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The Immunogenicity and Protective Efficacy of Bovine Herpesvirus 1 Glycoprotein D plus Emulsigen Are Increased by Formulation with CpG Oligodeoxynucleotides†

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The immunogenicity and protective efficacy of a bovine herpesvirus 1 (BHV-1) subunit vaccine formulated with Emulsigen (Em) and a synthetic oligodeoxynucleotide containing unmethylated CpG dinucleotides (CpG ODN) was determined in cattle. A truncated, secreted version of BHV-1 glycoprotein D (tgD) formulated with Em and CpG ODN at concentrations of 25, 2.5, or 0.25 mg/dose produced a more balanced immune response, higher levels of virus neutralizing antibodies, and greater protection after BHV-1 challenge compared to tgD adjuvanted with either Em or CpG ODN alone. In contrast, tgD formulated with Em and either 25 mg of a non-CpG ODN or another immunostimulatory compound, dimethyl dioctadecyl ammonium bromide, induced similar immunity and protection compared to tgD formulated with Em alone, a finding which confirms the immunostimulatory effect of ODN to be CpG motif mediated. Our results demonstrate the ability of CpG ODN to induce a strong and balanced immune response in a target species.

Adjuvants play an important role in the efficacy of vaccines. In addition to increasing the strength and kinetics of an immune response, adjuvants also play a role in determining the type of immune response generated. Aluminum compounds, including aluminum hydroxide and aluminum phosphate, are widely used in human vaccines. These adjuvants skew the immune response toward a T-helper type 2 (Th2) response, which is characterized by the secretion of Th2 type cytokines such as interleukin-4 (IL-4) and IL-5 and the generation of immunoglobulin G1 (IgG1) and IgE, but weak or absent cytotoxic-T-lymphocyte responses (4, 6, 10). Development of the appropriate type of immune response is essential for effective immunization. Immunity associated with a Th1-type immune response is thought to be essential for the control of intracellular pathogens, whereas immunity associated with mixed Th1/Th2 type immune responses appears to be essential for the control of extracellular pathogens (9). Synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG ODN) are novel adjuvants, which in mice, promote Th1-type immune responses characterized by enhanced secretion of IFN-γ, tumor necrosis factor alpha, and IL-12 cytokines, opsonizing antibodies such as those of the IgG2a isotype, and strong cytotoxic-T-lymphocyte induction (7, 20). Moreover, CpG ODN is not associated with adverse injection site reactions or other side effects and is generally well tolerated by humans and animals (51; H. L. Davis et al., Abstr. 2nd Int. Symp. Activating Immunity with CpG Oligos, 2001).

Bovine herpesvirus 1 (BHV-1), a member of the *Alphaherpesvirinae* subfamily, is associated with a variety of clinical disease manifestations, including rhinotracheitis, vulvovaginitis, abortions, conjunctivitis, encephalitis, and generalized systemic infections (13, 55). Infections occur even though live attenuated and killed vaccines are available. At present, the greatest potential for combined efficacy, safety, antigenic specificity, and protection against BHV-1 resides in subunit vaccines consisting of one or more of the viral glycoproteins (glycoprotein B [gB], gC, and gD). We have previously shown that a subunit vaccine containing a truncated, secreted form of glycoprotein D (tgD) is as efficient as the authentic full-length gD in protecting calves against BHV-1 disease (48). However, conventional adjuvants used for subunit vaccines such as Emulsigen combined with dimethyl dioctadecyl ammonium bromide (DDA/Em) not only generate a Th2-like immune response but are not metabolized and leave injection site reactions (27, 53). Indeed, in humans, DDA is known to induce a host of inflammatory reactions, including swelling, pain, and delayed-type hypersensitivity at the site of injection (50). Because of its inflammatory tendencies, DDA is also used to induce experimental arthritis in rats (31). Since such reactions are unacceptable for human or veterinary vaccines, this clearly indicates the need for improved adjuvants.

Recently, it was shown that formulating hepatitis B surface antigen with both CpG ODN and alum augments immune responses in mice without causing significant tissue damage compared to the effects of other adjuvants or adjuvant combinations (51). However, despite the promising results in mice, BHV-1 tgD formulated with CpG ODN and alum induced similar immune responses to tgD formulated with CpG ODN alone and failed to completely protect calves from BHV-1 challenge (35). In addition, BHV-1 subunit vaccines formulated with Freund incomplete adjuvant also failed to protect calves from BHV-1 challenge (18). These observations further indicate a need for better veterinary adjuvants.

Since CpG ODN is an innocuous alternative to DDA, the

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purpose of the present study was to determine whether CpG ODN could augment and modulate the immune response to BHV-1 tgD formulated with Em, a licensed veterinary adjuvant. Em and CpG ODN were evaluated either alone or in combination for their ability to modulate humoral and cellular immune responses to tgD and to protect animals from challenge with BHV-1. CpG ODN and Em adjuvant combinations (CpG/Em) were compared to DDA/Em or non-CpG ODN combined with Em (non-CpG/Em). In contrast to tgD formulated with Em or CpG ODN alone, tgD formulated with the CpG/Em combinations induced a balanced and fully protective immune response in cattle. Since tgD formulated with non-CpG/Em or DDA/Em failed to fully protect against viral challenge, the immune stimulatory effect of CpG/Em was due to the presence of the CpG motifs. Since most vaccination experiments depend on inbred mouse models, our data have significant implications for the vaccination of outbred target species.

MATERIALS AND METHODS

Cells and virus. The Cooper and 108 strains of BHV-1 were propagated in Madin-Darby bovine kidney (MDBK) cells as previously described (48). Strain 108 was used to challenge cattle, whereas strain Cooper was used in plaque titration and virus neutralization assays.

Production, processing, and purification of BHV-1 gD, tgD, and tgB. A truncated version of BHV-1 gD (tgD) was constructed by terminating the protein at amino acid 355, immediately upstream of the transmembrane anchor. It was expressed in MDBK cells under regulation of the bovine heat shock 70A (hsp70) gene promoter (21) and purified as previously discussed (48). Authentic gD (45) and tgB (24) were produced, processed, and purified as described elsewhere.

CpG and non-CpG ODN. A synthetic oligodeoxynucleotide (ODN) containing unmethylated CpG dinucleotides (Qiagen-GmbH, Hilden, Germany) was used as an adjuvant in the present study. The CpG ODN we used was ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT; CpG motifs are underlined). We have shown that this ODN stimulates bovine peripheral blood mononuclear cells (PBMC) in vitro (33, 36). To determine whether immune responses were induced by the CpG dinucleotides, we also used a non-CpG ODN, 2041 (CTGG TCTTTCTGGTTTTTTTCTGG). The CpG and non-CpG ODN were modified with phosphorothioate to increase resistance to nuclease degradation (39).

Immunization. Nine-month-old, BHV-1-seronegative Angus and Hereford cross calves were normalized for sex distribution and average body weight and allotted into eight groups of seven animals. Both breeds are susceptible to infection with BHV-1, and cross-bred calves have routinely been used in BHV-1 studies (18, 47, 54). The animals were immunized with 50 μg of BHV-1 tgD formulated with 30% (vol/vol) Em (MVP Laboratories, Ralston, Nebr.); 30% (vol/vol) DDA/Em (Em containing 24 mM DDA); 25 mg of CpG ODN (CpG); a combination of 30% Em and 25 (high), 2.5 (medium), or 0.25 (low) mg of CpG ODN (H CpG/Em, M CpG/Em, L CpG/Em, respectively); or a combination of Em and 25 mg of non-CpG ODN (non-CpG/Em). The vaccines were administered subcutaneously (s.c.) in a 2-ml volume. Placebo animals were immunized with 2 ml of phosphate-buffered saline (PBS) only. After 39 days, the animals were reimmunized and then challenged 2 weeks after the secondary immunization.

Experimental challenge and clinical evaluation. Two weeks after secondary immunization, animals were moved into an isolation pen, weighed, and examined clinically. The calves were then individually exposed for 4 min to an aerosol of 10^7 PFU of BHV-1 as previously described (26, 45). After challenge, the calves were weighed and clinically evaluated daily for 11 consecutive days. Clinical evaluation was performed at the same time each day by a veterinarian who was unaware of the vaccine status of the animals. The clinical signs evaluated included fever, depression, rhinitis, and conjuctivitis.

Sampling and virus isolation. Serum for assessment of antibody responses was collected on days 0, 14, 39, 47, 53, 57, 61, 64, and 67 after primary vaccination. Blood with anticoagulant (EDTA to a final concentration of 0.2%) was collected on days 50 and 61 after primary immunization for assessment of in vitro proliferation and IFN-γ production by enzyme-linked immunospot (ELISPOT) assay and by enzyme-linked immunosorbent assay (ELISA). Nasal tampons containing up to 5 ml of nasal fluid were collected every second day postchallenge and processed the same day to measure virus shedding. Virus recovered from nasal tampons was quantified by plaque titration in microtiter plates with an antibody overlay as previously described (38).

ELISA. In order to determine antibody responses before and after challenge, 96-well polystyrene microtiter plates (Immulon 2; Dynatech, Gaithersburg, Md.) were coated overnight with 0.05 μg of either purified tgD or purified tgB (23) per well and incubated for 2 h at room temperature with serially diluted bovine sera, starting at 1:10 in threefold dilutions. Alkaline phosphatase (AP)-conjugated goat anti-bovine 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 (Sigma Chemical Co., Oakville, Ontario, Canada). Absorbance was read at 405 nm with a reference wavelength of 490 nm.

Immunoglobulin isotyping ELISA. In order to determine the gD-specific IgG1 and IgG2 titers, 96-well polystyrene microtiter plates were coated overnight with 0.05 μg of purified tgD per well and blocked for 30 min at 37°C with 1% heat-inactivated horse serum. Serially diluted bovine sera, starting at 1:10 in threefold dilutions, were incubated overnight at 4°C in the tgD-coated plates, and bound antibodies were detected with mouse monoclonal IgG specific for bovine IgG1 and IgG2 (provided by K. Nielson, Agriculture Canada, Animal Disease Research Institute, Nepean, Ontario, Canada) as described previously (15, 32). The IgG1- and IgG2-specific antibodies were used at 0.05 and 0.5 μg of IgG/ml, respectively, and detected with AP-conjugated goat anti-mouse IgG (Kirkegaard & Perry) at a dilution of 1:10,000. The reaction was visualized as described for the ELISA assays. The results were expressed as total titers, as well as ratios of IgG1 to IgG2.

Virus neutralization assays. The virus neutralization titers of the bovine sera were determined as described previously (2). Titers were expressed as the reciprocal of the highest dilution of antibody that caused a 50% reduction of plaques relative to virus control.

In vitro proliferation of PBMC. PBMC were isolated by layering on Ficoll-Paque Plus (Pharmacia, Mississauga, Ontario, Canada) and cultured in triplicate in a 96-well tissue culture plate at 3.5×10^5 cells/well in minimum essential medium (Gibco-BRL, Grand Island, N.Y) supplemented with 10% (vol/vol) fetal bovine serum (Sigma), 2 mM L-glutamine (Gibco-BRL), 500 mg of gentamicin/ml, 5×10^{-5} M 2-mercaptoethanol, and 1 mg of dexamethasone/ml. Dexamethasone was added to the cultures since it reduces the level of spontaneous lymphocyte proliferative response without interfering with antigen-induced lymphocyte proliferative response (34). The mechanism of action is thought to be through an inhibition of cytokine secretion by cortisol acting on the cortisol response elements that are upstream from many cytokine genes. This inhibition is not effective when memory T cells are restimulated. PBMC were stimulated with 1 μg of gD/ml or cultured in medium alone. After 72 h in culture, the cells were pulsed with 0.4 μCi of [*methyl*-^{3}H]thymidine (Amersham, Oakville, Ontario, Canada)/well. The cells were harvested 18 h later by using a semiautomatic cell harvester (Skatron, Sterling, Va.), and radioactivity was determined by scintillation counting. Proliferative responses were calculated as the means of triplicate wells and are expressed as a stimulation index (SI), where SI represents the counts per minute (cpm) in the presence of gD divided by the cpm in medium alone.

IFN-γ ELISPOT assays. Nitrocellulose plates (Whatman, Inc., Clifton, N.J.) were coated overnight at 4°C with bovine gamma interferon (IFN-γ)-specific monoclonal antibody (clone 2.2.1) diluted to 2.5 μg of IgG/ml (34). Unbound antibody was washed off with 0.05% (vol/vol) PBS-Tween 20 (PBS-T) with a final wash in PBS. PBMC were isolated as for proliferation assays and cultured in 96-well tissue culture plates at 10^6 cells/well in the presence of 0.4 μg of gD/ml. Control cells were cultured with medium only. After 24 h, the cells were washed, resuspended in culture medium, transferred to nitrocellulose plates, and incubated for a further 24 h at 37°C, after which cells were washed with PBS-T. Subsequently, the plates were incubated for 2 h at room temperature with a rabbit serum specific for bovine IFN-γ (lot 92-131) at a dilution of 1:100 (34) and then for 2 h at room temperature with biotinylated rat anti-rabbit IgG (Zymed, San Francisco, Calif.), followed by streptavidin-AP (Gibco-BRL), each at a 1:1,000 dilution. Bound IFN-γ was visualized by using BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium substrate tablets (Sigma). The plates were washed in distilled water and air dried; spots were then counted with an inverted microscope. The number of IFN-γ-secreting cells was expressed as the difference between the number of spots per 10^6 cells from gD-stimulated cultures, and the number of spots per 10^6 cells from control cultures.

IFN-γ ELISA. Bovine PBMC were cultured as for the ELISPOT assays. After 24 h, the culture supernatants were harvested and serially diluted in 96-well plates coated with bovine IFN-γ-specific monoclonal antibody (clone 2.2.1) diluted to 2.5 μg of IgG/ml (34). To calculate the concentration of IFN-γ in the supernatants, purified recombinant bovine IFN-γ (Ciba Geigy, Basel, Switzerland) was used to generate a standard curve ranging from 2,000 to 7.8 pg/ml (*r*

> 0.98) (34). Samples and standards were assayed by using eight twofold dilutions in PBS-T at 100 μl/well. Bound IFN-γ was detected by using a rabbit serum specific for bovine IFN-γ (lot 92-131) (34), which was in turn detected by using AP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry). The reaction was visualized as described for tgD-specific antibody ELISAs. The absorbance of the substrate was measured at 405 and 490 nm. An ELISA reader program (Microplate Manager 5; Bio-Rad Laboratories) was used to construct a standard curve and to compute the concentration of IFN-γ in the samples.

Depletion of T-lymphocyte subsets. To identify the T-lymphocyte subsets from CpG/Em-vaccinated animals responsible for secreting IFN-γ, $CD4^+$ and $CD8^+$ T cells were depleted prior to ELISPOT analysis. PBMC from two animals vaccinated with L-CpG/Em were isolated and resuspended at a concentration of 4×10^7 cells/ml in PBS. Separate cell suspensions were incubated for 30 min on ice with murine monoclonal antibodies (VMRD, Inc., Pullman, Wash.) specific for either bovine CD4 (clone ILA12) or CD8 (clone CACT80C) at a concentration of 1 μg/10^6 PBMC. Cells were washed and then incubated with goat anti-mouse IgG-coated magnetic beads (Dynabeads M-450; Dynal Biotech, Great Neck, N.Y.) at a bead/target cell ratio of 5:1. Bead-bound cells were removed by using a magnetic particle concentrator (MPC-L; Dynal Biotech). The depleted cells were resuspended in culture medium for ELISPOT assays. After immunomagnetic bead depletion, $CD4^+$ and $CD8^+$ T cells comprised <1% of the total cell population as determined by flow cytometric analysis. For ELISPOT assays, each depleted cell population was cultured on nitrocellulose plates in the presence of 0.4 μg of gD/ml as described above. Controls consisted of depleted cells cultured with medium only and nondepleted cells cultured both in the presence or absence of gD.

Phenotypic analysis of PBMC. To determine the phenotypic characteristics of PBMC before and after depletion, PBMC were washed twice with a PBS solution containing 0.5% bovine serum albumin and 0.1% NaN_3 (PBSF). Aliquots of 2×10^6 cells per well in 96-well polystyrene microtiter plates (Immulon 2; Dynatech) were stained for surface antigens with monoclonal antibodies specific for either bovine CD4 (clone ILA12) or CD8 (clone CACT80C). The level of specific monoclonal antibody staining was defined by setting the threshold with an irrelevant isotype (IgG1) and concentration matched (10 μg/ml) monoclonal antibody (clone 1D7). After two washes in PBSF, fluorescein isothiocyanate-conjugated, goat anti-mouse IgG1 (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) was used to detect cell-bound monoclonal antibody, after which the cells were washed and fixed with 2% (vol/vol) formaldehyde. A FACScan (Becton Dickinson, Mountain View, Calif.) flow cytometer with the LYSIS II program was used for all flow cytometric analyses.

Statistical analysis. Data were analyzed by using the statistical software program Systat 10 (SPSS, Chicago, Ill.). Distribution of data was determined by using descriptive statistics. Data which were not normally distributed were transformed by ranking. Differences in serum neutralization titers, isotype ratios, in vitro proliferative responses, ELISPOT, and IFN-γ ELISA data were investigated by using one-way analysis of variance (ANOVA) performed on the ranks. This ANOVA was highly significant, indicating that at least two of the means of the ranks were significantly different from each other. Means of the ranks were compared by using Tukey's multiple comparison test. Differences in the number of animals with signs of disease among vaccine groups (temperature increase, weight loss and virus shedding) and between tgD and tgB-specific antibodies in bovine serum before and after challenge, were determined by two-way ANOVA performed on ranked data. Means of the ranks were compared by using Tukey's honestly significantly different multiple comparison test.

RESULTS

Humoral immune responses to tgD. In order to assess the adjuvant capabilities of CpG ODN, calves were immunized with BHV-1 tgD formulated with CpG, Em, DDA/Em, non-CpG/Em, or H-, M-, or L-CpG/Em. With the exception of the DDA/Em group, all vaccinated groups had significantly higher levels of neutralizing antibodies than the placebo group 14 days after the primary immunization ($P < 0.001$) (Fig. 1a). Antibody levels in the H-CpG/Em group were significantly ($P < 0.001$) higher than those of the non-CpG/Em, Em, CpG, or DDA/Em groups. The antibody levels increased dramatically after secondary immunization (Fig. 1b) such that all three CpG/Em groups had significantly ($P < 0.05$) higher titers than the placebo, non-CpG/Em, Em, or CpG groups. These data confirm that CpG ODN enhanced the immune response but the adjuvant activity was not dose dependent within the range tested. Importantly, antibody titers of animals immunized with tgD formulated with non-CpG/Em were not significantly different from the titers of the Em, CpG, or DDA/Em groups.

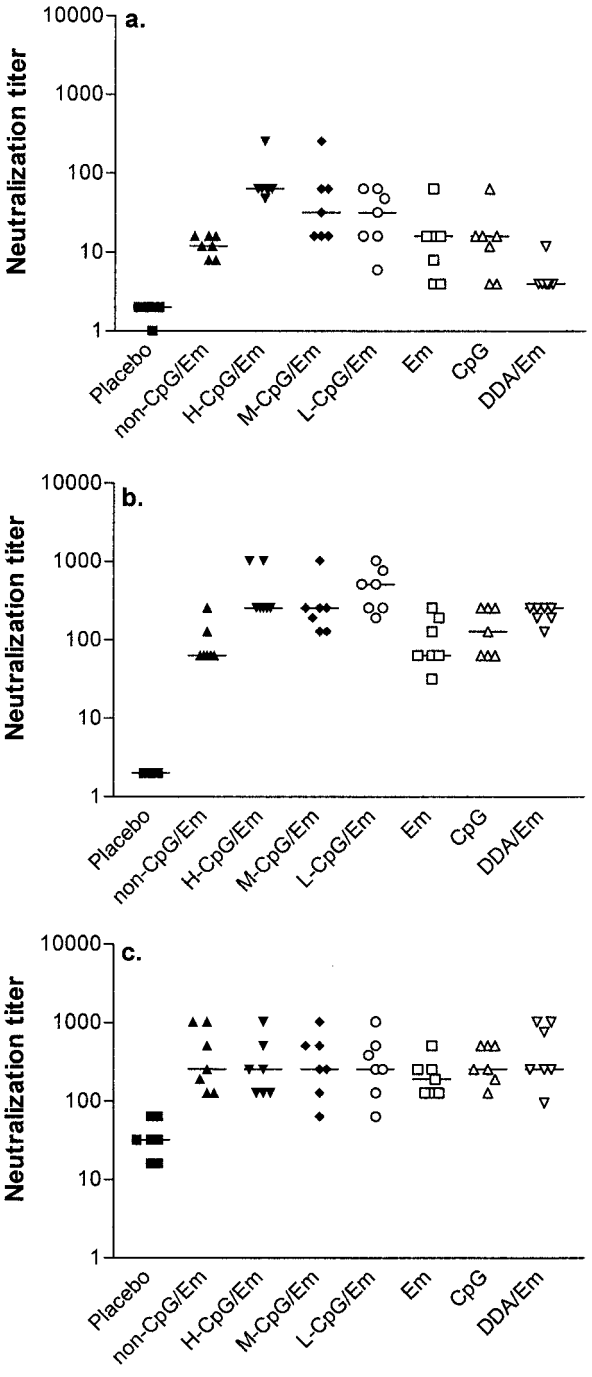

FIG. 1. BHV-1 neutralizing antibody responses in the serum of vaccinated and control animals. Antibody titers are expressed as 50% endpoint titers with 100 PFU of BHV-1. Titers of seven individual animals per group are shown, with bars indicating the median value for each group. (a) 14 days after primary immunization; (b) 8 days after secondary immunization; (c) 11 days after viral challenge.

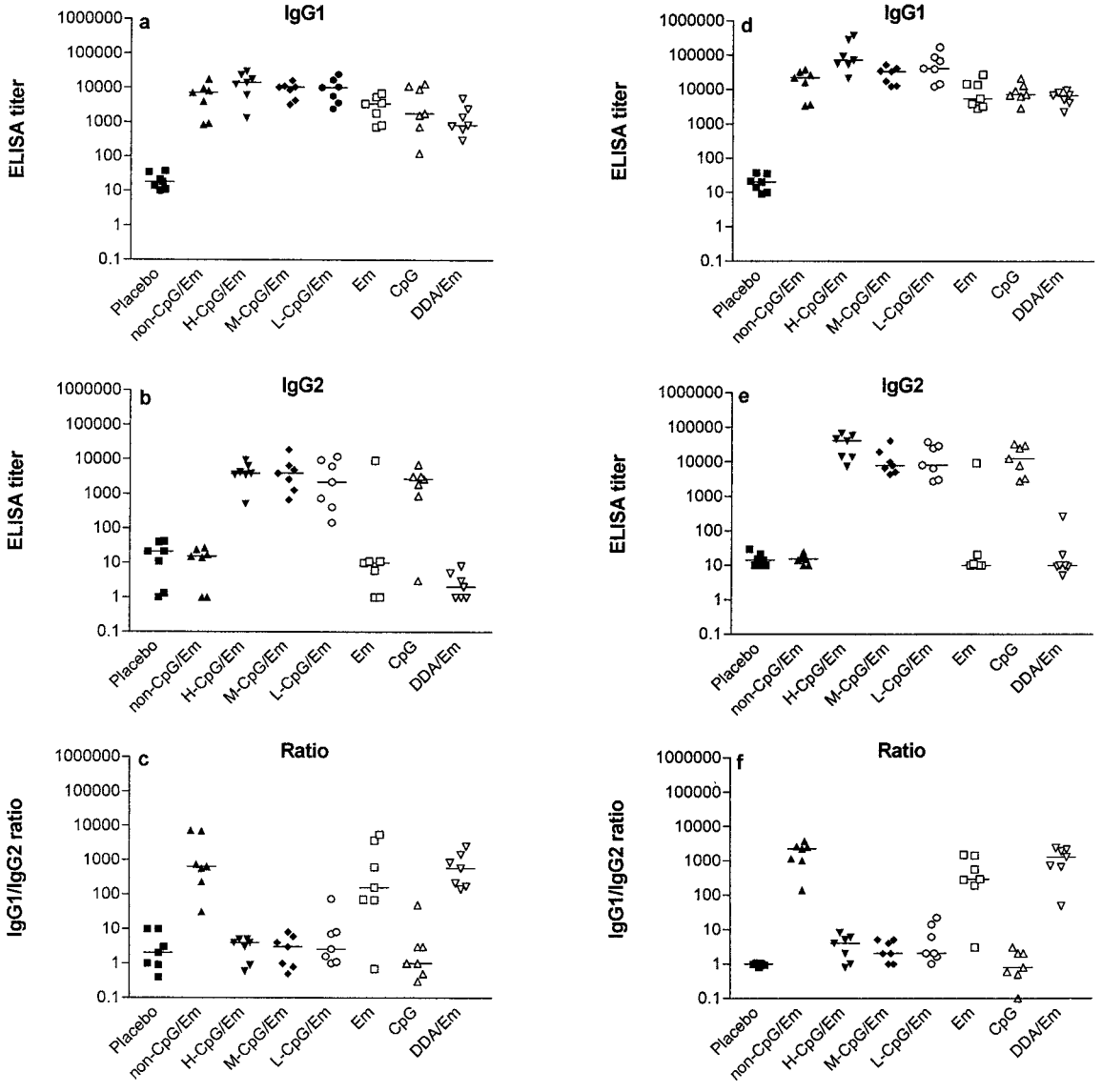

FIG. 2. BHV-1 tgD-specific IgG1 and IgG2 in bovine serum 14 days after primary immunization (a, b, and c) and 8 days after secondary immunization (d, e, and f). (a and d) IgG1; (b and e) IgG2; (c and f) IgG1/IgG2 ratio. ELISA titers specific for tgD are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the value of a negative control serum. Titers of seven individual animals per group are shown, with bars indicating the median value for each group.

This observation supports the conclusion that the phosphorothioate backbone of the CpG ODN did not contribute to the adjuvant activity and that the elevated immune response in the CpG/Em groups was directly related to the CpG motifs (28).

To characterize the type of immune response generated, tgD-specific IgG1 and IgG2 levels in the bovine sera were determined. Animals in the CpG/Em groups produced high levels of IgG1 and IgG2 after both primary (Fig. 2a and b) and secondary (Fig. 2d and e) immunization. Indeed, the IgG1/IgG2 ratio was ~5 in these groups (Fig. 2c and f), with no significant differences between H-, M-, and L-CpG/Em. Although the IgG2 titers were not significantly different between the CpG and CpG/Em groups, the IgG1 titers were significantly ($P < 0.05$) lower in the CpG group after the secondary immunization (Fig. 2d). Consequently, the IgG1/IgG2 ratio of animals in the CpG group was ~1. In contrast to the CpG and CpG/Em groups, animals in the Em, non-CpG/Em, and DDA/Em groups produced predominantly IgG1. Although the IgG1 and IgG2 titers were significantly (<0.05) lower than those in the CpG/Em groups, the IgG1/IgG2 ratios were significantly higher ($P < 0.01$) in the Em, non-CpG/Em, and DDA/Em groups than in the CpG or CpG/Em groups after both primary and secondary immunization. The IgG1/IgG2 ratios after challenge were not significantly different from the ratios after secondary immunization (data not shown).

Cell-mediated immune responses to tgD. Proliferative responses were determined as a measurement of cell-mediated immune responses induced by vaccination. Although proliferative responses of blood lymphocytes from the CpG/Em groups tended to be stronger than those in the other groups,

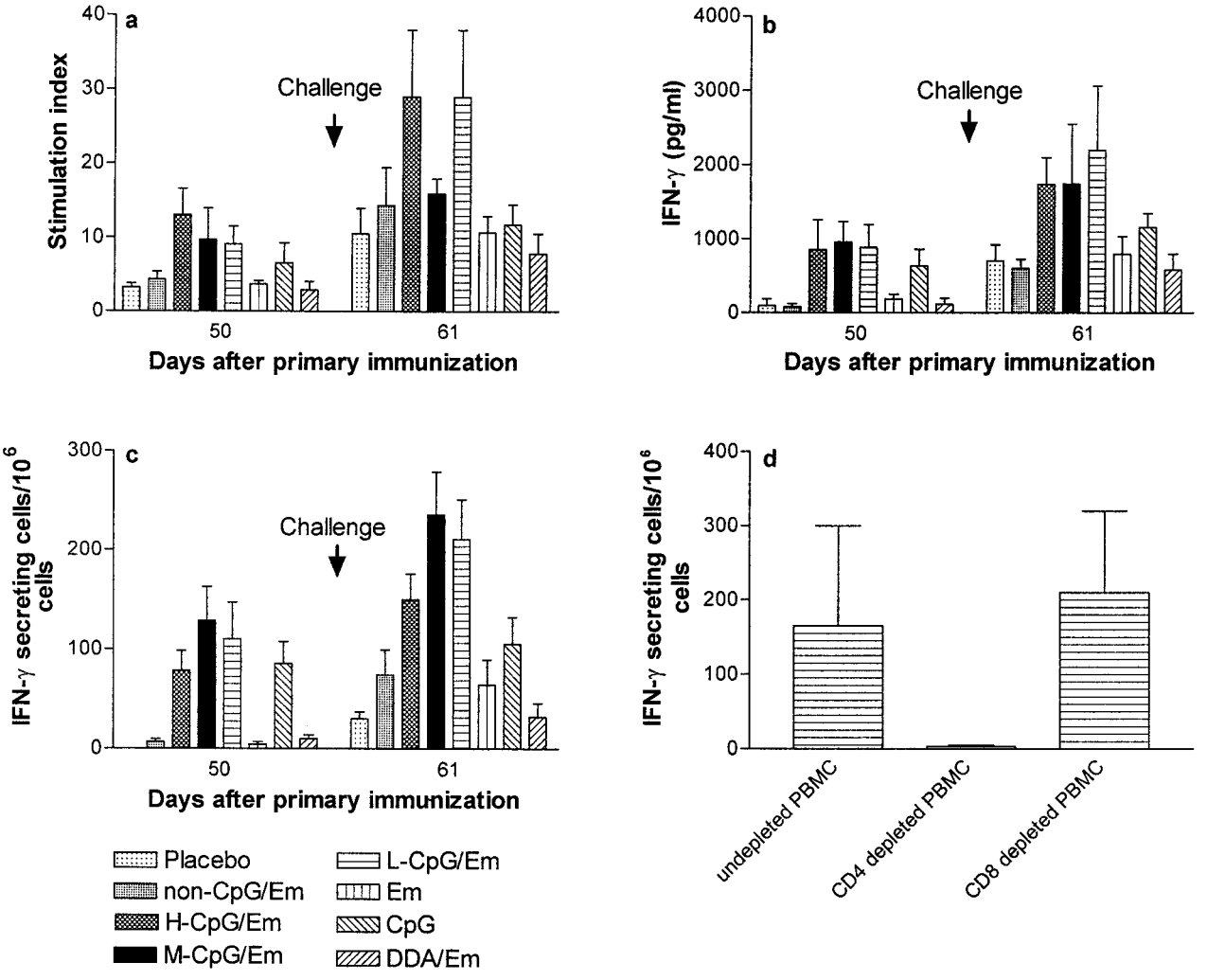

FIG. 3. Cellular immune responses after vaccination. Data are expressed as average ± the standard error of the mean of seven animals per group. (a) Antigen-specific proliferative responses of PBMC 3 days before (day 50 after primary immunization) and 8 days after (day 61 after primary immunization) BHV-1 challenge. SI represents the cpm of triplicate cultures in the presence of antigen divided by cpm of triplicate cultures in the absence of antigen. (b) Amount of IFN-γ secreted by PBMC in response to stimulation with BHV-1 gD for 24 h. (c) Number of IFN-γ secreting PBMC in response to stimulation with BHV-1 gD for 24 h. The difference in the number of IFN-γ-secreting cells per 10^6 cells in gD-stimulated wells and the number of IFN-γ-secreting cells per 10^6 cells in nonstimulated wells are shown. (d) Phenotype of T-cell subpopulations of PBMC secreting IFN-γ in response to gD. PBMC were isolated from animals vaccinated with L-CpG/Em. IFN-γ-secreting cells were calculated as the difference between the number of IFN-γ-secreting cells per 10^6 cells in gD-stimulated wells and the number of IFN-γ-secreting cells per 10^6 cells in nonstimulated wells.

differences were generally not significant (Fig. 3a). The responses of the H-CpG/Em and L-CpG/Em groups, however, were significantly ($P < 0.05$) higher than those of the DDA/Em and placebo groups. To further confirm T-cell activation, production of IFN-γ was assessed. The amount of IFN-γ measured in the supernatants of cultured PBMC from CpG/Em-vaccinated animals was significantly ($P < 0.05$) higher than IFN-γ measured in PBMC from the placebo, Em, non-CpG/Em, or DDA/Em groups (Fig. 3b). Similarly, the numbers of IFN-γ-secreting cells in the CpG/Em groups were significantly higher ($P < 0.001$) than the numbers of IFN-γ secreting cells in the non-CpG/Em, Em, DDA/Em, and placebo groups but were not statistically different from the CpG group or from each other (Fig. 3c). To identify which population of cells was secreting IFN-γ, $CD4^+$ or $CD8^+$ lymphocytes were depleted from PBMC and the number of IFN-γ-secreting cells was determined. Whereas depletion of $CD8^+$ lymphocytes did not affect the number of IFN-γ-secreting cells in the PBMC from animals in the L-CpG/Em group, the PBMC depleted of $CD4^+$ cells did not secrete IFN-γ in response to gD (Fig. 3d). These observations confirm that $CD4^+$ T cells were the primary source of gD-specific IFN-γ production.

Immune responses after BHV-1 challenge. To determine the level of cellular immunity after challenge, we measured tgD-induced proliferative responses and IFN-γ secretion from bovine PBMC. After BHV-1 challenge, proliferative responses in the H-CpG/Em and L-CpG/Em groups were significantly ($P < 0.05$) higher than those in the placebo and DDA/Em groups (Fig. 3a). Although proliferative responses in the H- and L-CpG/Em groups were also ~2-fold stronger than the responses

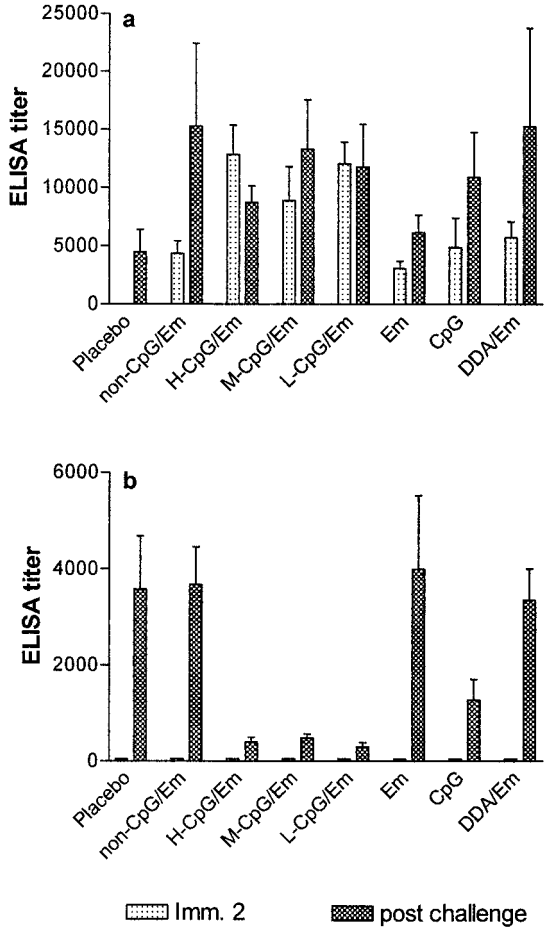

FIG. 4. Serum antibody titers specific for BHV-1 tgD and tgB 8 days after secondary immunization (Imm 2) and 11 days after viral infection (postchallenge.). (a) Antibodies specific for tgD; (b) antibodies specific for tgB. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the value of a negative control serum. Error bars show the standard error of the arithmetic mean of seven animals per group.

in the M-CpG/Em, non-CpG/Em, CpG, and Em groups, differences were not significant. The amount of IFN-γ measured in the culture media of PBMC was significantly ($P < 0.01$) higher for animals vaccinated with CpG/Em than for those vaccinated with placebo, Em, non-CpG/Em, or DDA/Em ($P < 0.001$) (Fig. 3b). In addition, the numbers of IFN-γ-secreting cells in the L-CpG/Em and M-CpG/Em groups were significantly ($P < 0.05$) higher than the numbers of IFN-γ-secreting cells in the Em, non-CpG/Em, DDA/Em, and placebo groups (Fig. 3c). Again, there was no difference between the three CpG/Em groups.

An increase in the levels of either BHV-1 neutralizing antibodies or antibodies against viral glycoproteins after challenge would be indicative of viral infection. Virus-neutralizing antibodies (Fig. 1c) and tgD-specific antibodies (Fig. 4a) measured in the sera of CpG/Em-vaccinated animals either did not increase significantly or actually decreased after challenge. In contrast, there was a significant ($P < 0.005$) increase in both virus neutralizing and tgD-specific antibodies in the sera of animals in the non-CpG/Em, CpG, Em, DDA/Em, and placebo groups. Since the vaccine antigen was tgD, an increase in antibodies against another viral glycoprotein would further confirm infection. Since gB-specific antibodies develop very early after BHV-1 infection, we used a truncated secreted version of gB (i.e., tgB) as a control antigen. Whereas no tgB-specific antibodies were measured before challenge, there was a significant ($P < 0.01$) increase after challenge in all but the CpG/Em-vaccinated animals (Fig. 4b). Collectively, these results suggest the induction of protective immunity in the CpG/Em-vaccinated animals.

Protection from challenge with BHV-1. The best measure of vaccine efficacy is the ability to protect animals from challenge. The absence of fever is one indicator of protection from infection. Whereas the mean rectal temperature of most animals increased between days 1 and 2 postchallenge and remained high until day 6, the mean temperature of the animals in the M-CpG/Em and H-CpG/Em groups remained at <39.5°C over the entire 12-day postinfection period (Fig. 5a). Although the mean rectal temperature of calves in the L-CpG/Em group increased steadily to 39.5°C by day 6, the temperature in this group remained significantly ($P < 0.001$) lower than the temperature measured for the placebo group. Another objective assessment of protection from infection is the extent of weight loss after viral challenge. Whereas animals in the H-, M-, and L-CpG/Em groups experienced minimal or no loss in weight, those in the placebo, CpG, non-CpG/Em, DDA/Em, or Em groups experienced a significant ($P < 0.05$) weight loss of up to 4 kg on day 4 after challenge (Fig. 5b).

To further determine the level of protection from BHV-1 infection, we assessed viral shedding in nasal secretions. Whereas animals in the placebo, CpG, Em, and non-CpG/Em groups began shedding virus on day 2 after challenge and continued to shed at least until day 6 postinfection, no virus was recovered from the nasal fluids of animals in any of the three CpG/Em groups (Fig. 5c). Although all vaccinated groups shed significantly ($P < 0.001$) less virus than the placebo group, the three CpG/Em groups were statistically different from the non-CpG/Em ($P < 0.01$) and placebo ($P < 0.001$) groups. These results suggest that vaccinating with both CpG and Em had significantly reduced the level of BHV-1 infection in the upper respiratory tract. Such a feature in veterinary vaccines is important for herd immunity.

DISCUSSION

We demonstrate here that BHV-1 tgD formulated with CpG/Em-induced higher neutralizing antibody titers than tgD formulated with CpG or Em alone, or with DDA/Em or non-CpG/Em. The addition of CpG ODN to Em not only influenced the magnitude of the antibody response but also altered the immunoglobulin subtypes, with the antigen-specific IgG1/IgG2 ratios being significantly lower in the CpG/Em groups. Furthermore, tgD formulated with CpG ODN in the presence or absence of Em induced higher levels of antigen-specific T-cell proliferation and IFN-γ secretion compared to tgD formulated with Em, DDA/Em or non-CpG/Em. Most importantly, tgD formulated with both Em and CpG ODN completely protected animals against BHV-1 challenge.

Although T-cell responses are considered to be critical for recovery from BHV-1 infection (43), humoral immune re-

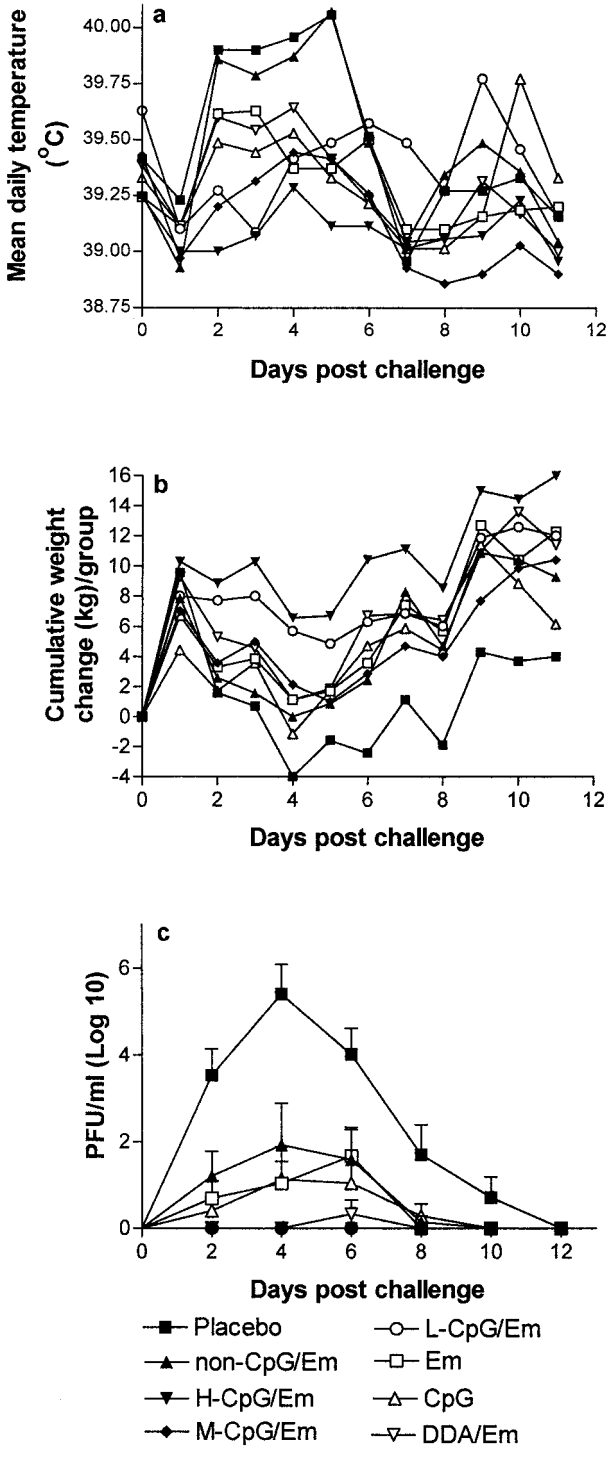

FIG. 5. Protection from BHV-1 infection. (a) Effect of various vaccine formulations on mean rectal temperature of BHV-1-challenged animals; (b) effect of immunization on average cumulative weight change per group after BHV-1 challenge; (c) virus shedding in nasal secretions after BHV-1 challenge. On the day of challenge and on alternative days thereafter, virus titers were determined in the nasal secretions of the challenged animals. Error bars show the standard error of the geometric mean of seven animals per group.

sponses appear to be very important for protection against BHV-1 infection in cattle. Indeed, strong cellular responses in the absence of high antibody titers do not fully protect against infection (25, 26). In contrast, calves fed freeze-thawed colostrum containing high levels of neutralizing antibodies to BHV-1 but no viable immune cells were fully protected against fatal multisystemic infectious rhinotracheitis (29). However, since it is not possible with active immunization to induce one type of immune response without the other, the role of humoral and cellular immunity in protection from BHV-1 infection is not fully understood. Thus, the induction of both cellular and strong humoral immune responses should be considered as the best strategy for vaccination against BHV-1. Previous studies indicate that significant protection from BHV-1-induced disease can be achieved by subunit vaccines containing one or more of the viral glycoproteins (1, 3, 12, 48, 56). However, due to inefficient humoral immune responses, some BHV-1 subunit vaccines induce little or no protection from challenge (18). The induction of protective immunity requires not only appropriate antigens but also effective vaccine adjuvants (17, 42, 46). Conventional adjuvants such as Freund complete adjuvant and DDA/Em generate strong immune responses, but they leave undesirable injection site reactions. The immunostimulatory agent in DDA/Em is an inflammatory compound, DDA. In humans, DDA is known to induce a host of inflammatory reactions, including swelling, pain, and delayed-type hypersensitivity at the site of injection (50).

Formulating with CpG ODN is a safer nontoxic alternative to DDA and is generally well tolerated by humans and animals (51; Davis et al., Abstr. 2nd Int. Symp. Activating Immunity CpG Oligos). In cattle, the use of 10 to 200 mg of CpG ODN as an adjuvant does not induce significant inflammatory responses, as measured by temperature differences, observations of injection site reactions, and serum haptoglobin levels (X. P. Ioannou et al., unpublished data). However, specific immune responses were low when CpG ODN was used as an adjuvant alone. Therefore, the objective of this trial was to increase immune responses by combining CpG ODN with Em.

Em (an oil in water emulsion) is believed to act, at least in part, by creating an antigen depot at the site of inoculation from which the antigen is slowly released, thus providing prolonged stimulation to the immune system. CpG ODN is a nonspecific immune activator that can augment immune responses in an antigen-specific manner by synergizing with signals delivered through the B-cell receptor (22) and by activating antigen-presenting cells (APC) including monocytes, macrophages, and dendritic cells (41). Maturation of immature APC such as dendritic cells is thought to play a central role in the adjuvanticity of CpG ODN. The synergy with Em is likely due to its depot effect which could increase the period of time that both the antigen and the CpG ODN are available. This adjuvant synergy supports the results of Weeratna et al. (51), who suggest that CpG ODN has a greater synergistic effect with depot-forming adjuvants than with those that act through similar mechanisms.

Because of its effects on APC, CpG ODN is an excellent candidate for immunization via the s.c. route. All of the conventional and most subunit BHV-1 vaccines are delivered intramuscularly (i.m.). However, s.c. administration is a pre-

ferred alternative in both veterinary and human practice because of its ease of administration and the immunocompetence of the skin. The skin is known to be rich in Langerhans cells, dendritic cells, keratinocytes, and other immune cells (5, 30, 44). Therefore, antigens can be efficiently taken up by professional APC (8, 11, 19, 52) and subsequently processed and presented to $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes (40). Muscle cells do not normally present antigens since they appear to express only a small number of major histocompatibility complex class I and no class II molecules (11, 16), so it is suggested that after i.m. immunization, leaking of antigen exposes the skin lymphoid tissue to injected antigen, leading to the observed immunity (37). Previously, we used CpG ODN as adjuvant in mice, sheep, and cattle and demonstrated that the immune responses induced by i.m. and s.c. immunization are equivalent (Ioannou et al., unpublished). In this report, we confirm that s.c. immunization is effective for tgD formulated with CpG ODN, resulting in protective immunity to BHV-1.

Modulation of antibody subclass distribution is an important property for adjuvants used in veterinary and human vaccines (14, 49); therefore, the ability of CpG/Em to produce a more balanced immune response compared to Em alone indicates a potential for CpG-based adjuvants in human or veterinary medicine. Indeed, recent clinical trials with CpG ODN to adjuvant human hepatitis B virus vaccines appear promising (Davis et al., Abstr. 2nd Int. Symp. Activating Immunity CpG Oligos). In the present study we show that protection against BHV-1 is associated with both a balanced and an augmented immune response. Indeed, a balanced response in the absence of high levels of neutralizing antibodies such as that produced by CpG ODN did not completely protect cattle against challenge. In contrast, a balanced and augmented immune response such as that produced with CpG/Em was associated with high neutralizing antibody titers and protection. Since the formulations inducing the highest antibody titers also induced a balanced response, the role of either antibody subtype in protection from BHV-1 infection is not clear.

In conclusion, our results indicate that high levels of both IgG1 and IgG2 as well as neutralizing antibodies and cellular immune responses, such as those produced in animals immunized with CpG and Em but not in animals immunized with non-CpG and Em, are linked to protection from viral infection.

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