). Prior to and, in particular, after BRSV challenge, mice immunized with FI-BRSV, FI-BRSV/Em, or FI-BRSV/Em/non-CpG produced substantial amounts of IgE. Formulation of FI-BRSV with CpG ODN significantly ($P < 0.0005$) reduced the serum IgE levels in vaccinated mice. Although the mice immunized with FI-BRSV/Em tended to have higher serum IgE titers, no significant difference was observed between this group and the one that received FI-BRSV/Em/CpG. However, although before challenge there was no difference in IgE titers, mice vaccinated with K-BRSV/CpG produced significantly ($P < 0.01$) less serum IgE than the animals that received K-BRSV alone after challenge.

In order to further evaluate the biological relevance of the BRSV-specific antibody responses, a virus neutralization assay was performed. As shown in Fig. 3, all vaccinated groups developed neutralizing antibody titers, whereas no neutralizing antibodies were detected in the saline group. Furthermore, the two groups immunized with CpG ODN-formulated FI-BRSV tended to develop higher neutralizing antibody titers than the corresponding groups immunized with FI-BRSV without CpG ODN or with FI-BRSV and non-CpG ODN. Similarly, K-BRSV/CpG appeared to induce higher neutralizing antibody titers than K-BRSV. After challenge, these differences became more obvious, with a three- to eightfold increase in neutralizing antibody titers upon addition of CpG ODN to FI-BRSV, FI-BRSV/Em, or K-BRSV. The neutralizing antibody titers were very similar in the two trials.

Cell-mediated immune response to FI-BRSV and K-BRSV. To further study the type of immune response elicited by the various vaccine formulations, BRSV-induced secretion of IFN-γ and IL-4 by splenocytes was measured after viral challenge (Fig. 4). Low numbers of IFN-γ-secreting cells were induced by immunization with FI-BRSV or FI-BRSV/Em. The addition of CpG ODN to FI-BRSV and FI-BRSV/Em enhanced the number of BRSV-specific IFN-γ producing splenocytes ($P < 0.05$), whereas non-CpG ODN did not have any effect. Although there was no significant difference, the K-BRSV-vaccinated group also had lower numbers of IFN-γ-secreting cells than the K-BRSV/CpG-immunized group. In contrast, the number of BRSV-specific IL-4-secreting cells was higher in the K-BRSV-vaccinated mice than in the K-BRSV/CpG-immunized animals ($P < 0.05$) (Fig. 4). These results confirm that the addition of CpG ODN to the vaccine formulations shifted the response from a Th2-biased to a balanced or Th1-type response.

Cytokine and chemokine production and cell populations in the lungs. The effect of CpG ODN on the type of immune response induced was further examined by measuring the amount of IL-5 produced in the lungs of the mice. Mice vaccinated with FI-BRSV or FI-BRSV/Em produced significant amounts of IL-5 compared to animals that received the same vaccines formulated with CpG ODN (Fig. 5A). Indeed, IL-5 production was significantly reduced in the groups vaccinated with FI-BRSV/CpG and FI-BRSV/CpG/Em compared to the FI-BRSV ($P < 0.01$)- and FI-BRSV/Em ($P < 0.0001$)-immu-

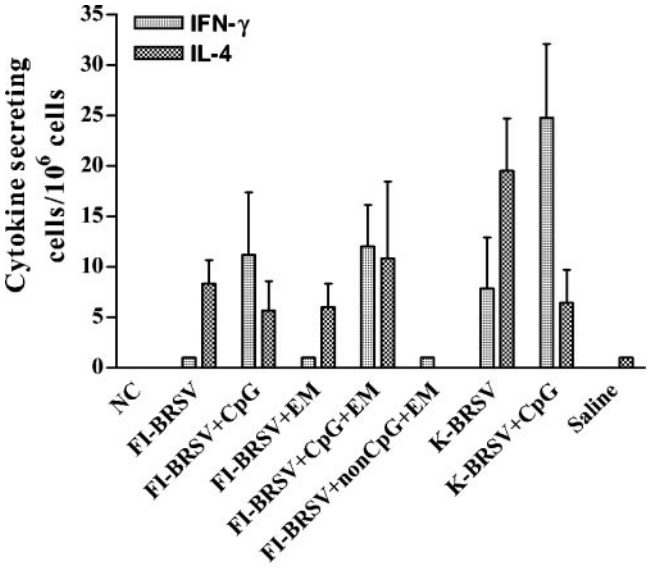

FIG. 4. Numbers of IFN-γ- and IL-4-secreting splenocytes in response to in vitro stimulation with BRSV-positive and -negative cell lysate for 24 h. Mice were vaccinated twice with FI-BRSV, FI-BRSV/CpG, FI-BRSV/EM, FI-BRSV/CpG/EM, FI-BRSV/nonCpG/EM, K-BRSV, K-BRSV/CpG or saline. The differences in the numbers of IFN-γ-secreting and IL-4-secreting cells per 10^6 cells in BRSV-stimulated wells and the IFN-γ-secreting and IL-4-secreting cells per 10^6 cells in nonstimulated wells are shown. Results are expressed as the mean ± standard error of the mean. NC, mice injected with saline but not challenged; all other groups were challenged with BRSV.

nized groups, respectively. Mice vaccinated with K-BRSV/CpG also tended to produce lower amounts of IL-5 than the animals that received K-BRSV, even if no statistical differences were observed. There was no effect of the non-CpG ODN on the amount of IL-5 in the lungs, which confirms that the modulatory effect is due to the CpG motifs in the ODN.

Chemokines are considered to be one of the inflammatory factors playing an important role in mediating the extravasation and accumulation of selective leukocyte subsets in the process of inflammation. The level of eotaxin, a chemokine member of the inflammatory family, tended to be higher in the lungs of mice vaccinated with FI-BRSV, FI-BRSV/Em, FI-BRSV/nonCpG/Em, or K-BRSV than that in the animals that received CpG ODN-formulated FI-BRSV or K-BRSV, but no significant difference was observed between the groups (Fig. 5B).

The efficacy of CpG ODN in modulating the immune response was further studied by measuring the percentages of various immune cells in the lung lavages from the mice. The percentage of eosinophils was 21 to 33% higher in the groups vaccinated with FI-BRSV, FI-BRSV/Em, or FI-BRSV/nonCpG/Em than in the groups that received FI-BRSV or FI-BRSV/Em formulated with CpG ODN. Furthermore, cells present in the lung lavages of the mice vaccinated with K-BRSV alone consisted of 15% eosinophils, while this was reduced to 1% in the group vaccinated with K-BRSV/CpG. The percentage of neutrophils was higher in the mice immunized with FI-BRSV/CpG/Em compared to the animals vaccinated with FI-BRSV/Em or FI-BRSV/nonCpG/Em. Similarly, when formulated with CpG ODN, K-BRSV produced 22% more

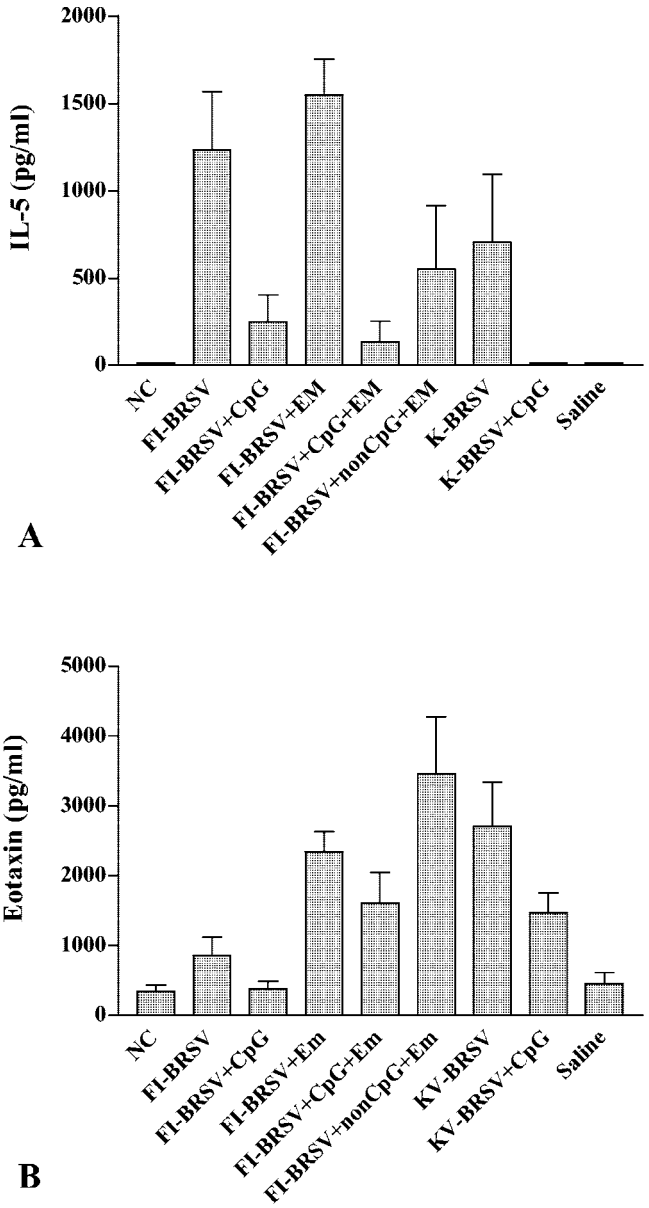

FIG. 5. Amounts of IL-5 and eotaxin secreted in the lung following two immunizations and challenge with BRSV. Mice were vaccinated twice with FI-BRSV, FI-BRSV/CpG, FI-BRSV/EM, FI-BRSV/CpG/EM, FI-BRSV/nonCpG/EM, K-BRSV, K-BRSV/CpG or saline. (A) Amounts of IL-5 measured in lung homogenates and expressed as picograms per milliliter ± standard error of the mean. (B) Amounts of eotaxin measured in lung homogenates and expressed as mean picograms per milliliter ± standard error of the mean. NC, mice injected with saline but not challenged; all other groups were challenged with BRSV.

neutrophils (Fig. 6). The number of lymphocytes was low in all of the groups, while the percentage of alveolar macrophages was high. Very similar cell numbers were observed in the second trial, with a decrease in eosinophils of between 22 and 28% when CpG ODN was added to the FI-BRSV or K-BRSV formulations.

Protection from BRSV challenge. In order to assess whether any of the vaccination strategies resulted in protection from virus infection, all groups of mice, except the negative control

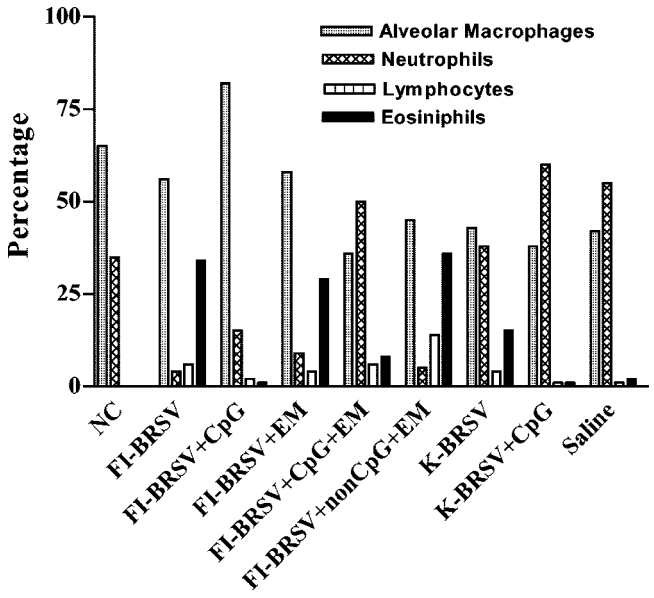

FIG. 6. Percentages of various immune cells in the lung lavages of vaccinated and challenged mice. Mice were vaccinated twice with either FI-BRSV, FI-BRSV/CpG, FI-BRSV/EM, FI-BRSV/CpG/EM, FI-BRSV/nonCpG/EM, K-BRSV, K-BRSV/CpG or saline. NC, mice injected with saline but not challenged; all other groups were challenged with BRSV.

group, were challenged with BRSV after two vaccinations. The virus titers in the lungs were determined 4 days later. The group that did not receive any vaccine had the highest virus titer (Fig. 7). Some of these mice had a titer as high as 10^7 PFU/ml, while others had titers of 10^5 PFU/ml. The efficacy of the vaccines was demonstrated by a significant decrease in virus in the lungs of the vaccinated groups. Compared to the saline-immunized group, the most efficient inhibition was observed in the groups that received FI-BRSV/CpG, FI-BRSV/CpG/Em, or K-BRSV/CpG ($P < 0.0001$). Although the other groups of vaccinated mice also shed less virus than the saline-immunized group ($P < 0.01$), they were not as well protected. These results were confirmed in a second trial, in which we observed a significant decrease in virus titer in the groups immunized with CpG ODN-formulated BRSV vaccine.

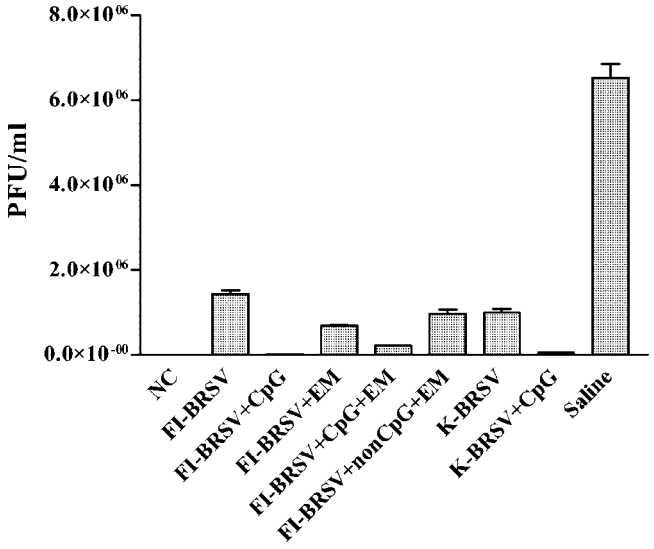

FIG. 7. Virus isolation from lungs after challenge with BRSV. The amount of virus isolated is calculated as mean PFU per milliliter ± standard error of the mean. Mice were vaccinated with FI-BRSV, FI-BRSV/CpG, FI-BRSV/EM, FI-BRSV/CpG/EM, FI-BRSV/nonCpG/EM, K-BRSV, K-BRSV/CpG or saline. NC, mice injected with saline but not challenged; all other groups were challenged with BRSV. Geometric means are shown.

DISCUSSION

Since the failure of the formalin-inactivated HRSV vaccine in the 1960s, which was characterized by massive lung lymphocyte infiltration and eosinophilia, as well as a Th2-biased response, a search for new RSV vaccines has been hampered by several challenges, including the difficulty of inducing protection early in life and the risk of exacerbating disease upon natural infection. Most adjuvants that have been used in vaccines against RSV, such as aluminum hydroxide and aluminum phosphate, promote a Th2-type immune response, which is characterized by the activation of T cells that express IL-4, IL-5, IL-9, and IL-13 and the induction of allergic inflammation due to IgG1 and IgE class switching (5, 14).

CpG DNA is a novel adjuvant that induces secretion of Th1 cytokines, including IFN-γ and tumor necrosis factor beta, and production of antigen-specific IgG2a by B cells (10, 14, 30). In the present study, CpG ODNs presented all the characteristics of an adjuvant appropriate to balance or shift the immune response. FI-BRSV elicited a Th2-biased immune response regardless of whether Emulsigen was present in the formulation. However, when FI-BRSV was formulated with ODN 1826, a balanced or Th1-type immune response was demonstrated based on serum antibody isotypes and cytokine production. Although the immune response induced by K-BRSV was not as strongly Th2 biased, the addition of CpG ODN to this commercial vaccine also resulted in a more Th1-type response. This suggests that coadministration of CpG ODN with FI- or K-BRSV resulted in a balanced or Th1-type immune response in vaccinated mice. Furthermore, the enhanced neutralizing antibody responses in the groups of mice vaccinated with FI-BRSV/Em/CpG, FI-BRSV/CpG, or K-BRSV/CpG compared to the mice immunized with FI-BRSV or K-BRSV without CpG ODN confirm that the addition of CpG ODN to the FI-BRSV, FI-BRSV/Em, and K-BRSV vaccine formulations results in an improved immune response.

IgE production is considered a hallmark of a type 2 immune response. HRSV-specific IgE has been detected in the nasal secretions of infants during the recovery phase of RSV bronchiolitis (62, 63). Indeed, IgE levels were high in infants with bronchiolitis and correlated with the degree of hypoxemia during acute RSV disease (15). In infected calves, BRSV has also been associated with production of BRSV-specific IgE, and IL-4 production has been commonly found in lymph node cells (18). In the present study, the groups of mice that were vaccinated with FI-BRSV without CpG ODN also had serum IgE titers prior to challenge. The IgE levels increased dramatically in the FI-BRSV groups and also developed in the K-BRSV group after challenge, which confirms that exacerbation of disease occurred. CpG ODNs are known to play a key role in

the shift of the immune response toward a Th1-type response, and therefore would be expected to reduce IgE levels. Indeed, CpG ODNs have been previously shown to reduce serum IgE in a murine model of asthma (1, 39). The reduced IgE levels in the mice vaccinated with FI-BRSV or K-BRSV formulated with CpG ODN correlated with previous observations (23) and supported the ability of the CpG ODN to reduce IgE levels.

IFN-γ was increased in the mice vaccinated with FI-BRSV or K-BRSV formulated with CpG ODN, whereas IL-4, a Th2 cytokine, was significantly decreased in the K-BRSV/CpG-vaccinated mice, which further confirms the role of CpG ODN as an immune modulator (10, 33, 41, 42, 49, 60). Results from several studies have demonstrated the importance of a cell-mediated immune response in protection from BRSV. The development of a Th1-biased immune response is accompanied by a high level of antigen-specific T-cell proliferation as well as by the secretion of Th1 cytokines in an antigen-specific manner. In contrast, IL-4 secreted by Th2 cells selectively induces immunoglobulin gene switching to IgE and IgG1, whereas IFN-γ secreted by Th1 cells inhibits IL-4 and causes production of IgG2a. IFN-γ is also very important in viral clearance because it induces macrophage activation and increases expression of major histocompatibility complex class I and II molecules (29). Vaccination of calves with FI-BRSV prior to challenge appears to result in diminished IFN-γ production by T cells in response to BRSV infection, and decreased IFN-γ production may contribute to enhanced disease severity in FI-BRSV-vaccinated calves (64).

The efficacy of CpG ODN in reducing IL-5 in the lungs is also very important, as IL-5 induces the proliferation of eosinophils both in vitro and in vivo (6). In the present study, as well as a previously reported study using the HRSV F protein vaccine (23), IL-5 levels and pulmonary eosinophilia were both significantly reduced when CpG ODN was added to the vaccines. Indeed, in the groups of mice vaccinated with FI-BRSV or K-BRSV, the amounts of IL-5 were 5 to 20 times higher than those in the groups immunized with FI- or K-BRSV formulated with CpG ODN.

Chemokines are important factors that control leukocyte function and are essential in mediating leukocyte trafficking and orchestrating cell activation and cytokine expression. They are also involved in RSV-mediated lung inflammation and pathology (21). To evaluate the role of CpG ODN in reducing eotaxin, a chemokine member of the inflammatory family, we measured its level in the lungs of the vaccinated and control mice. Overall, the level of eotaxin followed the same pattern as IL-5 in the lungs, which correlates with previous work in which IL-5 and eotaxin were associated with acute exacerbation of disease in humans (45). Indeed, the groups of mice that received CpG ODN appeared to produce less eotaxin in the lung than the groups without CpG ODN, even if no statistical differences were observed.

Eosinophils play a prominent role in promoting pathophysiologic conditions such as respiratory allergy and airway hyperreactivity (8). Enhanced disease and pulmonary eosinophilia associated with FI-HRSV were reported in the 1960s, when a disease-exacerbating FI vaccine was administered to infants and children in the United States (9, 17, 35, 61), and were also demonstrated in mice (12, 20, 25, 28, 44). In the present study, the percentage of eosinophils was 34% in the lungs of mice vaccinated with FI-BRSV, while this was reduced to 1% when CpG ODN was added. The same pattern was observed when FI-BRSV was codelivered with Em (29% eosinophils), in comparison to Em and CpG ODN (8% eosinophils), which correlated with the production of IL-5 and eotaxin, as previously reported (47). The effects of CpG were further confirmed when the non-CpG ODN did not reduce the amount of eosinophils when formulated with FI-BRSV and Emulsigen (36% eosinophils). While the percentage of eosinophils decreased significantly in the presence of CpG ODN, the increase in numbers of neutrophils indicates a possible role of these cells in the clearance of the virus (59).

We also determined whether the CpG ODN-mediated modulation of the immune responses induced by vaccination would correlate with inhibition of virus replication. The coadministration of CpG ODN with FI- or K-BRSV vaccine resulted in a significant reduction in infectious virus in the lungs after BRSV challenge, so in this study, as in a previous study using the HRSV F protein vaccine (23), CpG ODN contributed to the reduction in virus replication, while non-CpG ODN did not.

The results presented here demonstrate for the first time the ability of CpG ODN-formulated inactivated BRSV vaccines to generate balanced or Th1-biased immune responses. Indeed, the increases in serum IgG2a titers and IFN-γ levels, followed by reduction of eosinophilia and IL-5 in the lung, in vaccinated mice correlated with reduced pulmonary immunopathology and ultimately led to protection from virus infection.

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