Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic T-lymphocyte responses in mice and cattle

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Virus-specific cytotoxic T lymphocytes (CTLs) are considered to be important in protection against and recovery from viral infections. In this study, several approaches to induce cytotoxicity against bovine herpesvirus 1 (BHV-1) were evaluated. Vaccination of C57BL/6 mice with BHV-1 induced a strong humoral, but no CTL, response, which may be due to downregulation of major histocompatibility complex class I molecules. In contrast, vaccinia virus expressing glycoprotein B (gB) elicited a weaker antibody response, but strong cytotoxicity, in mice. As an approach to inducing both strong humoral and cellular immune responses, a plasmid vector was then used to express gB. Both antibody and CTL responses were induced by the plasmid encoding gB in C57BL/6 and C3H mice, regardless of the type of vector backbone. This demonstrated that DNA immunization induces a broad-based immune response to BHV-1 gB. Interestingly, removal of the membrane anchor, which resulted in secretion of gB from transfected cells, did not result in reduced cytotoxicity. Here, it is shown that, compared with the cell-associated counterpart, plasmid-encoded secreted protein may induce enhanced immune responses in cattle. Therefore, calves were immunized intradermally with pMASIAtgB, a plasmid encoding the secreted form of gB (tgB), using a needle-free injection system. This demonstrated that pMASIAtgB elicited both humoral responses and activated gamma interferon-secreting CD8+ CTLs, suggesting that a DNA vaccine expressing tgB induces a CTL response in the natural host of BHV-1.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) is associated with a variety of clinical syndromes in cattle, including respiratory, genital, nervous and multisystemic infections (Wyler et al., 1989). Rhinotracheitis is often followed by secondary bacterial infections, which lead to high morbidity and mortality (Griebel et al., 1990; Yates, 1982). BHV-1 uses a variety of mechanisms to evade the host’s immune system. By spreading intracellularly, it can exist in the presence of virus-specific antibodies (Fuller & Lee, 1992; Kühn et al., 1990; Miethke et al., 1995). Furthermore, depressed cell-mediated immune responses have been observed as a result of BHV-1 infection (Eskra & Splitter, 1997). Attempts to stimulate activated T cells with live BHV-1 have resulted in cell death (Griebel et al., 1990). Recent studies have shown that proteins produced by BHV-1 interfere with major histocompatibility complex (MHC) class I-mediated antigen presentation, which results in the prevention of cytotoxic T-lymphocyte (CTL) activity (Hariharan et al., 1993; Nataraj et al., 1997). Nevertheless, CTL epitopes have been identified for BHV-1, in both H-2d and H-2k mice (Zatechka et al., 1999).

Conventional attenuated and inactivated BHV-1 vaccines do not efficiently prevent the transmission of BHV-1 or the establishment of latency, although they may protect individual animals against clinical disease. Furthermore, live-attenuated vaccines are not entirely safe, because they may cause abortions and latency (Whetstone et al., 1992). Because of these limitations, there is a need for novel vaccines that contain as few viral products as possible, but induce sufficient cell-mediated and humoral immune responses.

DNA vaccination is based on direct injection of an expression plasmid that encodes an antigen. The 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 (APCs). 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 CD8+ CTL responses to 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 CTL responses.

The targets of CTLs are frequently conserved proteins. A CTL response against such conserved proteins would have the potential advantage of protecting the host against a variety of virus strains. This makes these proteins strong candidates for vaccine components (Franco et al., 1994). BHV-1 gB, a 932 aa molecule, is one of the major glycoproteins found in the virion envelope and plasma membrane of virus-infected cells (Misra et al., 1981). Homologues of gB have been detected in all herpesviruses studied to date. Furthermore, the gB homologues exhibit the highest level of conservation amongst all glycoproteins, which has been suggested to be due to the importance of gB in virus infectivity. All gB homologues analysed thus far are essential for attachment and penetration of free virions (Byrne et al., 1995), involving fusion between the virion envelope and the cellular plasma membrane (Li et al., 1997), as well as direct transmission of infectivity from primary infected cells to neighbouring non-infected cells (Fuller & Lee, 1992; Kühn et al., 1990; Miethke et al., 1995). In this report, we have presented evidence that a plasmid encoding either BHV-1 gB or a truncated, secreted form of gB (tgB) induced a specific CTL response in both mice and cattle.

**METHODS**

**Reagents and media.** Enzymes for DNA cloning were purchased from Pharmacia. Chemicals for DNA cloning and protein analysis were purchased from Sigma. Cell media were obtained from Gibco-BRL. Immunological reagents were obtained from Dimension Laboratories. Radioisotopically labelled compounds and fluorographic reagents were purchased from Amersham.

**Cells and virus.** The Cooper strain of BHV-1 was grown in Madin–Darby bovine kidney (MDBK) cells (Madin et al., 1956) in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). Recombinant vaccinia virus containing the BHV-1 gB gene (Vac-gB) was constructed as described previously (van Drunen Littel-van den Hurk et al., 1990), and propagated in Vero cells in MEM containing 5% FBS. Viruses titres of 1 × 10^6 p.f.u. ml^{-1} were obtained for both BHV-1 and Vac-gB. LMTK^{-} and RSVgB LTMK^{-} cells, which are syngeneic LTMK^{-} cells permanently transfected with the gB gene (Fitzpatrick et al., 1990), were grown in MEM containing 10% FBS.

**Immunofluorescence.** Virus-infected MDBK cells and transformed LMTK^{-} cells were trypsinized and resuspended at a concentration of 2 × 10^6 cells ml^{-1} in MEM containing 5% FBS. The cell suspension was spun onto slides in a Cytospin4 centrifuge (Thermo Fisher Scientific) and fixed with 3% paraformaldehyde for 30 min. Slides were washed in PBS followed by ddH2O. Subsequently, cells were incubated with a gB-specific monoclonal antibody (mAb) cocktail (van Drunen Littel-van den Hurk et al., 1984, 1985) for 1 h in a moist chamber. After washing, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zymed) was added to the cells for 1 h. Slides were washed, air-dried and covered with Citifluor mounting medium (BioLabs) and the cells were observed with a fluorescence microscope (Microscope CK; Olympus).

**Plasmids.** Construction of pSLIAgB, encoding full-length BHV-1 gB, and pSLIAtgB, which encodes a truncated, secreted form of BHV-1 gB (Li et al., 1996), has been described previously (Braun et al., 1997). The pMASIA vector was constructed previously (Pontarollo et al., 2002). The pSLIA vector has a pSL301 backbone, whereas the pMASIA vector has a PUC19 backbone with reduced numbers of immune-inhibitory motifs (Krieg et al., 1998). BamHI–BglII fragments bearing gB or tgB coding sequences were excised from pSLIAgB or pSLIAtgB and inserted into the BamHI site of pMASIA to generate pMASIAgB and pMASIAtgB, respectively. Plasmids were grown in Escherichia coli DH5α, purified on Qiagen EndoFree anion-exchange resin columns and analysed on the basis of 260/280 nm absorbency ratios with an Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech). The DNA constructs were confirmed by restriction digestion followed by agarose gel electrophoresis. Endotoxin levels in DNA stocks were verified to <0.1 endotoxin units (μg DNA)\(^{-1}\) [<10 pg (mg DNA)\(^{-1}\)], using the Limulus amoeboa cyt lysate QLC-1000 kit (BioWhittaker).

**Immunization of mice and cattle.** Inbred 6–8-week-old C57BL/6 (H-2^b^) and C3H/HeNcBr (H-2^d^) mice were purchased from Charles River Laboratories and injected intraperitoneally with 10^7 p.f.u. BHV-1 or 2 × 10^7 p.f.u. Vac-gB, or with 100 μg plasmid encoding gB or tgB, in 0.85% saline (n = 6). The plasmids were injected in two 25 μl doses into both quadriceps muscles. Mice were revaccinated 2 weeks later and euthanized by Halothane (MTC Pharmaceuticals) overdose 10 days after the secondary immunization. Blood samples were taken 0, 14 and 24 days after the primary immunization. Splenocytes were collected 10 days after the second immunization. All sets of experiments were repeated twice to validate the results.

Seven- to nine-month-old BHV-1-seronegative Hereford calves were purchased from a local ranch and allocated randomly to two groups of five animals. The experimental group was immunized intradermally in the hip with 500 μg pMASIAgB in 500 μl saline, using a Biojector 2000 needle-free injection system (Biojector Medical Technologies). The control group was injected with 500 μl saline. The calves were immunized on days 1, 29 and 43. Blood samples were taken prior to each immunization and 2 weeks after the third immunization. All animals were handled according to the guidelines of the Canadian Council on Animal Care.

**ELISA.** To determine gB-specific antibody titres in mouse sera, polystyrene microtitre plates (Immulon 2; Dynatech Laboratories) were coated with 50 ng purified tgB (Li et al., 1996) per well. After overnight incubation at 4 °C, the plates were incubated for 2 h at room temperature with murine sera diluted fourfold in PBS containing 0.05% Tween 20 and 0.5% gelatin (PBST-g). Subsequently, affinity-purified biotinylated goat anti-mouse IgG (Zymed) diluted 1:5000 in PBST-g was added for 1 h. Finally, the plates were incubated for 1 h with streptavidin–alkaline phosphatase (AP) (Gibco-BRL) diluted 1:2500 in PBST-g. Antibody-isotyping ELISAs were carried out essentially in the same manner. Bound antibodies were detected with biotinylated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Caltag Laboratories) at a dilution of 1:5000, followed by streptavidin–AP diluted 1:2000. To determine gB-specific antibody titres in bovine sera, microtitre plates were coated with 50 ng purified gB per well and incubated overnight at room temperature. Bovine sera diluted fourfold in PBST were added to the plates, which were then incubated at room temperature for 2 h. Subsequently, the plates were incubated for 1 h at room temperature with AP-conjugated goat anti-bovine IgG (Zymed) diluted 1:15000 in PBST. All reactions were visualized with 0.01 M p-nitrophenyl phosphate diluted in 0.1 M diethanolamine, 0.5 mM MgCl\(_2\). After 45 min, the reactions were stopped with 0.3 M EDTA. A\(_{405}\) / A\(_{490}\) was read in a Microplate reader 3550 (Bio-Rad).

**Virus-neutralization assays.** Virus-neutralization titres in sera were determined as described previously. Titres were expressed as the highest dilution of antibody that caused a 50% reduction in plaques relative to the virus control (van Drunen Littel-van den Hurk et al., 1998).
CTL assay. Splenocytes were isolated as described previously (Baca-Estrada et al., 1996) and resuspended at a final concentration of $1 \times 10^6$ cells ml\(^{-1}\) in RPMI 1640 supplemented with 10% FBS, 100 U penicillin ml\(^{-1}\), 100 U streptomycin ml\(^{-1}\), 1 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 5 x 10\(^{-5}\) M 2-mercaptoethanol (complete medium).

Splenocytes were restimulated in vitro to assess CTL effector function. Cells were cultured at 37°C and 5% CO\(_2\) for 4 days in complete medium in the presence of syngeneic stimulators that had been irradiated with 5000 rads ratios of 1:50. Cell-mediated cytotoxicity was assayed against target cells labelled with 100 μCi \(^{51}\)CrNa\(_2\)CrO\(_4\) (Amersham). Target cells were incubated at 10\(^4\) cells per well in triplicate for 4 h at 37°C with twofold serial dilutions of effector cells at a ratio of 100:1 to 25:1. The supernatant was then removed for counting in a γ-radiation counter (Gamma 5500; Beckman Instruments). Target cells incubated with complete medium only (spontaneous release) or with 5% Triton X-100 (total release) were included in the assay. Spontaneous release: total release ratios were <30%. Percentage specific release was calculated as specific lysis (%) = [experimental release (c.p.m.) – spontaneous release (c.p.m.)] / total release (c.p.m.) – spontaneous release (c.p.m.).

Proliferation assay. Blood was collected into EDTA-treated vacutainers (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll-Paque PLUS (Pharmacia) and resuspended at a concentration of 3 x 10\(^5\) cells ml\(^{-1}\) in MEM containing 10% FBS, 2 mM L-glutamine, 50 μg gentamicin ml\(^{-1}\), 1 ng dexamethasone ml\(^{-1}\) and 5 x 10\(^{-5}\) M 2-mercaptoethanol.

Dexamethasone was added to the cultures because it reduces the level of spontaneous lymphocyte proliferation without interfering with antigen-induced lymphocyte proliferative responses (Raggo et al., 2000). Subsequently, 10 μl volumes were dispensed into the wells of microtitre plates and the PBMCs were cultured in the absence or presence of 0.1 μg BHV-1 gB at 37°C in 5% CO\(_2\). After 3 days in culture, cells were incubated with [methyl-\(^3\)H]thymidine (Amersham) at a concentration of 0.4 μCi per well. The cells were harvested 18 h later and thymidine uptake was measured. Plates were harvested on a Filtermate harvester and counted on a TopCount NXT microplate scintillation and luminescence counter (Packard, PerkinElmer Life and Analytical Sciences).

Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay. PBMCs were isolated from peripheral blood and cultured for 24 h in the presence of 1 μg BHV-1 gB. Following in vitro restimulation, cells were resuspended at a concentration of 1 x 10\(^6\) cells ml\(^{-1}\) in culture medium. Nitrocellulose plates (Millipore Multiscreen-HA) were coated for 2 h at room temperature with bovine IFN-γ (BoIFN-γ)-specific mAb 2-2-1 (Raggo et al., 2000) diluted 1:400. After incubation with culture medium for 2 h, 100 μl of each suspension was added to triplicate wells. Plates were incubated overnight at room temperature. Subsequently, plates were incubated for 2–4 h at room temperature with BoIFN-γ-specific rabbit serum (Raggo et al., 2000) diluted 1:100. Biotinylated anti-rabbit IgG (Zymed) was added at a dilution of 1:1000 and plates were incubated for 2 h at room temperature. Finally, plates were incubated with a 1:1000 dilution of streptavidin–AP (BIO/CAN Scientific) in 0.1% BSA in PBS for 2 h at room temperature. The reaction was visualized with NBT/BCIP. The number of IFN-γ-secreting cells was expressed as the difference between the number of spots per 10\(^6\) cells in gB-stimulated wells and the number of spots per 10\(^6\) cells in non-stimulated wells.

Phenotypic analysis of PBMCs. PBMCs were resuspended at a concentration of 4 x 10\(^5\) cells ml\(^{-1}\) in FACola (Becton Dickinson). Aliquots of 50 μl per well in 96-well polystyrene microtitre plates (Immuron 2) were stained for surface antigens with 50 μl mAb specific for CD4 (CALT83B; VMRD) or CD8 (CALT80C, CD8alpha; VMRD). The level of specific mAb staining was defined by setting the threshold with an irrelevant isotype (IgG1) and concentration-matched mAb. After 30 min on ice, 150 μl FACola was added to each well to resuspend the cells. Cells were then spun at 1400 r.p.m. in a Beckman GRP centrifuge for 2 min at 4°C and washed in FACola. One hundred microlitres of 0.02 mg FITC-conjugated goat anti-mouse IgG1 (Becton Dickinson) ml\(^{-1}\) was added to detect cell-bound antibodies. After 30 min at 4°C, cells were washed with FACola, followed by fixation with 200 μl 2% formaldehyde. Samples were analysed on a FACScan (Becton Dickinson) flow cytometer using the LYSYS II program.

Magnetic cell sorting. PBMCs were isolated from peripheral blood, counted by trypan blue exclusion and resuspended at a concentration of 1 x 10\(^6\) cells ml\(^{-1}\). Separate cell suspensions were incubated for 30 min on ice with murine mAbs specific for either bovine CD4 (CALT83B) or CD8 (CALT80C) at a concentration of 1 μg (10\(^6\) PBMCs\(^{-1}\)). Cells were washed and incubated with goat anti-mouse IgG-coated magnetic beads (Dynabeads M-450) at a bead:target cell ratio of 5:1. Bead-bound cells were recovered by using a magnetic particle concentrator (Miltenyi Biotec). After immunomagnetic bead selection, CD4\(^+\) and CD8\(^+\) T cells comprised >95% of the total cell population as determined by flow-cytometric analysis.

Adherent cells from the PBMCs were cultured for 7 days with bovine granulocyte–macrophage colony-stimulating factor (BoGM-CSF) (kindly provided by Dr Philip Griebel, Vaccine and Infectious Disease Organization, SK, Canada) at a concentration of 80 ng ml\(^{-1}\) and ovine interleukin 4 (OvIL4) and then also in the presence of gB for an additional day. OvIL4 was derived from supernatant medium from COS-7 cells transfected with an OvIL4-expressing plasmid, pOvIL4 (R. Pontarollo, personal communication). Bioactivity of OvIL4 was confirmed by using the method described by Chan et al. (2002). The cells showed diminishing CD14 marker, dendritic cell-like morphology and the ability for antigen presentation. Because no CTL epitopes have been identified for gB, we used pure gB protein for stimulation. For ELISPOT assays, each cell population was cultured on nitrocellulose plates in the presence of autologous gB-pulsed adherent cells. Controls consisted of cells cultured with medium only.

Statistical analysis. Data were analysed with the aid of Graphpad Prism 3.0 software. One-way analysis of variance was used to measure the differences between selected groups.

RESULTS

BHV-1 induces a gB-specific antibody response, but no CTL response, in C57BL/6 mice

To establish stimulators for the CTL assay, B6/wt19 syngeneic cells were infected with either BHV-1 or Vac-gB at different m.o.i. for different periods of time. The presence of gB on the cell surface was confirmed by immunofluorescence (data not shown). Cells infected with BHV-1 at an m.o.i. of 5 for 12 h and cells infected with Vac-gB at an m.o.i. of 0.5 for 6 h were chosen as stimulators and irradiated.

To test whether gB in BHV-1 induced a CTL response, C57BL/6 mice were immunized intraperitoneally with BHV-1. BHV-1 induced high total IgG titres after primary and secondary immunizations (Fig. 1a) and a mean virus-neutralization titre of 75 after the second immunization. The co-existence of all isotypes after the last immunization, neutralization titre of 75 after the second immunization.

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response. Fig. 1(b) shows the relative ratios of IgG1 to IgG2a after both primary and secondary immunizations. The IgG1 and IgG2a titres were similar, which confirmed the development of a balanced response. To measure the induction of a CTL response, CTL effectors were expanded in vitro and then tested with BHV-1-infected B6/wt19 cells as target cells. However, no CTL-mediated killing was observed when splenocytes were stimulated with Vac-gB (Fig. 1d). This could be due to inefficient priming of CD8$^+$ T cells or downregulation of MHC class I molecules by BHV-1, which would be consistent with previous reports (Hariharan et al., 1993).

**Recombinant vaccinia virus expressing gB induces a CTL response, but a relatively low antibody response, in C57BL/6 mice**

As vaccination with BHV-1 did not elicit a CTL response, we evaluated whether the viral vector Vac-gB could induce gB-specific cytotoxicity in C57BL/6 mice. Compared with the immune responses induced by immunization with BHV-1, the IgG titres induced by Vac-gB were lower and no obvious enhancement was observed after the secondary immunization (Fig. 2a). No virus-neutralization titres were detected, which agreed with the fact that gB induces relatively low virus-neutralization titres (van Drunen Littel-van den Hurk et al., 1990). IgG isotypes induced after the last immunization are shown in Fig. 2(c) and the relative ratios of IgG1 to IgG2a are shown in Fig. 2(b) after both primary and secondary immunizations. The antibody-isotype profile was suggestive of a Th1-type response, as the IgG2a titres were significantly higher than the IgG1 titres ($P < 0.01$; Fig. 2b). To measure CTL-mediated killing, BHV-1-infected syngeneic B6/wt19 cells were used as stimulators and Vac-gB-infected B6/wt19 cells were used as target cells. In contrast to the BHV-1-induced immune responses, a strong CTL response was demonstrated by vaccination with Vac-gB (Fig. 2d).

**A plasmid encoding gB induces cell-mediated and antibody responses in both C57BL/6 and C3H mice**

Although we established that gB could induce a CTL response in mice when expressed by a viral vector, the
gB-specific antibody responses were relatively low. Therefore, we investigated the possibility of eliciting gB-specific cytotoxicity by DNA immunization.

In both C57BL/6 and C3H mice, gB-specific total IgG, but no virus-neutralization, titres were observed after immunization with pSLIAgB (Fig. 3a and b). The IgG titres appeared to be twofold higher than those induced by Vac-gB, but lower than the titres elicited by BHV-1. Although in C57BL/6 mice, the IgG1 and IgG2a titres were similar after the first immunization, more IgG2a than IgG1 was observed after the second immunization (Fig. 3c, $P < 0.05$), which is suggestive of a Th1-biased response. In C3H mice, the predominance of IgG2a over IgG1 ($P < 0.05$) also indicated a Th1-biased response after both primary and secondary immunizations (Fig. 3d). The IgG2b and IgG3 isotypes induced after the last immunization (Fig. 3e and f) confirmed this Th bias.

To determine the ability of the gB DNA vaccine to elicit a CTL response in C57BL/6 mice, splenocytes were harvested and restimulated with Vac-gB-infected B6/wt19 syngeneic cells, and CTL-mediated killing of BHV-1-infected B6/wt19 cells was measured. As shown in Fig. 3(g), the plasmid encoding gB induced antigen-specific cytotoxicity in C57BL/6 mice. To determine the induction of a CTL response in C3H mice, we used RSVgB cells, which are syngeneic LTMK2 cells permanently transfected with the gB gene, both as stimulators and as targets. The constitutive expression of membrane gB was confirmed by immunofluorescent staining (data not shown). This demonstrated that plasmid encoding gB also elicits a gB-specific CTL response in C3H mice (Fig. 3h).

By comparing the two mouse strains, we observed that pSLIAgB elicited equivalent IgG responses in C57BL/6 and C3H mice. Furthermore, the isotype profiles suggested the
Fig. 3. Antibody and CTL responses induced by immunization of C57BL/6 and C3H mice with pSLIAgB. (a) gB-specific IgG titres of C57BL/6 mice. (b) gB-specific IgG titres of C3H mice. ELISA titres were calculated as the highest dilution resulting in a reading of 2 SD above the value of a negative-control serum and shown as mean titre + SEM. (c) gB-specific IgG1 and IgG2a titres of C57BL/6 mice. (d) gB-specific IgG1 and IgG2a titres of C3H mice. (e) gB-specific isotypes after secondary immunization of C57BL/6 mice. (f) gB-specific isotypes after secondary immunization of C3H mice. (g) CTL-mediated killing by effectors from pSLIAgB-immunized C57BL/6 mice. Vac-gB-infected B6/wt19 cells were used as stimulators and BHV-1-infected (●) or mock-infected (○) B6/wt19 cells were used as targets in the CTL assay. (h) CTL-mediated killing by effectors from pSLIAgB-immunized C3H mice. RSVgB cells were used as stimulators and RSVgB (●) and LMTK- (○) cells were used as targets in the CTL assay. E : T ratio, effector : target cell ratio.
response to be Th1-biased in both mouse strains and the CTL activity was at least as strong in the C3H mice as in the C57BL/6 mice. Finally, the gB-transformed LMTK$^-$ cells were a more consistent source of stimulators and target cells. Thus, the C3H mouse was chosen as the model system to perform further studies.

Plasmid encoding membrane-anchored or soluble forms of gB with either a pSLIA or pMASIA backbone induces cell-mediated and antibody responses in C3H mice

In the case of BHV-1 gD, the removal of the membrane anchor of glycoproteins has resulted in stronger immune responses when expressed from a plasmid, specifically in cattle (van Drunen Littel-van den Hurk et al., 1998). However, it is not clear how the lack of an anchor affects the induction of a CTL response. To evaluate this, we expressed a truncated version of gB (tgB) that lacked the transmembrane anchor and was therefore secreted from transfected cells (Li et al., 1996, 1997). We inserted the genes encoding BHV-1 gB and tgB into two plasmid backbones, pSLIA and pMASIA, to obtain four DNA constructs, pSLIAgB, pSLIAtgB, pMASIAgB and pMASIAtgB.

C3H mice were immunized with either saline or one of these four plasmids. A robust antibody response was elicited by all of the plasmids (Fig. 4a). There were no significant differences among the groups. There was no difference among either the overall IgG isotype levels at the end of the trial (Fig. 4d) or the IgG1:IgG2a ratios after primary or secondary immunization (Fig. 4b and c) of the four groups of vaccinated mice. Similarly, there appeared to be no differences in the CTL responses induced by plasmids encoding gB or tgB and no effect of the plasmid backbone (Fig. 4e and f).

These results demonstrated that, in the C3H mouse model, a plasmid encoding either the membrane-anchored or the soluble form of gB induced both antibody responses and CTL-mediated killing, regardless of the type of backbone used.

Plasmid encoding tgB induces cell-mediated and antibody responses in cattle

Once we had confirmed that plasmid encoding tgB induced a CTL response in mice, this plasmid was administered to cattle, which allowed us to assess the immune responses in the natural host of BHV-1. Two groups of calves were immunized with either pMASIAtgB or saline. Serum IgG titres specific for gB started to increase ($P<0.05$) 4 weeks after the primary immunization in the pMASIAtgB-vaccinated group compared with the saline group. After the second and third immunizations, the difference between groups became even more obvious ($P<0.01$) (Fig. 5a). After the third immunization, PBMCs were collected to measure gB-specific cellular immunity. The pMASIAtgB-immunized group showed a significantly stronger proliferative response ($P<0.05$) than the saline group (Fig. 5b).

There also tended to be more ($P=0.07$) IFN-γ-producing cells in the pMASIAtgB-immunized group than in the saline group (Fig. 5c). As a measurement of CTL response, we performed a phenotypic and functional characterization of the PBMCs. Upon gB stimulation, the CD8$^+$ population in the PBMCs was significantly higher ($P<0.001$) in the pMASIAtgB-immunized animals than in the saline-immunized calves (Fig. 5d). It is now accepted that the activated CD8$^+$ population that secretes IFN-γ is an indication of the CTL population (Badovinac & Harty, 2000; Butz & Bevan, 1998; Doherty, 1998; Kostense et al., 2001; Murali-Krishna et al., 1998). Furthermore, the CTL assay is not easily performed in large animals. Therefore, in order to determine which subpopulation secreted IFN-γ, CD4$^+$ and CD8$^+$ lymphocytes were purified from the PBMCs with a magnetic particle concentrator. Autologous adherent cells from the PBMCs were cultured with BoGMI-CSF and OvIL4 for 7 days and with gB for an additional day, and then were used as APCs in culture with the pure populations of CD4$^+$ or CD8$^+$ cells in BoIFN-γ-coated ELISPOT plates. Compared with the saline group, the pMASIAtgB-immunized group developed larger populations of both activated gB-specific CD4$^+$ T-helper cells (Fig. 5e, $P<0.01$) and activated gB-specific CD8$^+$ T cells (Fig. 5f, $P<0.01$), which are indicative of CTL effectors.

DISCUSSION

Virus-specific CTLs are activated when viral proteins synthesized in host cells are processed into peptides by cellular enzymes and are considered to be important in protection against and recovery from viral infections (Vasilakos & Michael, 1993). However, cytotoxicity is one of the more difficult immune responses to induce by vaccination, because most types of vaccines, including killed whole virus, subunit proteins and peptides, tend to elicit Th2-type responses.

The currently used inactivated and modified live BHV-1 vaccines do not confer adequate protection against infection with, and spread of, the virus (Zateckla et al., 1999). Moreover, the ability of the virus to downregulate the expression of MHC class I molecules (Nataraj et al., 1997) and to induce apoptosis of CD4$^+$ T lymphocytes (Eskra & Splitter, 1997) results in defective priming of cytotoxic T lymphocytes. The virus could thus survive in individuals with high antibody titres in the absence of a cell-mediated immune response. As we observed in our experiments, immunization of mice with BHV-1 induced antibody responses, but no CTL-mediated killing. This confirmed the need to develop methods to induce effective cell-mediated immune responses to clear the virus.

gB, as one of the most conserved and functionally important herpesvirus proteins, was an attractive candidate to test the induction of BHV-1-specific CTL responses. In herpes simplex virus (HSV), there are several CTL epitopes in gB, and gB is a major target recognized by HSV-specific CTLs in several strains of mice (Mikloska & Cunningham, 1998;
Vasilakos & Michael, 1993; Witmer et al., 1990). Indeed, approximately 10% of the entire CTL response is directed against gB. In equine herpesvirus-1, gB also induces CTL responses (Smith et al., 1998). We compared the CTL responses induced by BHV-1 gB when expressed by vaccinia virus, a viral vector, or by two different plasmid vectors. In

**Fig. 4.** Immune responses induced by pSLIAgB, pSLIAtgB, pMASIAgB and pMASIAtgB in C3H mice. (a) gB-specific serum IgG titres. ELISA titres were calculated as the highest dilution resulting in a reading of 2 SD above the value of a negative-control serum and shown as mean titre + SEM. (b) gB-specific IgG1 and IgG2a titres in pSLIAgB- and pSLIAtgB-immunized mice. (c) gB-specific IgG1 and IgG2a titres in pMASIAgB- and pMASIAtgB-immunized mice. (d) CTL-mediated killing by pSLIAgB- and pSLIAtgB-immunized mice. (e) CTL-mediated killing by pMASIAgB- and pMASIAtgB-immunized mice. RSVgB cells were used as stimulators and RSVgB and LMTK* cells were used as targets in the CTL assay. E:T ratio, effector:target cell ratio.
contrast to BHV-1, which induced high gB-specific antibody titres, but no CTL killing, Vac-gB elicited a strong CTL response, but low gB-specific antibody titres. This low antibody response may be due to an increase in immune response to the viral vector after each immunization, which generally is a problem with delivery of vaccine antigens by
viral vectors. A gB DNA vaccine elicited a twofold stronger humoral response than Vac-gB, as well as cell-mediated immune responses.

We tested two mammalian expression vectors, two mouse models and two forms of gB and confirmed that BHV-1 gB does indeed induce CTL responses. According to the literature, soluble viral proteins usually are not able to induce CTL responses in vivo (Bevan, 1989; Bangham & McMichael, 1990; Townsend et al., 1986). However, we observed that a plasmid encoding the secreted form of gB elicited a CTL response in mice that was as good as, if not better than, that elicited by the membrane-anchored form of gB. This is probably due to the fact that, after DNA immunization, membrane-anchored as well as truncated proteins are produced and processed intracellularly and then presented on the cell surface. Furthermore, truncated proteins are secreted from the transfected cells and taken up by other APCs, possibly inducing a strong humoral response. Moreover, it has been well established that HSV-1 gB, a homologue of BHV-1 gB, elicits a strong CTL response in mice (Mikloska & Cunningham, 1998; Witmer et al., 1990). Furthermore, Deshpande et al. (2002) reported that a DNA vaccine encoding gD, another major glycoprotein of BHV-1, induces a CTL response. Because of the difficulty of performing CTL assays in outbred animals, most cytotoxicity studies, like most DNA-vaccine trials in general, are performed in mice (Deshpande et al., 2002; Doe et al., 1996; Mikloska & Cunningham, 1998; Piali et al., 2001; Witmer et al., 1990), which are not the natural host for HSV-1 or BHV-1. Our report represents the first study on the induction of CTL responses by plasmid encoding BHV-1 gB or tgb, not only in mice, but also in cattle.

Recently, several lines of evidence for cross-presentation of antigen have been reported. Bone marrow-derived APCs have been shown to play a key role in the induction of the immune response after DNA vaccination. For example, transplantation of stably transfected myoblasts into F1 and bone marrow-chimeric mice demonstrated that APCs take up antigens transferred from muscle cells (Fu et al., 1997; Ulmer et al., 1996). Furthermore, adoptive transfer of APCs into immunodeficient mice can transfer the ability of CTL-mediated killing for up to 3 weeks (Doe et al., 1996). Corr et al. (1996) used a controllable plasmid expression system and adoptive transfer to show that most of the immune responses are induced by antigen expression in non-lymphoid cells and subsequent presentation on APCs. This process is defined as cross-presentation. In our study, we applied the principle of cross-presentation by following a previously published protocol (Truong et al., 1999). We obtained differentiated APCs from adherent PBMCs, pulsed the cells with gB antigen and used them as in vitro stimulators for T lymphocytes from tgb plasmid-immunized and control calves. As we observed gB-induced IFN-γ-secreting CD8+ T lymphocytes in the vaccinated calves, this confirmed our hypothesis that a tgb DNA vaccine not only induces a humoral response, but also activates CD8+ CTLs in the natural host. Interestingly, we also observed induction of high numbers of IFN-γ-secreting CD4+ T cells, which is a Th1 population.

The present report is significant, because this is the first demonstration of CTL activity induced by a BHV-1 gB DNA vaccine, not only in mice but also in cattle, the natural host. These results may contribute to the design of more effective BHV-1 vaccines. Clearly, there are many unanswered questions regarding the CTL responses against BHV-1, including the identification of important CTL epitopes. Identification of such epitopes may be of great value in developing novel therapeutic agents against BHV-1.

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