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Induction of specific cytotoxic activity for bovine herpesvirus-1 by DNA immunization with different adjuvants

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ABSTRACT

It is well documented that adjuvants improve the immune response generated by traditional viral vaccines; however, less is known about their effects on the immune response elicited by DNA vaccines.

In this study, we have investigated the use of adjuvants, and have analyzed the humoral and cellular specific immune responses elicited by DNA vaccines based on the BoHV-1 glycoprotein D (secreted version) in pCIneo vector with and without Montanide ISA25 (O/W), ISA206 VG (SEPPIC) and Cliptox™ (natural microparticles of clinoptilolite).

The comparison of the immune response induced in mice by pCIgD formulated with or without adjuvants showed that the immunomodulators affect the total specific humoral and cellular response. The isotypes induced by these adjuvants were of the type Th1/Th2. A significant increase in the mac-3+ and F4/80+ populations of the groups receiving pCIneo with ISA25, ISA206; and an increase in CD4+ populations of the group receiving pCIneo ISA25, in comparison with the pCIneo group was observed.

On the other hand, mice vaccinated with pCIgD/ISA25, pCIgD/ISA206, or pCIgD/Cliptox developed a significantly higher specific cytotoxic activity against BoHV-1 than the pCIgD and pCIneo groups. In this report we propose the use of ISA25, ISA206 or Cliptox as adjuvants in a DNA vaccine since they are able to induce not only a specific humoral immune response but also a specific cellular immune response.

1. Introduction

Bovine herpesvirus-1 (BoHV-1), an alphaherpesvirus, is an important etiological agent of the bovine respiratory disease complex. This virus causes a wide variety of clinical manifestations that include infectious rhinotracheitis, pustular vulvovaginitis/balanoposthitis; and may predispose animals to secondary bacterial infections which lead to high morbidity and mortality (Griebel et al., 1990; Wyler and Schwyzer, 1989; Yates, 1982). BoHV-1 uses a variety of mechanisms to elude the host's immune system. By spreading intracellularly, it can exist in the presence of anti-viral specific antibodies (Fuller and Lee, 1992; Kuhn et al., 1990; Miethke et al., 1995). For this reason, cytotoxic T-lymphocytes (CTL) are critical for the elimination of the virus (Tikoo et al., 1995). Although in European countries the control of the disease frequently depends on removal of latently infected animals and maintaining infection-free status of sero-negative herds, eradication of Infectious Bovine Rhinotracheitis (IBR) will always include the destruction of a great number of healthy, seropositive animals, which are considered to represent the virus reservoir because they are persistently infected with BoHV-1. Europe has a long history of fighting against BoHV-1 infections. However, only a small number of countries has achieved the goal of IBR-eradication. In contrast, in other countries the control strategy against this virus has been vaccination with conventional attenuated or inactivated vaccines. These vaccines cannot prevent either the efficient transmission of the virus or the establishment of latency, although they may protect individual animals against clinical disease. Additionally, live-attenuated vaccines are not entirely safe, because they may cause abortion and latency (Whetstone et al., 1992, 1986) with subsequent reactivation and shedding. Most importantly, the available vaccine strains, based on wild-type virus, may down-regulate the cell surface expression of major histocompatibility complex (MHC) class I molecules (Hariharan et al., 1993; Nataraj et al., 1997), which likely compromises the development of cytotoxic T lymphocytes (CTL) against not only BoHV-1, but also other viruses and intracellular pathogens. On the other hand, inactivated viral vaccines may not provide complete protection due to a low antigen

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load or a loss of important epitopes during the inactivation procedure. They are generally poor inducers of cellular immune responses and have a relatively short duration of immunity (Deshpande et al., 2002). Because of these limitations, there is a need for novel vaccines that contain as few viral products as possible, but at the same time, induce efficient cell-mediated and humoral immune responses.

DNA vaccines have emerged as an attractive approach for the generation of antigen-specific immunity. DNA is taken up and expressed by cells resident in the tissue, and the protein is likely to be processed and presented by local antigen presenting cells (APC). This obviates the need to purify recombinant protein and has the added benefit of intracellular expression of the antigen, which may be targeted to the class I MHC for efficient induction of cellular immune responses (Iwasaki et al., 1997; Torres et al., 1997). Thus, in addition to the practical advantages of construction, purification and stability, plasmid DNA has the potential to induce both humoral and cellular immune response against the expressed antigen. Furthermore, in contrast to recombinant viral vectors, no immune responses are developed against a plasmid vector. Therefore, DNA immunization may be one of the best approaches to induce cytotoxic responses. However, the potency of naked DNA vaccines is limited by their inability to amplify and spread *in vivo*, for this reason the adjuvant incorporation could be a good option to increase the magnitude and direction of the immune response.

Several studies have demonstrated the induction of antibody response against BoHV-1 in mice and cattle immunized with plasmids encoding BoHV-1 glycoprotein D (gD) (Cox et al., 1993; van Drunen Littel-van den Hurk et al., 1998). Futhermore, recent studies determined that gD is the best antigen to be used in a DNA vaccine against BoHV-1 (Toussaint et al., 2005) and Deshpande et al. (2002) demonstrated that genetic immunization of full-length gD induced the production of CTL. Interestingly, a vector encoding for the secreted form of the glycoprotein induced a different immune response as compared with one encoding full-length gD (Lewis et al., 1997, 1999) because of the cellular compartment to which the expressed antigen is delivered determines the type of immune response (type 1 or type 2). However, these studies did not analyze the induction of CTL response against BoHV-1 by genetic immunization of secreted gD.

The aim of this study was to evaluate the type of immune response elicited in mice by a plasmid encoding BoHV-1 secreted gD without and with different adjuvants: Cliptox™, Montanide ISA 25 and Montanide ISA 206 VG. Zeolites are microparticles of silica, silicates, and aluminosilicates which act as non-specific immunostimulators similarly to superantigens with the ability to activate a relatively large fraction (5–20%) of the T cell population, as well as humoral immunity (Ueki et al., 1994). Recently, we demonstrated that the incorporation of natural microparticles of the zeolite clinoptilolite (Cliptox™) into an inactivated Foot-and-Mouth Disease Virus (iFMDV) vaccine induces an increase of the protective immune response in mice and an up-regulation of dendritic cells and macrophages (Batista et al., 2010). This type of adjuvant has been shown to be an effective delivery system for vaccine formulations, inducing potent cellular and humoral immune responses. Furthermore, they can protect antigens against aggressive conditions such as low pH, biliary salts and enzymes.

ISA 25 and ISA 206 (Montanide™) are "ready-to-formulate" mineral oil adjuvants. Their mode of action is not yet clear but could be explained by (i) a depot effect and the slow release of the antigen from the injection site; (ii) Protection of the antigen from rapid degradation by enzymes by the emulsion; (iii) generation of an inflammatory reaction and stimulation of the recruitment of APC such as macrophages and dendritic cells; (iv) facilitation of the uptake of antigens by APC and due to interactions between the surfactant and cellular membrane.

The results presented in this work show that Cliptox, ISA25 and ISA206 adjuvants increase the basal specific humoral and cellular immune responses elicited by a genetic BoHV-1 secreted gD vaccine, and are of relevance for the development of control measures against the bovine respiratory disease complex.

2. Materials and methods

2.1. Plasmid construction

Plasmid pCIgD (Fig. 1) was constructed by inserting a PCR product encoding the secreted version of the BoHV-1 glycoprotein D (gD) in EcoRI-digested pCIneo vector (Promega). Forward primer 5′-AAGAATTCCGGCTGCTGCGAGCGGGCGAACA-3′ and reverse primer 5′-AAGAATTCTCAGGCGTCGGGGGCCGCGGGCGTA-3′ both containing an EcoRI restriction site were used to amplify a 1121 bp DNA fragment, which corresponds to 118,874 bp to 119,975 bp of BoHV-1 gD gene (GenBank Accession number Emb AJ004801) and aditional 19 bp corresponding primers desing. DNA from infected MDBK cells was extracted when extensive cytopathogenic effect was observed and the supernatant was collected and cell debris was removed by centrifugation at 3000*g* for 30 min. The virus suspension was diluted to 10^5 cell-culture infective dose (CCID50) per ml. Then this virus was used as template. The plasmid was amplified in transformed *Escherichia coli* DH5α and purified using anion exchange columns (Qiagen Plasmid Purification Mega Kit). All plasmid preparations used for immunizations had A260/280 ratios higher than 1.7. After purification, plasmid concentration was adjusted as needed with phosphate-buffered saline (PBS). Non recombinant plasmid (pCIneo) was produced and purified in a similar way.

2.2. Adjuvants

Montanide™ ISA 25, a mineral oil in water emulsion (O/W) and Montanide™ ISA 206, a mineral oil, were provided by Seppic Inc., France. The other adjuvant, Cliptox™, is a variety of zeolite named clinoptilolite. All adjuvants were used according to the Manufacturer's instructions. Cliptox was dissolved in PBS and used at 2 μg of Cliptox per μg of plasmid.

According to our knowledge and to the toxicological tests made on MONTANIDE™ ISA range (Berlin test, Oral LD 50, IP LD 50, ocular irritation test, dermal irritation test, pyrogenicity) we conclude to the non-toxicity and favourable tolerance of these adjuvants. Montanide™ adjuvants and their components have been considered as safe by the Committee for Veterinary Medical Products (CVMP) for use in immunological products and are included as authorized substances in the annex of the European Council Regulation n° 470/2009 (previously 2377/90/EC) needing no further MRL studies, or included in already registered veterinary commercial products.

In order to evaluate the safety of Cliptox™, toxicity was assessed by the HET-CAM test at the Centro de Toxicología y Biomedicina (TOXIMED), Santiago de Cuba, Cuba. This procedure has been validated by the European Center for the Validation of

Fig. 1. Construction of pCIgD plasmid. The PCR product encoding for the secreted version of gD was inserted in *Eco*RI-digested-pCIneo vector (Invitrogen). pCMV, cytomegalovirus promoter; s, signal sequence; SV40 poly A: polyadenylation sequence from SV40.

Alternative Methods (ECVAM), Ispra, Italy. Cliptox was classified as safe.

2.3. Vaccine formulations and animal treatment

Eight week-old male BALB/c mice were used. The animals (obtained from INTA –Castelar, Buenos Aires, Argentina) were acclimatized and randomly distributed into experimental groups. Mice were kept in conventional animal facilities and received water and food *ad libitum*. At day 0, groups of 10 mice were intradermically (i.d.) inoculated in the back with 15 μg DNA (pCIgD) formulated without and with Montanide ISA 25 or Cliptox in a final volumen of 0.2 ml. In the case of Montanide ISA 206 VG, animals were inoculated with the adjuvant alone and 20 h later they were inoculated again in the same area with 15 μg of non-emulsified pCIgD. The same dose was used for a booster at day 20, and seven days before the cellular immune response studies (130 dpv). Negative control groups were inoculated with the same amount of pCIneo without and with the different adjuvants. Serum samples were taken at days 20, 35, 75, and 160.

2.4. Virus preparation

BoHV-1 strain LA (Los Angeles) was propagated in Madin Darby bovine kidney (MDBK) cells, supplemented with 10% fetal bovine serum (FBS, Gibco). Confluent monolayers in roller bottles were infected with the virus at a multiplicity of infection (moi) of 0.1 and the virus was allowed to adsorb for 45 min at 37 °C before the addition of fresh culture medium. When extensive cytopathogenic effects were observed, the supernatant was collected and cell debris was removed by centrifugation at 3000*g* for 30 min. The virus suspension was stored at −70 °C. Exposure to UV light for 30 min was used for viral inactivation.

2.5. Cloning of gD recombinant protein and expression vector assembly

The sequence coding for full-length gD was amplified by PCR from BoHV-1 DNA, using the following forward and reverse primers: 5′-AAGAATTCATGTTGCCTACACCCGCGCCCGCGGGT-3′, containing an *Eco*RI restriction site followed by bp 138 to 161 of gD and 5′-AAGAATTCTCAGGCGTCGGGGGCCGCGGGCGTA-3′ complementary to bp 1164 to 1143 of gD (emb AJ004801 BHV1CGEN) followed by an *Eco*RI restriction site. The 1005 bp fragment was digested with *Eco*RI and inserted into plasmid pRSET-B (Invitrogen) digested with the same restriction enzyme to origin pRSETgD. The integrity of the gD sequence and the correct in-frame orientation with the 6× his tag was confirmed by restriction analysis and nucleotide sequencing (data not shown). Recombinant plasmid was introduced into *E. coli* BL21 cells by standard methods and bacterial cultures were induced for 2 h with 1 mM IPTG (Sigma) when the absorbancy at 600 nm was 0.3. Total proteins were extracted with denaturing buffer (6 M Guanidine HCl and 0.5 M NaCl) and gD-his protein was purified by Ni–nitrilotriacetic acid (Ni–NTA) Agarose columns (Invitrogen) by standard procedures.

2.6. Enzyme-linked immunosorbent assay for detection of anti-gD antibodies

Briefly, Immulon 2 (Dynatech Laboratories) microtiter plates were coated with 1.4 μg/well of purified recombinant gD in 0.1 M carbonate-bicarbonate buffer, pH 9.6 and incubated overnight (ON) at 4 °C. Plates were blocked with PBS/0.05% Tween 20 (PBST) containing 1% ovalbumin (PBST-OVA). Serial dilutions of mice sera were prepared in PBST-OVA and dispensed in 50 μl/well. Plates were washed three times with PBST and incubated with anti-mouse IgG peroxidase conjugate (KPL) for 1 h at 37 °C. After extensive washing, ortho-phenylene-diamine (1,2-benzenediamine) dihydrochloride (OPD, SIGMA) and H_2SO_4 were added as substrate and absorbance was measured at 492 nm.

2.7. Immunoglobulin isotyping ELISA

In order to determine the IgG1, IgG2a, IgG2b and IgG3 subtypes in immunized mice, Immulon II HB microtiter plates were coated with gD and dilutions were conducted as described in 2.6. Bound antibodies were detected with biotinylated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Caltag Laboratories, San Francisco, CA). After incubating for 60 min, plates were washed with PBST, and a dilution of streptavidin/alkaline phosphatase (SIGMA), was added to each plate. The reaction was visualized as described in 2.6.

2.8. T-cell proliferation

Seven days after the third booster, spleens were removed from mice and splenocytes were obtained by gentle homogeneization of spleens in supplemented RPMI 1640 medium/10% fetal calf serum (FCS, GIBCO)/1% ampicillin–streptomycin. Splenocytes were plated in 96-well flat-bottom plates at a concentration of 2.5×10^5 cells per well, in 100 μl of complete medium, consistent of RPMI 1640/10% FCS/2 mM L-glutamine (SIGMA)/1 mM sodium pyruvate (GIBCO), 5 mM HEPES (GIBCO)/50 mM 2-mercaptoethanol/1% ampicillin–streptomycin/10 mM MEM Non-Essential Amino Acids Solution (GIBCO). Triplicate wells were incubated for 3 days at 37 °C either with 100 μl of gD (28 μg/ml in complete medium) or with the same volume of complete medium. One μCi of [methyl-3H] Thymidine (20 Ci/mmole, Amersham) was added to each well, and after 18 h, cells were harvested using a Semiautomatic cell harvester (Skatron) and the amount of radioactivity incorporated into cellular DNA was measured by liquid scintillation counting (Wallac, Winspectral). Proliferative responses are expressed as the stimulation index (cpm in the presence of antigen/cpm in medium alone).

2.9. Cytotoxic T-cell assay (JAM test)

Cytotoxicity assay was performed as previously described by Matzinger et al. (Matzinger, 1991; Usharauli et al., 2006). Briefly, splenocytes were isolated as described above and resuspended in complete medium. Splenocytes were re-stimulated *in vitro* to assess CTL effector function. Cells were cultured at 37 °C and 5% CO_2 for 5 days in complete medium in the presence of gD protein or medium alone. Cell-mediated cytotoxicity was assayed against target cells (P815 cells) infected with moi of 5 of BoHV-1 and labelled with [methyl-^{3}H] thymidine. Target cells were incubated at 10^4 cells per well in triplicate for 3 h at 37 °C with dilutions of effector cells at effector:target ratios of 100:1 to 10:1. Cells were harvested using a Semiautomatic cell harsvester (Skatron) and radioactivity was measured by liquid scintillation counting. Target cells incubated with complete medium only were included in the assay determine spontaneous release. Results were calculated as percentages of specific lysis using the formula:

$$\%\text{specific lysis} = 100 \times [(\text{spontaneous release} - \text{experimental release})/\text{spontaneous release}]$$

Spontaneous release was calculated from wells containing targets cells and medium alone, and experimental release was calculated from wells containing targets cells plus effector cells.

2.10. Immunofluorescent staining and flow cytometry

Splenocyte preparations were stained for surface markers using the following labelled monoclonal antibodies (Mabs): fluorescein isothiocyanate (FITC) anti-mouse CD4 (helper T lymphocyte marker), allophycocyanin (APC) anti CD8β (cytotoxic/suppressor T lymphocyte marker), phycoerythrin (PE) anti-mouse CD45R/B220 (pan B-cell marker), FITC-anti Mac-3 (macrophage marker), FITC-anti CD11c (dendritic cell marker), PE-anti MHCII (MHC class II marker), PE-anti F4/80 (pan macrophage cell marker) and FITC, APC and PE-conjugated rat Ig isotype controls. All Mabs and isotype controls were purchased from Pharmingen. Briefly, cells were incubated for 20 min at 4 °C with Mabs, washed with PBS containing 1% FCS and fixed with 0.2% paraformaldehyde. Flow cytometry was performed in an BD FacsCalibur and analyzed with CellQuest software.

2.11. Statistical analysis

One-way analysis of variance (ANOVA) and Bonferroni post-tests were used to compare data between three or more groups. Log_{10} transformation was done when necessary. P value < 0.05 was considered as an indicator of significant difference.

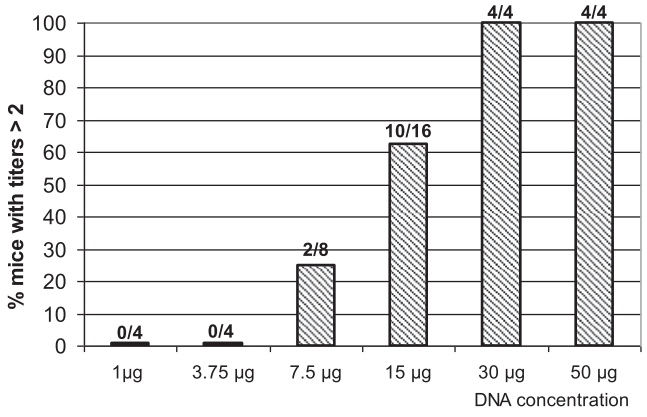

Fig. 2. Humoral response in mice vaccinated with different pCIgD DNA concentrations. Groups of 4 to 16 mice were vaccinated with 1, 3.75, 7.5, 15, 30, and 50 µg per animal of pCIgD and serum antibodies were evaluated by ELISA at 40 dpv. Titers were calculated as the highest serum dilution resulting in a reading of 3 standard deviations above the pCIneo control mean value. The figure represents percentages of mice with titers higher than 2.

3. Results

3.1. Selection of the amount of DNA for vaccination to test the adjuvant action. Antibody response induced by DNA immunization

To determine the immunization doses, mice were vaccinated and boosted 20 days later with different concentrations (1, 3.75, 7.5, 15, 30 or 50 µg) of non emulsified pCIgD and their sera analyzed with anti-gD ELISA at 40 days post vaccination (dpv). Anti gD specific antibodies could be detected in mice inoculated with pCIgD doses of 7.5 µg and higher. Sixty percent of animals (n = 16) sero-converted in the group of 15 µg pCIgD, while 100% of animals showed antibody titers in the group inoculated with 30 and 50 µg doses (n = 4 in both cases) (Fig. 2). Thus, a dose of 15 µg pCIgD was chosen for the following experiments. The specificity of the antibody response was also confirmed by western blot to gD protein and immunofluorescence to BoHV-1 using sera obtained at 15 dpv (data not shown).

3.2. DNA vaccines with ISA 25, ISA 206 and Cliptox elicit higher specific humoral immune response than DNA vaccine alone

In order to compare the immunogenicity of secreted antigen expressed *in vivo* after DNA immunization formulated without and with ISA 25, ISA 206 and Cliptox adjuvants, groups of mice were immunized as described in Section 2. Analysis of sera by anti-gD ELISA (Fig. 3) showed that immunization with pCIgD and adjuvants was able to induce specific gD antibodies in all cases. Antibody levels were significantly higher (p < 0.05) in pCIgD/ISA25 and pCIgD/Cliptox compared to pCIgD without adjuvants at 75 and 160 dpv. Although the data of antibody levels at 75 and 160 dpv of mice inoculated with pCIgD/ISA 206 are not available, the results obtained at 35 dpv show the same trend of an increased humoral response elicited by this adjuvant as in the case of the other two. None of the pCIneo or pCIneo plus adjuvants immunized control animals produced any specific immune response. The sera of animals vaccinated with pCIgD or pCIgD plus adjuvants were also reactive to the virus using ELISA and immunofluorescence (data not shown). When the neutralizing activity was measured at 160 dpv, pCIgD/ISA25, pCIgD/ISA206 and pCIgD/Cliptox sera presented positive neutralizing activity but not the pCIgD group (data not shown).

At 35 dpv, IgG1 was the main IgG isotype in the sera of all groups, except in pCIgD/ISA206 where the IgG1/IgG2a ratio was 1:1. At 75 dpv, the difference within IgG isotypes decreased in all

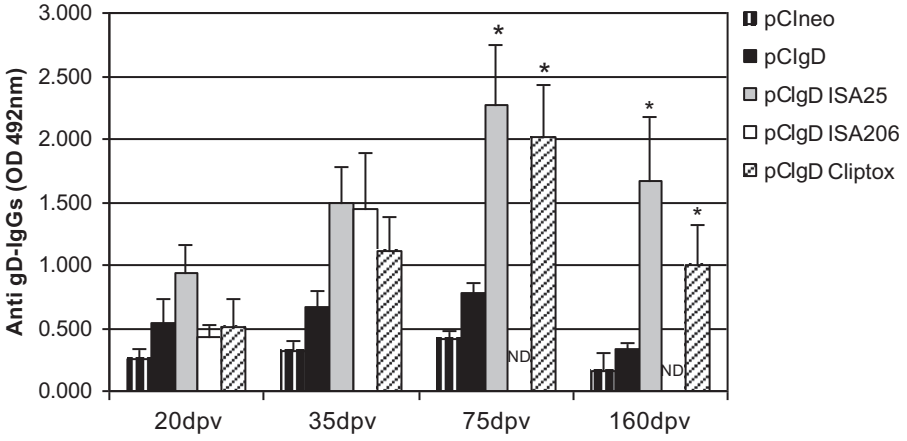

Fig. 3. Serum IgG antibody levels specific for gD in mice after vaccination. The values represent the average A_{492} ± standard error of the mean (SEM) at a serum dilutions of 1:100 at 20, 35, and 75 dpv and of 1:200 at 160 dpv. ND: not determined. Significant differences (p < 0.05) from the mean of the pCIgD group are indicated as ∗.

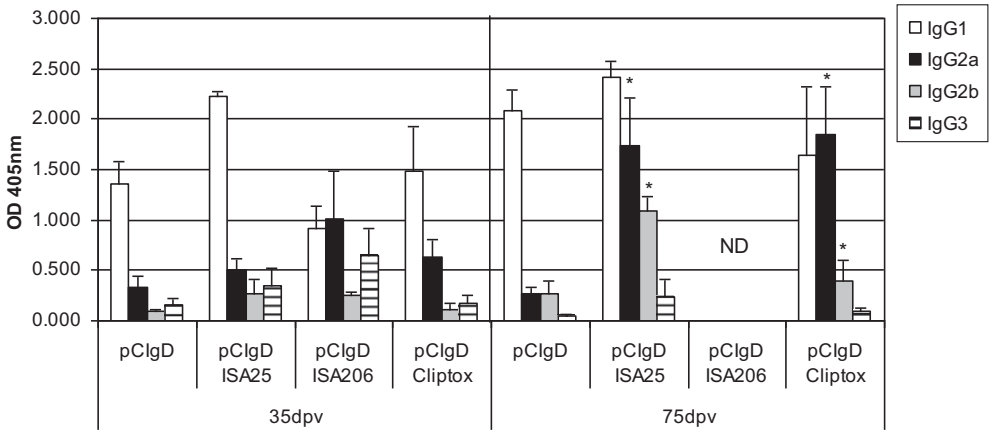

Fig. 4. Serum IgG subclass levels generated by injection of plasmid with ISA 25, ISA 206 and Cliptox. gD specific ELISAs were carried out on serum collected at 35 and 75 dpv. Results show the average A_{492} (after subtracting control values) ± SEM of seven individual mice at a serum dilution of 1/100. ND: not determined.

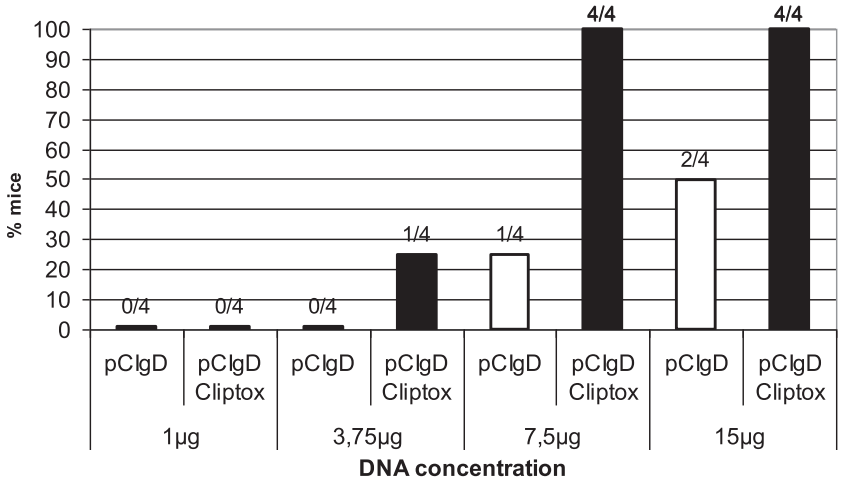

Fig. 5. Humoral immune response to Cliptox. Group of mice ($n = 4$) were vaccinated with 1, 3.75, 7.5, and 15 µg per mouse of pCIgD with or without Cliptox. Serum IgG antibody levels specific for gD after vaccination were measured by ELISA test. Titers are calculated as the highest dilutions resulting in a reading of 3 standard deviations above the pCIneo control mean value. The figure represents the percentages of mice with titers higher than 2.

formulations because IgG2 showed higher levels. Animals vaccinated with pCIgD/ISA25 or pCIgD/Cliptox had a significant increase ($p < 0.05$) of IgG2a and IgG2b when compared to pCIgD (Fig. 4).

To test if the presence of adjuvants allowed to decrease the minimum dose of antigen needed to elicit an immune response, groups of four mice were vaccinated with different doses of pCIgD formulated with Cliptox. The results showed that 7.5 µg pCIgD/Cliptox induced specific gD antibodies in 100% of the animals, while pCIgD was only effective in 25% (Fig. 5). This result indicates that the use of this adjuvant increased the capacity of DNA to induce a humoral immune response.

3.3. Specific proliferative responses following immunization

To compare the ability of the pCIgD vaccine with or without adjuvants to induce cell-mediated immune responses, splenocytes from immunized mice were stimulated *in vitro* with gD protein. Antigen-specific responses were measured by a T-cell proliferation assay. No specific proliferation was observed in splenocytes from pCIgD-inoculated mice, while a significant antigen-specific proliferative response was observed in the group vaccinated with pCIgD/ISA25 ($p < 0.001$) and in the pCIgD/Cliptox group ($p < 0.01$) (Fig. 6).

3.4. Induction of CTL in vivo

The success of an anti-viral vaccine against BoHV-1 depends on its ability to induce CTL that recognize and lyse virus-infected cells.

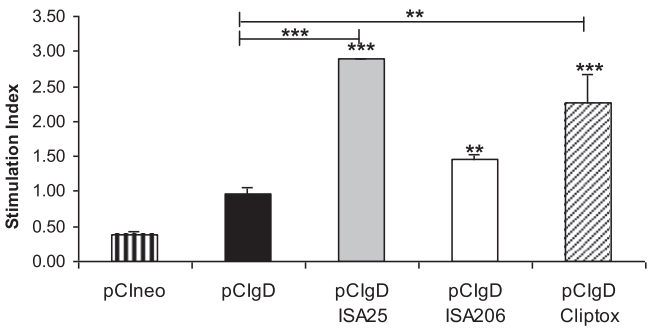

Fig. 6. T cell proliferation in response to adjuvants co-inoculated with pCIgD. Seven days after the third booster, spleen cells were isolated from animals ($n = 2$) inoculated with pCIgD with adjuvants and *in vitro* stimulated with gD protein. The results represent the average of triplicate wells and are expressed as mean stimulation index (SI) ± SEM.

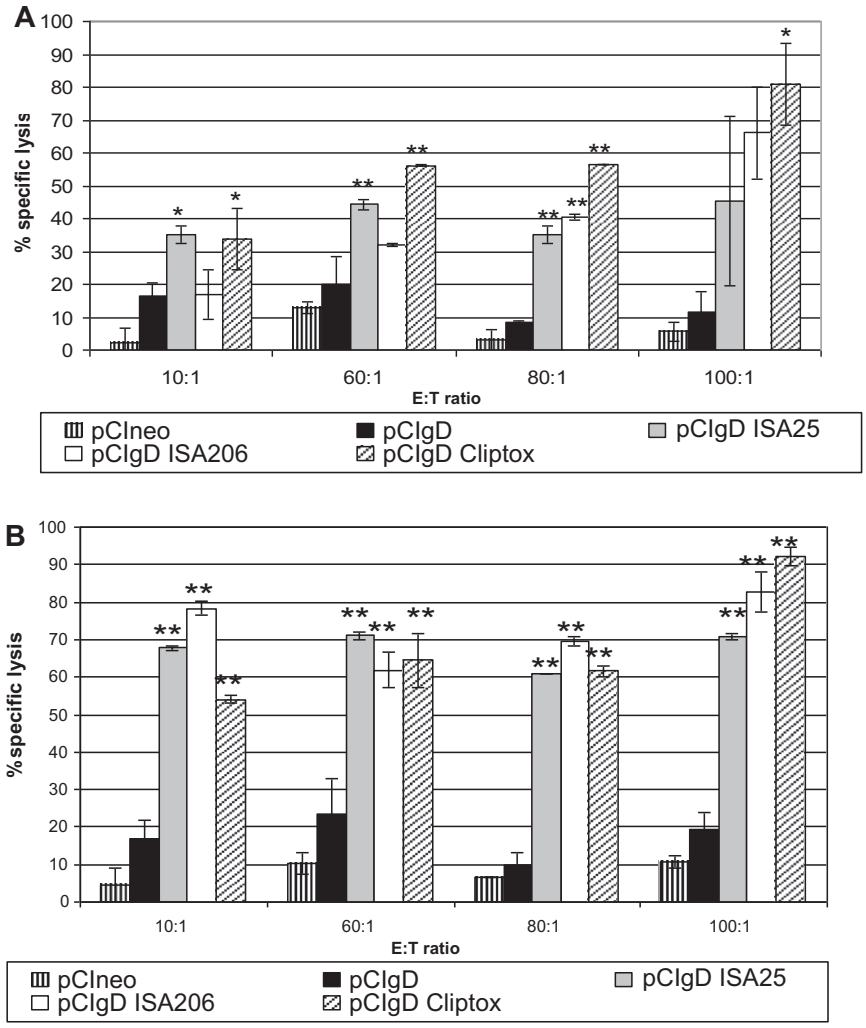

Fig. 7. Immunization with pCIgD with adjuvants induces a higher CTL response as compared to pCIgD alone. Splenocytes collected 1 week after immunization were tested for CTL response by cytotoxicity assay against BoHV-1 without stimulation (A) or re-stimulated *in vitro* with gD protein (B). The percent specific lysis was calculated as described in Section 2. Representative results are shown at various effector-to-target (E:T) ratios. The results shown (average of 3 parallel samples ± SEM) are representative of three independent experiments. Significant differences with respect to the pCIgD group are shown with asterisks ($*p < 0.05$, $**p < 0.01$).

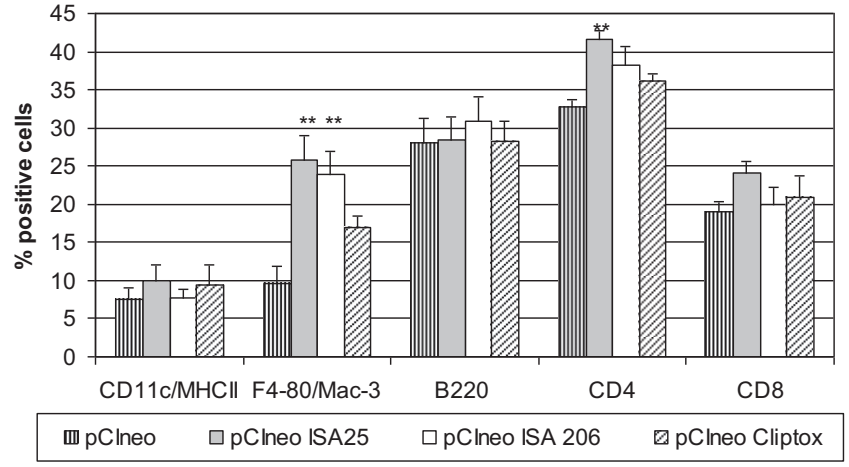

Fig. 8. Cellular subpopulations in spleens from mice vaccinated with pCIneo without and with ISA 25, ISA 206 or Cliptox at 3 dpv. Results are expressed as percentages (means ± SEM of 6 determinations) of dendritic cells (CD11c/MHCII), macrophages (F4/80/Mac-3), B lymphocytes (B220), CD4 lymphocytes (CD4) and CD8 lymphocytes (CD8β). (**) Significant differences ($p < 0.01$) with respect to the pCIneo group.

Therefore, syngeneic target cells (P815 cells, H-2d) were infected with BoHV-1, and tested for recognition of epitopes presented in MHC class I context by murine CTL. To examine CTL levels, splenocytes of pCIgD mice with or without adjuvants and pCIneo groups

were incubated with radioactively-labeled BoHV-1-infected P815 cells.

CTL activities of the three groups that received pCIgD plus adjuvants were significative higher ($p < 0.05$) than those of the pCIgD and pCIneo groups (Fig 7A). This significant increase in the percentage of lysis associated to these cells indicates that they were recognized and lysed by the CTL induced by immunization with gD-encoding plasmid plus adjuvants. Nevertheless, when splenocytes were re-stimulated with gD protein *in vitro*, the groups of pCIgD/ISA25, pCIgD/206 and pCIgD/Cliptox developed higher levels of cytolytic activity ($p < 0.01$) than non-stimulated cells, while the control groups maintained the same low levels (Fig. 7B) as prior to stimulation. Although the average cytotoxic activity obtained with the pCIgD group was clearly higher than that of the pCIneo group, the differences were not significant.

3.5. Adjuvant effects on spleen cell populations

Spleen cells obtained from vaccinated mice with pCIneo with and without adjuvants were used in order to determine the effect produced by these adjuvants in the main cellular populations involved in early immune response.

A significant ($p < 0.01$) expansion in the Mac3+ F4/80+ macrophage population was seen after immunization with pCIneo/ISA 25 and pCIneo/ISA 206, as compared with immunization with pCIneo without adjuvants. In the pCIneo/Cliptox group, a clear increase in this macrophage population can also be observed, but the differences with the pCIneo group were not significant (Fig. 8). Although ISA 25 induced a significant increase ($p < 0.01$) in the CD4+ cells (Fig. 8). Other populations were measured but no significant differences were observed.

4. Discussion

The ability of BoHV-1 to undergo latency and to induce immune evasion in infected cattle hampers the control of infections by this virus. It is well known that BoHV-1 immune evasion mechanisms include apoptosis of CD4+ T-lymphocytes, B-cells and monocytes (Eskra and Splitter, 1997) and down-regulation of the expression of MHC class I molecules (Nataraj et al., 1997). For this reason the development of immunization strategies that do not involve live virus is needed.

Antibodies can neutralize the entry of virus into the host and play an important role in prevention of and defense against infection. However, once the virus enters the host and replicates, its elimination requires antibody-dependent cell specific cytotoxicity (ADCC) or CTL. The ADCC is a relatively inefficient process (Oldstone, 1994), whereas CTL play a vital role regarding the defense against viral pathogens such as herpesviruses. For this reason, CTL induction is highly critical to host defense against BoHV-1, where intracellular dispersion occurs before the extracellular one, and viral infection continues even in the presence of neutralizing antibodies (Lodmell et al., 1973).

As an approach to induce both strong humoral and cellular immune responses against BoHV-1, Huang et al. (2005) used a plasmid vector to express gB from virus. Both antibody and CTL responses were induced by the plasmid in mice. They demonstrated that DNA immunization induces a broad-based immune response to BoHV-1 gB. When calves were immunized intradermally with a plasmid encoding the secreted form of gB (tgB) elicited both humoral responses and activated gamma interferon-secreting CD8+ CTL, suggesting that a DNA vaccine expressing tgB induces a CTL response in the natural host of BoHV-1. This results indicate that the mouse model can serve as a representative of the humoral and cellular immune response against BoHV-1 in bovines.

Glycoprotein D of BoHV-1 is the most important glycoprotein involves in the protective immune response (Babiuk et al., 1987; Hutchings et al., 1990). It has cytotoxic epitopes (Denis et al., 1993; Deshpande et al., 2002), and induces neutralizing antibodies (Babiuk et al., 1987; Deshpande et al., 2002; Oliveira et al., 2000; van Drunen Littel-van den Hurk et al., 1990, 1993; Wyler and Schwyzer, 1989). Apparently gD is responsible of the penetration of the virus in the host cell (Chase and Letchworth, 1994; Fehler et al., 1992) with participation in the viral adsorption and membrane fusion (Marshall et al., 1986; Spear et al., 2000; Tikoo et al., 1990; van Drunen Littel-van den Hurk et al., 1990). A secreted form of BoHV-1 gD was chosen for these experiments since previous work performed in mice and bovines showed that this version of gD induced higher antibody titers than the membrane-anchored form (Lewis et al., 1999; van Drunen Littel-van den Hurk et al., 1998).

Since adjuvants play a key role in increasing vaccine efficiency, the immunomodulatory effects on a DNA vaccine of three adjuvants ISA 25, ISA 206 and Cliptox was analyzed in this work.

Incorporation of any of these adjuvants to vaccine formulations yielded an increased humoral response towards a gD DNA vaccine. Antibodies were able to recognize the gD protein and the virus as well, and important they showed neutralizing activity even after 160 days post vaccination (data not shown). Similar results with the use of ISA 25 were reported by Yung-Fu Chang et al. (1995) who were able to induce, at 180 dpv, high levels of protective antibodies in dogs using a recombinant vaccine against *Borrelia burgdorferi*.

However, analysis of the isotype profiles revealed that the IgG1 levels were similar for all the immunized animals and the variations in overall antibody titers induced by the different groups with adjuvant were mostly accounted for by a variation in IgG2a and IgG2b. A higher proportion of IgG2a has been described as a typical feature of DNA immunization (Raz et al., 1996) and suggests a T-helper 1 bias. The humoral protective immune responses to viral infections in mice are characterized by predominance in IgG2a. In these animals, this isotype is clearly more efficient at complement fixation (Coutelier et al., 1991, 1988; Klaus et al., 1979; Nguyen et al., 1994) and as opsonizing agent than IgG1 in mice (Abbas et al., 1996; Janeway, 1999). The detection of an *in vitro* lymphocyte increase stimulated by the gD protein and the B-cell switch evidenced by antigen-specific IgG2a secretion are indicating a modulation of the T CD4+ cell response to a Th1/Th2 profile (Coutelier et al., 1991; , 1988; Mosmann and Coffman, 1989; Stevens et al., 1988).

Moreover, when evaluating mouse spleen cell populations at 3 days after immunization with pCIneo/ISA 206 or pCIneo/ISA 25, we observed a significant increase of macrophages in comparison with the pCIneo group. This could be explained by the "depot" and inflammatory effects that are considered important characteristics of these adjuvants, generating inflammation-attracted cells, including APC (Cox and Coulter, 1997). Even though the mechanism of action of these formulations remains unclear, it is possible to suggest that these DNA-oil formulations are more efficiently taken up by macrophages, which play an important role in the immune response. The oil emulsions such as ISA 25 and ISA 206 can be efficiently endocytosed by APC. This generates an increase in the proliferative response of macrophages and other cells (Hamilton et al., 2000). In addition, an increase of macrophage numbers was observed when animals were vaccinated with pCIneo/Cliptox, as described by Pavelic et al. (2002) who observed a high concentration of activated macrophages in the peritoneal cavity 4 h after zeolite administration through the intraperitoneal route. In agreement with this, we recently demonstrated an increase in macrophage numbers in mice vaccinated with iFMDV/Cliptox in comparison with those

vaccinated with the virus in the absence adjuvant (Batista et al., 2010).

DNA immunizations have been used to induce immune responses against BoHV-1 both in mice and cattle (Caselli et al., 2005; Cox et al., 1993; van Drunen Littel-van den Hurk et al., 1998). Nevertheless, these studies have been focused in the antibody-mediated and T-cell proliferative responses. Induction of specific CTL against the virus have only been investigated by Deshpande et al. (2002), who demonstrated that a BoHV-1-specific CTL response could be elicited by immunization with a plasmid encoding the membrane-anchored form of gD. For this reason, the capacity of DNA vaccines formulated with ISA 206, ISA 25 and Cliptox to induce specific CTL against BoHV-1 was tested in this work. Hence, it was necessary to determine if the vaccine-induced CTL could lyse BoHV-1-infected cells. Cell cytotoxicity assays with herpesvirus-infected target cells demonstrated the presence of a specific CTL response against the virus elicited by immunization with the genic vaccine. Furthermore, the specific CTL response was boosted by the adjuvants, even in a splenocyte:target ratio of 10:1. It was possible to observe the existence of memory CTL that recognized and lysed infected cells, even without being *in vitro* re-stimulated with gD protein.

The data obtained up to the moment is very encouraging, since previous studies involving DNA vaccines encoding for BoHV-1 gD in mice used doses of 100 μg/mouse (Caselli et al., 2005; Cox et al., 1993) whereas we were able to induce good immune responses, including CTL, only with 15 μg/animal. Because of this we can suggest that these vaccines should be tested in bovines since they seem to be good candidates to induce a protective immune response against BoHV-1. This is the first time that the improvement of a specific cytotoxic immune response against BoHV-1 is described, using a DNA vaccine encoding for the secreted gD plus ISA 25, ISA 206 and Cliptox adjuvants.

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References

Abbas, A.K., Murphy, K.M., Sher, A., 1996. Functional diversity of helper T lymphocytes. Nature 383, 787–793.

Babiuk, L.A., L'Italien, J., van Drunen Littel-van den Hurk, S., Zamb, T., Lawman, J.P., Hughes, G., Gifford, G.A., 1987. Protection of cattle from bovine herpesvirus type I (BHV-1) infection by immunization with individual viral glycoproteins. Virology 159, 57–66.

Batista, A., Quattrocchi, V., Olivera, V., Langellotti, C., Pappalardo, J.S., Di Giacomo, S., Mongini, C., Portuondo, D., Zamorano, P., 2010. Adjuvant effect of Cliptox on the protective immune response induced by an inactivated vaccine against foot and mouth disease virus in mice. Vaccine 28, 6361–6366.

Caselli, E., Boni, M., Di Luca, D., Salvatori, D., Vita, A., Cassai, E., 2005. A combined bovine herpesvirus 1 gB-gD DNA vaccine induces immune response in mice. Comp. Immunol. Microbiol. Infect. Dis. 28, 155–166.

Chang, Y.F., Appel, M.J., Jacobson, R.H., Shin, S.J., Harpending, P., Straubinger, R., Patrican, L.A., Mohammed, H., Summers, B.A., 1995. Recombinant OspA protects dogs against infection and disease caused by *Borrelia burgdorferi*. Infect. Immun. 63, 3543–3549.

Chase, C.C., Letchworth, G.J., 1994. Bovine herpesvirus 1 gIV-expressing cells resist virus penetration. J. Gen. Virol. 75, 177–181.

Coutelier, J.P., van der Logt, J.T., Heessen, F.W., 1991. IgG subclass distribution of primary and secondary immune responses concomitant with viral infection. J. Immunol. 147, 1383–1386.

Coutelier, J.P., van der Logt, J.T., Heessen, F.W., Vink, A., van Snick, J., 1988. Virally induced modulation of murine IgG antibody subclasses. J. Exp. Med. 168, 2373–2378.

Cox, G.J., Zamb, T.J., Babiuk, L.A., 1993. Bovine herpesvirus 1: Immune responses in mice and cattle injected with plasmid DNA. J. Virol. 67, 5664–5667.

Cox, J.C., Coulter, A.R., 1997. Adjuvants – a classification and review of their modes of action. Vaccine 15, 248–256.

Denis, M., Slaoui, M., Keil, G., Babiuk, L.A., Ernst, E., Pastoret, P.P., Thiry, E., 1993. Identification of different target glycoproteins for bovine herpes virus type 1-specific cytotoxic T lymphocytes depending on the method of in vitro stimulation. Immunology 78, 7–13.

Deshpande, M.S., Ambagala, T.C., Hegde, N.R., Hariharan, M.J., Navaratnam, M., Srikumaran, S., 2002. Induction of cytotoxic T-lymphocytes specific for bovine herpesvirus-1 by DNA immunization. Vaccine 20, 3744–3751.

Eskra, L., Splitter, G.A., 1997. Bovine herpesvirus-1 infects activated CD4+ lymphocytes. J. Gen. Virol. 78, 2159–2166.

Fehler, F., Herrmann, J.M., Saalmuller, A., Mettenleiter, T.C., Keil, G.M., 1992. Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant. J. Virol. 66, 831–839.

Fuller, A.O., Lee, W.C., 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. 66, 5002–5012.

Griebel, P.J., Ohmann, H.B., Lawman, M.J., Babiuk, L.A., 1990. The interaction between bovine herpesvirus type 1 and activated bovine T lymphocytes. J. Gen. Virol. 71, 369–377.

Hamilton, J.A., Byrne, R., Whitty, G., 2000. Particulate adjuvants can induce macrophage survival, DNA synthesis, and a synergistic proliferative response to GM-CSF and CSF-1. J. Leukoc. Biol. 67, 226–232.

Hariharan, M.J., Nataraj, C., Srikumaran, S., 1993. Down regulation of murine MHC class I expression by bovine herpesvirus 1. Viral Immunol. 6, 273–284.

Huang, Y., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2005. Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic T-lymphocyte responses in mice and cattle. J. Gen. Virol. 86, 887–898.

Hutchings, D.L., van Drunen Littel-van den Hurk, S., Babiuk, L.A., 1990. Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cattle. J. Virol. 64, 5114–5122.

Iwasaki, A., Stiernholm, B.J., Chan, A.K., Berinstein, N.L., Barber, B.H., 1997. Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. J. Immunol. 158, 4591–4601.

Janeway, C. Jr., Walport, M., Capra, J.D., 1999. T-cell mediated immunity. In: Immunobiology: The Immune System in Health and Disease, Elsevier, New York, pp. 262–305.

Klaus, G.G., Pepys, M.B., Kitajima, K., Askonas, B.A., 1979. Activation of mouse complement by different classes of mouse antibody. Immunology 38, 687–695.

Kuhn, J.E., Kramer, M.D., Willenbacher, W., Wieland, U., Lorentzen, E.U., Braun, R.W., 1990. Identification of herpes simplex virus type 1 glycoproteins interacting with the cell surface. J. Virol. 64, 2491–2497.

Lewis, P.J., Cox, G.J., van Drunen Littel-van den Hurk, S., Babiuk, L.A., 1997. Polynucleotide vaccines in animals: enhancing and modulating responses. Vaccine 15, 861–864.

Lewis, P.J., van Drunen Littel-van den Hurk, S., Babiuk, L.A., 1999. Altering the cellular location of an antigen expressed by a DNA-based vaccine modulates the immune response. J. Virol. 73, 10214–10223.

Lodmell, D.L., Niwa, A., Hayashi, K., Notkins, A.L., 1973. Prevention of cell-to-cell spread of herpes simplex virus by leukocytes. J. Exp. Med. 137, 706–720.

Marshall, R.L., Rodriguez, L.L., Letchworth, G.J., 1986. Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus 1) by biochemical and immunological methods. J. Virol. 57, 745–753.

Matzinger, P., 1991. The JAM test. A simple assay for DNA fragmentation and cell death. J. Immunol. Methods 145, 185–192.

Miethke, A., Keil, G.M., Weiland, F., Mettenleiter, T.C., 1995. Unidirectional complementation between glycoprotein B homologues of pseudorabies virus and bovine herpesvirus 1 is determined by the carboxy-terminal part of the molecule. J. Gen. Virol. 76, 1623–1635.

Mosmann, T.R., Coffman, R.L., 1989. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7, 145–173.

Nataraj, C., Eidmann, S., Hariharan, M.J., Sur, J.H., Perry, G.A., Srikumaran, S., 1997. Bovine herpesvirus 1 downregulates the expression of bovine MHC class I molecules. Viral Immunol. 10, 21–34.

Nguyen, L., Knipe, D.M., Finberg, R.W., 1994. Mechanism of virus-induced Ig subclass shifts. J. Immunol. 152, 478–484.

Oldstone, M.B., 1994. The role of cytotoxic T lymphocytes in infectious disease: history, criteria, and state of the art. Curr. Top. Microbiol. Immunol. 189, 1–8.

Oliveira, S.C., Harms, J.S., Rosinha, G.M., Rodarte, R.S., Rech, E.L., Splitter, G.A., 2000. Biolistic-mediated gene transfer using the bovine herpesvirus-1 glycoprotein D is an effective delivery system to induce neutralizing antibodies in its natural host. J. Immunol. Methods 245, 109–118.

Pavelic, K., Katic, M., Sverko, V., Marotti, T., Bosnjak, B., Balog, T., Stojkovic, R., Radacic, M., Colic, M., Poljak-Blazi, M., 2002. Immunostimulatory effect of natural clinoptilolite as a possible mechanism of its antimetastatic ability. J. Cancer Res. Clin. Oncol. 128, 37–44.

Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J.A., Roman, M., Swain, S.L., Spiegelberg, H.L., Carson, D.A., 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc. Natl Acad. Sci. USA 93, 5141–5145.

Spear, P.G., Eisenberg, R.J., Cohen, G.H., 2000. Three classes of cell surface receptors for alphaherpesvirus entry. Virology 275, 1–8.

Stevens, T.L., Bossie, A., Sanders, V.M., Fernandez-Botran, R., Coffman, R.L., Mosmann, T.R., Vitetta, E.S., 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature 334, 255–258.

Tikoo, S.K., Campos, M., Babiuk, L.A., 1995. Bovine herpesvirus 1 (BHV-1): Biology, pathogenesis, and control. Adv. Virus Res. 45, 191–223.

Tikoo, S.K., Fitzpatrick, D.R., Babiuk, L.A., Zamb, T.J., 1990. Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol. 64, 5132–5142.

Torres, C.A., Iwasaki, A., Barber, B.H., Robinson, H.L., 1997. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. J. Immunol. 158, 4529–4532.

Toussaint, J.F., Coen, L., Letellier, C., Dispas, M., Gillet, L., Vanderplasschen, A., Kerkhofs, P., 2005. Genetic immunisation of cattle against bovine herpesvirus 1: Glycoprotein gD confers higher protection than glycoprotein gC or tegument protein VP8. Vet. Res. 36, 529–544.

Ueki, A., Yamaguchi, M., Ueki, H., Watanabe, Y., Ohsawa, G., Kinugawa, K., Kawakami, Y., Hyodoh, F., 1994. Polyclonal human T-cell activation by silicate in vitro. Immunology 82, 332–335.

Usharauli, D., Perez-Diez, A., Matzinger, P., 2006. The JAM Test and its daughter P-JAM: simple tests of DNA fragmentation to measure cell death and stasis. Nat. Protoc. 1, 672–682.

van Drunen Littel-van den Hurk, S., Gifford, G.A., Babiuk, L.A., 1990. Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. Vaccine 8, 358–368.

van Drunen Littel-van den Hurk, S., Parker, M.D., Massie, B., van den Hurk, J.V., Harland, R., Babiuk, L.A., Zamb, T.J., 1993. Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine 11, 25–35.

van Drunen Littel-van den Hurk, S., Braun, R.P., Lewis, P.J., Karvonen, B.C., Baca-Estrada, M.E., Snider, M., McCartney, D., Watts, T., Babiuk, L.A., 1998. Intradermal immunization with a bovine herpesvirus-1 DNA vaccine induces protective immunity in cattle. J. Gen. Virol. 79, 831–839.

Whetstone, C.A., Miller, J.M., Seal, B.S., Bello, L.J., Lawrence, W.C., 1992. Latency and reactivation of a thymidine kinase-negative bovine herpesvirus 1 deletion mutant. Arch. Virol. 122, 207–214.

Whetstone, C.A., Wheeler, J.G., Reed, D.E., 1986. Investigation of possible vaccine-induced epizootics of infectious bovine rhinotracheitis, using restriction endonuclease analysis of viral DNA. Am. J. Vet. Res. 47, 1789–1795.

Wyler, R., Schwyzer, M., 1989. Infectious bovine rhinotracheitis/vulvovaginitis (BoHV1). In: Wittmann Boston, G. (Ed.), Herpesvirus Diseases of Cattle, Horses, and Pigs. Kluwer, pp. 1–72.

Yates, W.D., 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. 46, 225–263.