Primbing by DNA immunization augments T-cell responses induced by modified live bovine herpesvirus vaccine


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DNA vaccines have several advantages over conventional vaccines. One of the most important characteristics is the presentation of antigen via both MHC class I and class II receptors. Although this generally results in strong T-cell responses, antibody production and protection achieved by DNA immunization are unfortunately not always adequate. In contrast, modified live virus (MLV) vaccines usually induce adequate antibody and moderate cellular responses, whereas killed vaccines tend to elicit weak immune responses in general. A DNA prime–MLV boost regimen should result in enhanced cellular immunity and possibly improved antibody production. To test this hypothesis, plasmids encoding bovine herpesvirus-1 (BHV-1) glycoproteins B and D were delivered by gene gun to the genital mucosa of cattle prior to immunization with modified live BHV-1 vaccine. The immune responses induced were compared to those of an MLV-vaccinated group and a negative control group. Although significantly enhanced T-cell responses were induced by priming with the DNA vaccine, there was no increase in antibody titres. Similar levels of protection were induced by the MLV vaccine alone and the DNA prime and MLV boost regimen, which suggests that there is no correlation between the induction of T-cell responses and protection from BHV-1 challenge.

Introduction

Due to the endogenous processing of antigen as a mechanism to induce an immune response, DNA vaccines have several advantages over conventional vaccines, such as antigen presentation via individual MHC class I and II receptors. Furthermore, DNA vaccines have a high degree of safety and they elicit immune responses in neonates (Donnelly et al., 1997). Unfortunately, although DNA vaccines tend to induce strong T-cell responses, antibody and protection levels are not always adequate (Laylor et al., 1999; Braun et al., 1999; Johnson et al., 2000). In contrast, killed vaccines generally elicit weak immune responses and modified live virus (MLV) vaccines usually induce adequate antibody and moderate cellular responses. Consequently, DNA vaccines could be used as a method of priming before the use of killed or modified live vaccines, either to modulate or to enhance the immune response. In support of this concept, the most potent vaccination protocol to improve the level of human immunodeficiency virus (HIV)-specific T-cells has been a DNA prime and a boost with modified live vaccinia virus Ankara (MVA) expressing CTL epitopes (Hanke et al., 1999).

Bovine herpesvirus-1 (BHV-1) is an important pathogen in cattle. The respiratory form of BHV-1, also referred to as infectious bovine rhinotracheitis (IBR) virus, manifests itself as rhinotracheitis and conjunctivitis. Illness may be prolonged or death may occur in animals with secondary bacterial infections (Yates, 1982). Single vaccination with modified live BHV-1 vaccines, according to the label claim and proven efficacy testing, is commonly used in calves on feedlot entry and in the past it appeared to control clinical disease (Van Donkersgoed & Babiuk, 1991). However, recently outbreaks of IBR have been observed in feedlot calves a few months after entry in spite of vaccination on arrival with an MLV vaccine (Van Donkersgoed & Klassen, 1995), which suggests that priming with a DNA vaccine might be beneficial. In support of this
concept, when DNA encoding herpes simplex virus-2 glycoprotein D (gD) was used for priming followed by a protein boost, both antigen-specific antibodies and Th1-type cellular responses were enhanced in mice (Sin et al., 1999).

Previously we have demonstrated that intradermal immunization with DNA encoding gD of BHV-1 into the hip of calf resulted in partial protection from virus challenge. We also reported that long-lasting T-cell and antibody responses were induced by the DNA vaccine (Braun et al., 1999). In another study we immunized cattle with DNA encoding gB of BHV-1 using the gene gun either at the hip or at the genital mucosa, and found that mucosal immunization resulted in significantly stronger T-cell responses and antibody priming than intradermal delivery (Loehr et al., 2000). The enhanced immune responses induced by this method and route of delivery, the easily accessible genital tract in cattle and the availability of MLV vaccines provides an excellent model to study DNA immunization as a method to prime immune responses to existing vaccines.

In this study, we used plasmids encoding BHV-1 gB and gD and delivered them by gene gun to the genital mucosa of cattle prior to immunization with an MLV vaccine. The immune responses induced were compared to those of an MLV-vaccinated group and an unvaccinated control group. This demonstrated that even though significantly enhanced T-cell responses were induced by priming with the DNA vaccine, there was no difference in antibody titres and, likewise, the levels of protection induced by the MLV vaccine alone and the DNA prime and MLV boost regimen were similar.

Methods

- **Cells and viruses.** Bovine viral diarrhoea virus-free Madin–Darby bovine kidney cells were cultured in minimal essential medium (MEM; Gibco-BRL), supplemented with 5% foetal bovine serum (FBS; Gibco-BRL). The 108 strain of BHV-1 was propagated in these cells.

- **Plasmids.** Plasmids pSLIAgB, pSLIAgD, pSLIAgD and pSLIAgD were constructed by cloning the genes encoding gB and gD, and the truncated versions of gB (tgB) (Li et al., 1996) and gD (tgD) (Tikoo et al., 1990), into pSL301 (Invitrogen) as described previously (Braun et al., 1997, 1998). The truncated forms lack the transmembrane anchor and cytoplasmic domain and are therefore secreted from transfected cells. The plasmids were amplified in transformed Escherichia coli (DH5α strain) and purified using anion exchange resins (Qiagen). After the concentrations were determined, the plasmids were stored at −20°C. The A260/A280 ratios were typically 1.8 or higher. All plasmids were shown to be free of endotoxins with the Limulus amoebocyte lysate kit (Bio-Whittaker).

- **Preparation of gene gun bullets.** Bullets were prepared as recommended by the manufacturer. Each DNA preparation containing one of the four plasmids was coated separately onto 1·6 µm gold beads (Bio-Rad) to ensure only one kind of plasmid on each gold particle. Gold beads, 0·05 M spermidine and DNA were mixed, 1 M CaCl2 was added dropwise while vortexing and the mixture was left at room temperature for 10 min. Subsequently, the gold bead preparations coated with plasmid encoding full-length and truncated versions of each glycoprotein were combined in a 1:2 ratio, so that each shot would have a combination of plasmids encoding gD and tgD or gB and tgB. The gold beads were washed three times with 100% ethanol, suspended in polyvinylpyrrolidone–ethanol solution and coated to the inside of Teflon tubing to be used with the Bio-Rad Helios gene gun.

- **Immunizations.** BHV-1-seronegative cows balanced by age and weight were randomly allocated to three groups. Seven animals served as negative controls (control group). Five animals (MLV group) were immunized with a modified live BHV-1 vaccine (Boehringer-Ingelheim) according to the manufacturer’s instructions 3 weeks before challenge. Five other animals (DNA-MLV group) were immunized by gene gun into the most caudal part of the vulva mucosa with plasmids encoding gB, tgB, gD and tgD at 12 and 8 weeks before challenge and subsequently with MLV vaccine 3 weeks before challenge. The conditions for delivery of plasmid were 300 p.s.i. of helium (ca. 2070 kPa), 0·25 mg gold and 1·25 µg of plasmid per shot. The total amount of plasmid delivered per immunization was 7·5 µg in six shots. Three of the six shots delivered per animal contained 0·417 µg of pSLIAgB and 0·833 µg of pSLIAgB and the other three shots contained 0·417 µg of pSLIAgD and 0·833 µg of pSLIAgD. All animals were housed under the same conditions in accordance with the guidelines of the Canadian Council on Animal Care.

- **Challenge and clinical observations.** Three weeks after immunization with MLV vaccine, each calf was exposed for 4 min to an aerosol of 106 p.f.u./ml of BHV-1 strain 108, which was generated by a DeVilbis nebulizer, model 65. On the day of challenge and for 10 days afterwards the calves were clinically examined each morning by the attending veterinarian, who was blind to the identities of the vaccine groups. Body weights and rectal temperatures were also measured daily during the clinical assessment.

- **Sampling.** Sera were collected at each immunization. Nasal tampons were used to obtain up to 5 ml of nasal fluid from all animals 6 days before challenge. Sera and nasal fluids were collected again on days 2, 4, 6, 8, 10, 13 and 17 post-challenge. Blood was collected into tubes with anticoagulant 1 day before and 9, 30 and 72 days after challenge.

- **Virus isolation.** Virus was recovered from the nasal fluids and quantified by plaque titration in microtitre plates with an antibody overlay as previously described (van Drunen Littel-van den Hurk et al., 1998).

- **ELISAs.** Polystyrene microtitre plates (Immulon 2, Dynatech Laboratories) were coated with 0·05 µg of tgD (Kowalski et al., 1993) or tgB (Li et al., 1996) per well and incubated with serially diluted bovine sera, starting at 1:10 in threefold dilutions. Alkaline phosphatase-conjugated goat anti-bovine IgG (Kirkegaard and Perry Laboratories) at a dilution of 1:10000 was used to detect IgG, and biotin-labelled goat anti-bovine IgA (VMDR) at a dilution of 1:1000 was used to detect IgA. The reaction was visualized with p-nitrophenyl phosphate (Sigma).

- **Virus neutralization assays.** The virus neutralization titres in the sera were determined as previously described (van Drunen Littel-van den Hurk et al., 1990). The titre were expressed as the highest dilution of antibody that caused a 50% reduction of plaques relative to the virus control.

- **Proliferation assays.** Bovine blood was collected into citrate-dextran and peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Paque PLUS (Pharmacia). PBMCs were dispersed at 3×106 cells/ml of culture medium consisting of MEM (Gibco-BRL), 10% FBS (Sigma), 2 mM l-glutamine (Gibco-BRL), 500 mg/ml gentamycin, 5×10–5 M 2-mercaptoethanol and 1 mg/ml dexamethasone. Subsequently, 100 µl volumes were dispensed into the wells of microtitre
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Fig. 1. Cellular immune responses prior to and after challenge. Cattle were left untreated as negative controls (c), immunized with MLV vaccine 3 weeks before challenge (m) or immunized with plasmids encoding BHV-1 gB and gD 12 and 8 weeks before challenge followed by MLV vaccine 3 weeks before challenge (dm). Glycoprotein B-specific (a) and gD-specific (b) proliferation of PBMCs at various time-points before and after challenge. Each value is the average + standard error of the mean of the stimulation index. Number of IFN-γ-secreting cells per 10^6 cells in gB-stimulated (c) or gD-stimulated (d) wells.

Fig. 2. Humoral immune responses prior to challenge. Cattle were left untreated as negative controls (c), immunized with MLV vaccine 3 weeks before challenge (m) or immunized with plasmids encoding BHV-1 gB and gD 12 and 8 weeks before challenge (dm). Glycoprotein B-specific and gD-specific ELISA titres were determined prior to challenge and are expressed as the reciprocal of the highest dilution resulting in an absorbance reading greater than mean plus two standard deviations above the control value. Significance of differences from mean of the control group are indicated as: * P < 0.05; ** P < 0.01; *** P < 0.001.

plates. Purified gB or gD at 1 µg/ml was added in a 100 µl volume to triplicate wells. After 3 days in culture the cells were pulsed with [methyl-^3H]-thymidine (Amersham) at a concentration of 0.4 µCi (14.8 kBq) per well. The cells were harvested 18 h later and thymidine uptake was measured by scintillation counting. Proliferative responses were calculated as the mean of triplicate wells and expressed as a stimulation index (mean counts per min in the presence of antigen/mean counts per min in the absence of antigen). The stimulation indices per group were calculated as the arithmetic average stimulation index.

- ELISPOT assays. PBMCs were cultured for 24 h in the presence of 1 µg of gB or gD, washed twice and resuspended to the appropriate concentration in culture medium. Nitrocellulose plates (Millipore) were coated for 2 h at room temperature with a bovine IFN-γ-specific monoclonal antibody at a dilution of 1:400. Unbound antibody was removed and 100 µl of each cell suspension was added to triplicate wells. After an overnight incubation at 37 °C, the plates were incubated with rabbit serum specific for bovine IFN-γ at a 1:100 dilution for 2–4 h at room temperature. Subsequently, the plates were incubated for 2 h at room temperature with biotinylated rat anti-rabbit IgG (Zymed), followed by streptavidin–alkaline phosphatase (BIO/CAN Scientific), each at a 1:1000 dilution. The spots were visualized with substrate consisting of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Sigma), which was left on the plates for
Fig. 3. Humoral immune responses post-challenge. Cattle were left untreated as negative controls (c), immunized with MLV vaccine 3 weeks before challenge (m) or immunized with plasmids encoding BHV-1 gB and gD 12 and 8 weeks before challenge followed by MLV vaccine 3 weeks before challenge (dm). Glycoprotein B-specific (a, c, e) and gD-specific (b, d, f) IgG (a–d) and IgA (e, f) titres in serum (a, b, e, f) and nasal fluids (c, d). ELISA titres are expressed as the reciprocal of the highest dilution resulting in an absorbance reading greater than mean plus two standard deviations above the control value. (g) Virus neutralization titres in sera, expressed as a 50% end-point using 100 p.f.u. of BHV-1. Significance of differences from mean of the control group are indicated as: * P < 0.05; ** P < 0.01; *** P < 0.001. The tgB-specific serum IgG and nasal IgG titres of the DNA-MLV group were higher than the titres of the MLV group (P < 0.05).
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Results

Cellular immune responses

To assess antigen-specific cell-mediated immune responses, lymphocyte proliferation and IFN-γ ELISPOT assays were performed with isolated PBMCs. In the proliferation assay, the PBMCs were re-stimulated in vitro with either gB (Fig. 1a) or gD (Fig. 1b). Prior to challenge, the DNA-MLV group showed significantly stronger gB-specific (P < 0.01) and gD-specific (P < 0.001) lymphocyte proliferation than the control group, whereas the MLV-vaccinated and control groups were not different. There also was a significant difference (P < 0.01) in gD-specific lymphocyte proliferation between the MLV and DNA-MLV groups. After challenge the gB- and gD-specific lymphocyte proliferation of the DNA-MLV group increased strongly (P < 0.001) and remained significantly higher than that of the control group for at least 10 weeks (P < 0.05) (Fig. 1a, b). In contrast, the stimulation indices of the MLV group were not different from those of the control group at any of the sampling times. On days 9 and 30 post-challenge, both gB- and gD-specific proliferative responses were significantly higher (P < 0.01) in the DNA-MLV group than in the MLV group. In order to assess an additional parameter for T-cell activation, IFN-γ ELISPOT assays were performed after in vitro stimulation with gB (Fig. 1c) or gD (Fig. 1d). This assay confirmed the results of the proliferation assay, since before as well as after challenge significantly more IFN-γ-secreting cells were found in the PBMCs from the DNA-MLV group than in those of the control group, after re-stimulation both with gB (P < 0.05) and with gD (P < 0.01). In contrast, the number of IFN-γ-secreting cells in the MLV group was significantly different (P < 0.05) from the control group only before challenge and only when re-stimulated with gD (P < 0.05). After challenge there also were higher numbers of IFN-γ-secreting cells in the PBMCs from the DNA-MLV group than in those of the MLV group. This difference was significant on day 9 (P < 0.01), as well as on day 30 (P < 0.05) post-challenge for gB and on day 30 (P < 0.05) for gD.

Humoral immune responses

Prior to challenge and in contrast to the cell-mediated immune responses, the gB- and gD-specific serum IgG titres in both vaccinated groups were higher than those in the control group (P < 0.001) (Fig. 2). Similarly, the gB- and gD-specific IgG titres in the nasal fluids of both vaccinated groups were significantly higher (P < 0.001) than those of the control group, although the difference in gD-specific nasal IgG titres between the control and MLV groups was not as pronounced (P < 0.05). However, there was no difference in either serum 10–60 min at room temperature. The plates were washed in ddH2O and air-dried before counting the number of stained spots in the wells. IFN-γ-secreting cells were expressed as the difference between the number of spots per 10^6 cells in antigen-stimulated wells and the number of spots per 10^6 cells in non-stimulated wells.

Statistical analyses. All data were analysed with the aid of a statistical software program (Systat 7.0, SPSS Inc.). ELISA and virus neutralization data were transformed to normality by log transformation prior to performing the analysis. Differences between the groups were examined by performing one-way ANOVA and Tukey tests for ELISPOT counts and the two-way ANOVA and Tukey HSD multiple comparison for ELISA titres, virus neutralization titres, stimulation indices, temperatures, weights and virus shedding.

Fig. 4. Clinical course after challenge with BHV-1. Cattle were left untreated as negative controls (c), immunized with MLV vaccine 3 weeks before challenge (m) or immunized with plasmids encoding BHV-1 gB and gD 12 and 8 weeks before challenge followed by MLV vaccine 3 weeks before challenge (dm). (a) Average daily rectal temperatures. (b) Cumulative weight change. (c) Nasal shedding of BHV-1. Significance of differences from mean of the control group are indicated as: * P < 0.05; ** P < 0.01; *** P < 0.001. The two vaccinated groups were not different from each other in any clinical variable.

10–60 min at room temperature. The plates were washed in ddH2O and air-dried before counting the number of stained spots in the wells. IFN-γ-secreting cells were expressed as the difference between the number of spots per 10^6 cells in antigen-stimulated wells and the number of spots per 10^6 cells in non-stimulated wells.

Statistical analyses. All data were analysed with the aid of a statistical software program (Systat 7.0, SPSS Inc.). ELISA and virus neutralization data were transformed to normality by log transformation prior to performing the analysis. Differences between the groups were examined by performing one-way ANOVA and Tukey tests for ELISPOT counts and the two-way ANOVA and Tukey HSD multiple comparison for ELISA titres, virus neutralization titres, stimulation indices, temperatures, weights and virus shedding.
or nasal IgG titres between the two vaccinated groups. Neither
the vaccinated groups nor the control group had gB- or gD-
specific serum or nasal IgA titres at the time of challenge.

After challenge there was a significant interaction effect (P
< 0·001) of treatment and time on serum IgG levels. The gB-
and gD-specific serum IgG titres rapidly increased in both
immunized groups from day 4 onwards and reached maximal
levels on day 8, in contrast to the IgG titres of the control
group, which increased from day 8 onwards until they reached
a plateau between days 12 and 17 post-challenge (Fig. 3a,
b). The kinetics and magnitude of the gB- and gD-specific IgG
titres in the nasal fluids were also significantly different (P <
0·01) between the control group and the vaccinated groups.
Both vaccinated groups experienced a decrease in nasal IgG
around day 4, correlating to the peak in nasal virus shedding,
but a rapid increase in titre on day 6. In contrast, in the control
group the IgG titres in the nasal fluids increased later (Fig. 3c,
d). The gB- and gD-specific serum IgA titres of the vaccinated
groups also increased from day 4 onwards and reached
maximal levels by day 8. However, the control group never
attained as high a titre, so overall, the serum IgA titres in the
vaccinated groups were significantly higher (P < 0·05) than
those of the control group (Fig. 3e, f). There were no detectable
levels of nasal IgA in any of the groups. The only significant
differences (P < 0·05) between the DNA-MLV and MLV
groups were found in the gB-specific serum and nasal IgG
titres, which were lower in the MLV group at some time-points
(Fig. 3a, c). In order to assess the biological relevance of the
gB- and gD-specific antibodies, virus neutralization assays
were performed. The serum neutralization titres before as well
as after challenge agreed well with the ELISA titres, both
vaccinated groups being significantly different from the control
group (P < 0·001) (Fig. 3g).

Protection from BHV-1 challenge

On the day of challenge and for up to 2 weeks afterwards,
temperatures, weights and virus shedding were measured. A
comparison of rectal temperatures demonstrated a significant
difference between both vaccinated groups and the control
group (P < 0·01). In contrast to the control group, which
developed elevated temperatures between days 1 and 7, no
temperature rise occurred in the vaccinated groups, with the
exception of a slight increase on days 2 and 9 post-challenge
(Fig. 4a). A further objective indicator for the well-being of an
animal and the level of protection achieved is the amount of
weight loss measured post-challenge. After challenge, the two
groups of vaccinated cows experienced less weight loss than
the non-vaccinated animals (P < 0·001) (Fig. 4b). Indeed, the
vaccinated groups did not lose any weight, while the control
group lost up to an average of 15 kg within the first 6 days
post-challenge. Furthermore, the animals in the control group
shed significantly more virus than the vaccinated cows (P <
0·001). The virus shedding in the nasal secretions of the control
group reached maximal levels on day 6 and ended on day 13
after challenge. In contrast, virus shedding in the two
vaccinated groups peaked on day 4 and was barely detectable
on day 8 (Fig. 4c). Although it appeared that there might be a
difference in virus shedding on days 2 and 4 post-challenge,
there was no significant difference in any of these clinical
parameters between the two vaccinated groups.

Discussion

In order to assess how priming by DNA immunization
could modulate the immune response to a modified live BHV-
1 vaccine, we immunized a group of cattle with plasmids
encoding gB and gD prior to immunization with the MLV
vaccine. The plasmids were delivered by gene gun into the
genital mucosa, since this was shown to be a convenient and
effective site for DNA immunization (Loehr et al., 2000). As
was expected based on our previous results with plasmids
encoding gB and gD (Braun et al., 1999; Loehr et al., 2000),
the group primed by DNA immunization showed a strong T-cell
response in proliferation and IFN-γ ELISPOT assays. In
contrast, the group that had only received the MLV vaccine
did not show T-cell activation in comparison to the control
group. Previously we immunized cattle with plasmids encoding
gB and gD by gene gun and observed the induction of
moderate antibody and strong cellular immune responses
(Braun et al., 1999; Loehr et al., 2000). However, the cellular
immunity elicited by DNA immunization and MLV boost in
the current trial was even stronger. Although it is possible that
other factors contributed to the strong cellular response
observed here, this suggests that there may be a synergistic
effect between these two types of vaccine. Regardless of the
mechanism involved, this strategy results in a very strong T-
cell activation, which is very important in control of a variety
of pathogens.

Modulation of the immune response to a vectored or
protein vaccine after initial DNA immunization has also been
demonstrated for other infectious agents. Generally a DNA
prime and protein boost regimen leads to a strongly increased
T-cell response and better protection than other vaccination
strategies. For example, when various methods for immuni-
ization with DNA and MVA containing different CTL epitopes
were assessed, DNA prime and MVA boost was found to be
the most potent protocol to enhance the induction of HIV-
specific T-cells (Hanke et al., 1999). Intradermal DNA prime
and MVA boost also induced good CD8+ T-cells and
protection in malaria and influenza models in mice (Degano
et al., 1999). In another study, comparing eight different vac-
cination protocols in macaques, the most promising con-
tainment of HIV infection was again achieved by intradermal
priming with DNA followed by a boost with a recombinant
fowlpox virus, which resulted in neutralizing antibody-
-independent immunity (Robinson et al., 1999). Different DNA
prime and boost strategies using DNA, MVA and virus-like
particles have also been compared for Plasmodium falciparum.
The highest levels of CD8\(^+\) T-cells were obtained after DNA prime and MVA boost, which was also the only vaccination regime leading to 100% protection (Schneider et al., 1999). Prime and boost vaccination strategies with DNA vaccines have also been performed for herpesvirus infections. DNA prime and protein boost with herpes simplex virus-2 gD (Sin et al., 1999) or DNA prime and boost with recombinant baculovirus expressing equine herpesvirus-1 gD (Ruitenberg et al., 2000) resulted in enhanced T-cell responses as well as increased antibody titres in a mouse model.

In contrast to the numerous reports on primary DNA immunization and secondary immunization with a vectored or protein vaccine, very few prime–boost regimens have been reported using replication-competent organisms. One study demonstrated that DNA immunization with plasmid encoding the colonization factor I antigen of enterotoxigenic *E. coli* (CFA/I of ETEC) and two oral booster vaccinations with live recombinant *Salmonella typhimurium* expressing ETEC antigen had a synergistic effect that resulted in systemic IgG and mucosal IgA, which could not be attained by either immunization strategy alone (Lasaro et al., 1999). Because MLVs are widely used and yet have rarely been applied in prime–boost strategies, we determined the effect of priming with a DNA vaccine on the immune responses induced by a replication-competent virus.

In our study, the DNA-MLV group developed a significant T-cell response prior to challenge, whereas essentially no T-cell activation could be observed in the MLV group. In contrast, the vaccinated groups developed similar levels of IgG in sera and nasal fluids, regardless of whether they were primed with a DNA vaccine or not. As intranasal challenge with BHV-1 following DNA immunization with BHV-1 gD or gB led to a significant increase in antibody levels (Braun et al., 1999; Loehr et al., 2000), the DNA-MLV vaccination protocol was expected to reflect a similar situation. However, MLV is less virulent than the challenge strain of BHV-1, and it was delivered intramuscularly, which suggests that the level of virus replication and the route of delivery may influence the efficacy of the virus boost. Interestingly, with similar antibody titres in the MLV and DNA-MLV groups and little T-cell activation in the MLV group, this study suggests that the T-cell activation measured by lymphocyte proliferation and IFN-\(\gamma\) production in the DNA-MLV group contributed marginally to the protection from BHV-1 infection. After BHV-1 challenge, there also was little difference between the gB- and gD-specific IgG and IgA titres of the DNA-MLV and MLV groups and, despite the significant difference in T-cell activation, there was no difference in recovery rate between the two vaccinated groups. These data show that the T-cell activation did not improve antibody production, protection or recovery from BHV-1 challenge as we had anticipated based on the results with challenge infection after DNA immunization (Braun et al., 1999; Loehr et al., 2000). Despite the contention that T-cell responses help recovery after BHV-1 infection (Turin et al., 1999), this reveals that antibodies are necessary not only for protection, but also for recovery from BHV-1 infection (Babiuk et al., 1996).

After challenge, T-cell activation in the DNA-MLV group lasted for at least 10 weeks, while the MLV group did not show any significant T-cell activation. This demonstrates not only the longevity of T-cell stimulation after DNA immunization, which has also been shown with several other immunization strategies (Haddad et al., 1999; Braun et al., 1999; Johnson et al., 2000; Hasset et al., 2000), but more importantly it shows the potential to greatly increase the T-cell stimulation induced by live virus vaccination. This potential for further T-cell stimulation can be very valuable for vaccination against diseases where strong T-cell activation is required. For example, in infections with *Listeria monocytogenes*, where protective immunity is dependent on CD8\(^+\) T-lymphocytes and IFN-\(\gamma\) secretion, a type 1 response induced by DNA vaccination by gene gun can result in protective immunity (Fensterle et al., 1999). Other similar examples include HIV, malaria and influenza virus (Degano et al., 1999; Johnson et al., 2000).

A strong T-cell activation may be of great value not only for prevention, but also for treatment of disease, such as in tumour defence, where vaccination with DNA has been effective against mammary and lung tumors (Ohwada et al., 1999; Wei et al., 1999; de Zoeten et al., 1999), papilloma (Wei et al., 1999; Chen et al., 1999; Tan et al., 1999; Ji et al., 1999) and melanoma. For example, immunization with a plasmid encoding the melanoma antigen MAGE-1, which is expressed in several human tumours of various tissues, induced antibodies and CTL responses in mice (Park et al., 1999) and thus could be a promising immune therapy for cancer. As shown in this as well as other studies, administration of protein in a replication-deficient or -competent vector could further increase the T-cell stimulation following priming with DNA, without concern about existing immunity against the vector. Since the development of immunity against the vector is one of the impediments to successful gene therapy, the combination of DNA prime and vector boost is a promising approach to inducing strong cellular immunity.

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