CLINICAL AND VACCINE IMMUNOLOGY, Nov. 2006, p. 1246–1254 Vol. 13, No. 11
1556-6811/06/$08.00+0 doi:10.1128/CVI.00200-06
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Recombinant Bovine Herpesvirus 4 (BoHV-4) Expressing Glycoprotein D of BoHV-1 Is Immunogenic and Elicits Serum-Neutralizing Antibodies against BoHV-1 in a Rabbit Model[▽]

Gaetano Donofrio,[1]* Sandro Cavirani,[1] Alain Vanderplasschen,[2] Laurent Gillet,[2] and Cesidio Filippo Flammini[1]

Università di Parma, Facoltà di Medicina Veterinaria, Dipartimento di Salute Animale, Sezione di Malattie Infettive degli Animali, via del Taglio 8, 43100 Parma, Italy,[1] and Immunology-Vaccinology, B43 b Faculty of Veterinary Medicine, Boulevard de Colonster 20, University of Liège, B-4000 Liège, Belgium[2]

Received 29 May 2006/Returned for modification 30 July 2006/Accepted 4 August 2006

Several biological characteristics of bovine herpesvirus 4 (BoHV-4) make it a good candidate as a gene delivery vector for vaccination purposes. These characteristics include little or no pathogenicity, unlikely oncogenicity, the capability to accommodate large amounts of foreign genetic material, the ability to infect several cell types coming from different animal species, and the ability to maintain transgene expression in both undifferentiated and differentiated cells. Starting from BoHV-4 cloned as a bacterial artificial chromosome (BAC), we used MuA transposase-mediated in vitro transposition to generate recombinant BoHV-4 expressing the immunodominant glycoprotein D (gD) of BoHV-1, one of the most important pathogens of cattle. Although a *cis*-acting element from woodchuck hepatitis virus (the woodchuck hepatitis virus posttranscriptional regulatory element [WPRE]) in the 3′ end of the gD expression cassette was required for maximal gD expression from plasmids in transient transfection assays, this element was not necessary for efficient expression of gD from recombinant BoHV-4 genomes. BoHV-4 recombinants containing gD expression cassettes with or without the WPRE expressed gD at similarly high levels. Several cell lines originating from different animal species expressed gD when infected with BoHV-4 recombinants. When rabbits were immunized with one of the recombinants, high levels of serum neutralizing antibodies against BoHV-1 were generated. This work is one of the first demonstrations of the use BoHV-4 as a vector for vaccine purposes and may provide the basis for BoHV-1 vaccination of cattle with recombinant BoHV-4.

Bovine herpesvirus 4 (BoHV-4) has been isolated from a variety of samples and cells from healthy cattle and from cattle that have experienced abortion or with metritis, pneumonia, diarrhea, respiratory infection, and mammary pustular dermatitis (reviewed in reference 35). The virus was first isolated in Europe from cattle with respiratory and ocular diseases by Bartha et al. (2) and later in the United States by Mohanty et al. (26). Subsequently, distinct BoHV-4 isolates were obtained both in Europe and in the United States (11, 25, 31, 34). However, the pathogenic role of BoHV-4 remains unclear, and only a few investigators have successfully produced an experimental disease (reviewed in reference 35). Although BoHV-4 is classified as a gammaherpesvirus based on genome sequence (3, 13, 41), it differs from other *Gammaherpesviridae* members in important biological properties. Unlike most other gammaherpesviruses, BoHV-4 causes cytopathic effect (CPE) and replicates in a variety of primary cultures and cell lines of bovine and various other animal species (32). In addition, there is no evidence for oncogenicity or growth transformation by BoHV-4.

In contrast to BoHV-4, BoHV-1, an alphaherpesvirus, is a major viral pathogen of cattle and causes significant economic losses worldwide (39). Infection is accompanied by various clinical manifestations, such as infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, abortion, and generalized systemic infection. BoHV-1 is known to play an important role in the bovine respiratory disease complex, commonly referred to as shipping fever (39). Inflammation and necrosis of respiratory epithelia and immunosuppression often lead to increased susceptibility to secondary viral and bacterial infections, resulting in severe clinical disease.

Due to its biological characteristics, BoHV-4 has been suggested as a gene delivery vector (7, 9, 14). In the present work, we explored the feasibility of employing BoHV-4 as a vector to deliver the immunodominant glycoprotein D (gD) of BoHV-1 and generated a model for BoHV-1 vaccination by BoHV-4 expressing BoHV-1 gD.

MATERIALS AND METHODS

Viruses. Recombinants BoHV-4, wild-type BoHV-4 (strain LVR), and wild-type BoHV-1 (strain New York) were propagated by infecting confluent monolayers of Madin-Darby bovine kidney (MDBK) cells at a multiplicity of infection (MOI) of 0.5 50% tissue culture infectious doses ($TCID_{50}$) per cell and maintained in minimal essential medium (MEM) with 2% fetal bovine serum (FBS) for 2 h. The medium was then removed and replaced by fresh MEM containing 10% FBS. When approximately 90% of the cell monolayer exhibited CPE (approximately 72 h postinfection), the virus was prepared by freezing and thawing cells three times and pelleting the virions through 30% sucrose, as described previously (7). Virus pellets were resuspended in cold MEM without FBS. $TCID_{50}$ were determined with MDBK cells by limiting dilution (26).

Plasmid construction. pEGF1-C1 plasmid vector (Clontech; GenBank accession number U55763) was cut with BglII/AseI to remove the enhanced green

* Corresponding author. Mailing address: Dipartimento di Salute Animale, Sezione di Malattie Infettive, Facoltà di Medicina Veterinaria, via del Taglio 8, 43100 Parma, Italy. Phone: 00390521902677. Fax: 00390521902672. E-mail: gaetano.donofrio@unipr.it.

▽ Published ahead of print on 23 August 2006.

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fluorescent protein (EGFP) open reading frame (ORF) and the human cytomegalovirus (HCMV) enhancer-promoter, then blunt ended and ligated. A 2,116-bp XhoI/PstI fragment (corresponding to nucleotides 118423 to 120539 of GenBank accession number NC_001847) from the BoHV-1 strain New York genome, containing the full gD gene, including promoter and ORF, was cloned between the XhoI and PstI sites of pEGFP-C1, which had previously been deleted to generate pgD (see Fig. 1A). This XhoI/PstI fragment was subcloned between the XhoI and PstI sites in the multiple cloning site of pCMVEΔprom, obtained by deletion of the 1,105-bp NdeI/BglII fragment containing the TATA box and EGFP coding sequences from pEGFP-C1, to produce pEgD. For obtaining pCMVgDWPRE, a 600-bp woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence from a lentivirus vector (pCCLsin.PPT.prom.EGFP.WPRE, obtained from L. Naldini, University of S. Raffaele, Milano, Italy) was first cut out with SalI and KpnI and ligated between the SalI and KpnI sites of the multiple cloning site of pEGFP-C1. Subsequently, the EGFP ORF was removed by digestion with NheI and XhoI, and the 1,303-bp MaeI fragment containing the gD ORF from pgD was ligated into the vector containing WPRE by blunt-end ligation after repair of the ends with T4 DNA polymerase. pCMVgD was generated from pCMVgDWPRE by removal of the WPRE by digestion with PstI/KpnI, blunt ending with T4 DNA polymerase, and self-ligation.

pCMVgDWPRE-Kana and pCMVgD-Kana transposon vectors were made by cutting out the expression cassettes from pCMVgDWPRE and pCMVgD with AseI/MluI, repairing the ends with T4 DNA polymerase, and ligating into the XhoI site (also repaired with T4 DNA polymerase) of a MuA transposon vector, pMu-Kana, obtained by replacing the chloramphenicol resistance expression cassette of pEntraceposon(Cam) (Finnzyme) with a kanamycin resistance expression cassette. Minitransposons RI/RII-CMVgD-Kana-RII/RI and RI/RII-CMVgDWPRE-Kana-RII-RI were released from the vectors by cutting with BglII, purified by agarose gel electrophoresis, and directly used for transposition reactions.

Transposition reactions. Transposition reactions were assembled as previously described (17). Standard reaction mixtures (25 μl) contained 10 nM transposon (donor DNA, RI/RII-CMVgD-Kana-RII/RI or RI/RII-CMVgDWPRE-Kana-RII-RI), 5 μg target DNA (BAC-BoHV-4 DNA), 224 nM (0.4 μg) MuA (Finnzymes), 25 mM Tris-HCl (pH 8.0), 100 μg/ml bovine serum albumin, 15% (wt/vol) glycerol, 0.05% (wt/vol) Triton X-100, 126 mM NaCl, and 10 mM $MgCl_2$. Reactions were carried out for 1 h at 30°C. Transposition reactions were dialyzed against distilled H_2O and used to transform electrocompetent *Escherichia coli* cells.

Cell culture, viral infection, and cell extraction. MDBK (ATCC CCL-22), bovine embryo kidney (BEK) (obtained from E. Ferrari, Istituto Zooprofilattico, Brescia, Italy), DH82 (canine histiocyte cells, ATCC CRL-10389), RK13 (rabbit kidney, ATCC CCL-37), NBL-6 (equine fibroblasts, ATCC CCL-57), HC-11 (mouse epithelial cells; Roslin Institute, Edinburgh, United Kingdom), Vero (African green monkey, ATCC CRL-1586), RD-4 (human rhabdomyosarcoma, ATCC CCL-136), BRL (rat liver, ATCC CRL-1442), and PK-15 (pig kidney, ATCC CCL-33) cell lines were cultured in Dulbecco's modified essential medium (DMEM) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Confluent monolayers in 25-cm^2 flasks were infected with 1 $TCID_{50}$/cell of BoHV-4 recombinants. Cell extracts for use in Western blotting were prepared 72 h postinfection in 100 μl of cell extraction buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, and 1% NP-40).

Plasmid DNA preparation. For high-copy-number plasmid mini preparation, a modified alkaline lysis-sodium dodecyl sulfate (SDS) procedure and disposable spin columns were employed (plasmid purification kit; Genomed) according to instructions supplied by the manufacturer. For BAC-BoHV-4 plasmid mini preparation, we used the following protocol. A 5-ml overnight LB culture (15-ml Falcon tube) was pelleted for 5 min at 5,000 rpm, the supernatant was removed, and the pellet was resuspended in 250 μl of 50 mM Tris-HCl (pH 8)–10 mM EDTA–100 μg/ml RNase A and transferred to a 1.5-ml microcentrifuge tube. An equal volume of 0.2 M NaOH–1% SDS was added followed by mixing by inversion and incubation for <5 min at room temperature. Next, 250 μl of 3 M sodium acetate, pH 4.8, was added, followed by mixing and incubation on ice for 5 min. The supernatant was cleared by two rounds of centrifugation at 13,200 rpm for 5 min. Each time the supernatant was transferred to a new tube. DNA was precipitated by adding 750 μl isopropanol, mixing, and incubating on ice for 10 min, followed by centrifugation for 10 min at 13,200 rpm. The pellet was washed once in 70% ethanol, and the dry pellet was dissolved in 50 μl of 1 mM Tris-HCl (pH 7.8)–0.1 mM EDTA.

***E. coli* electroporation.** Five-microliter aliquots of dialyzed transposition reaction mixtures were electroporated into 40 μl of electrocompetent DH10B in cuvettes with a 1-mm gap by using an EquiBio Easyjet Plus apparatus with the following settings: capacitance, 25 μF; voltage, 2.5 kV; resistance, 201 Ω. After electroporation, 1 ml Luria medium per aliquot was added and bacteria were grown for 1 h at 37°C, collected by centrifugation, and plated on LB-kanamycin (50 μg/ml) selection plates. The day after plating, single colonies were picked and grown overnight in 1 ml of LB medium containing 50 μg/ml kanamycin for high-copy-number plasmids or in 5 ml of medium for BAC-BoHV-4 plasmids.

Restriction enzyme analysis and Southern hybridization. Fifteen microliters of DNA prepared from bacteria containing transposed BAC-BoHV-4 were digested with EcoRI, separated by electrophoresis overnight in a 0.8% agarose gel, stained with ethidium bromide, capillary transferred to a positively charged nylon membrane (Roche), and cross-linked by UV irradiation by standard procedures (8). The membrane was prehybridized in 50 ml of hybridization solution (7% SDS, 0.5 M phosphate [pH 7.2], and 1 mM EDTA) for 2 h at 65°C in a rotating hybridization oven (Techna instruments). Probe preparation and digoxigenin nonisotopic labeling was performed by PCR using a pair of primers (kana sense, 5′-TCG CGA CGC GTC CGG AAT TC-3′; kana antisense, 5′-CGA CGC GTG AAA TTG TAA GC-3′) amplifying the entire kanamycin expression cassette contained in the plasmid pTOPO (Invitrogen) and leading to an amplicon of 973 bp. PCR amplification was carried out in a final volume of 50 μl of 10 mM Tris-HCl, pH 8.3, containing 0.2 mM deoxynucleoside triphosphate, 0.02 mM alkaline labile digoxigenin-dUTP (Roche), 3 mM $MgCl_2$, 50 mM KCl, and 0.25 μM (each) primer over 35 cycles, each cycle consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and chain elongation with 1 U of *Taq* polymerase (Boehringer Diagnostics) at 72°C for 1 min. A parallel reaction omitting digoxigenin-dUTP was performed, because digoxigenin incorporation into the amplicon can be checked through the size shift of the amplicon in a gel electrophoresis. Five microliters of the probe was added to 100 μl of H_2O into a screw-cap tube, denatured in boiling water for 5 min, and cooled down on ice for another 2 min. Denatured probe was added to 50 ml of preheated 65°C hybridization solution to the prehybridized membrane and hybridized overnight at 65°C in a rotating hybridization oven (Techna Instruments). Following hybridization, the membrane was washed twice for 30 min with 100 ml of washing solution I (0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS) and twice for 30 min with 100 ml of washing solution II (40 mM phosphate, pH 7.2, and 0.05% SDS) at 65°C.

On a freshly washed dish, the membrane was incubated for 30 min at room temperature in 25 ml of blocking solution (100 mM maleic acid [pH 7.5], 150 mM NaCl, and 1% blocking reagent [Roche] or 5% skim milk). Anti-digoxigenin Fab fragment (150 U/200 μl [Roche]), diluted 1:15,000 in 25 ml of blocking solution, was applied to the membrane for 30 min under gentle shaking at room temperature and washed twice for 15 min with 100 ml of washing solution (100 mM maleic acid [pH 7.5], 150 mM NaCl, and 0.3% Tween 20). Detection was performed following equilibration of the membrane in detection buffer (100 mM Tris-HCl [pH 9.5]–1 mM EDTA) for 2 min at room temperature. Chemiluminescent substrate (CSPD; Roche) was added by scattering the drops over the surface of the membrane after placement of the membrane between two plastic sheets, and any bubbles present under the sheet were eliminated with a damp lab tissue to create a liquid seal around the membrane. Signal detection was obtained, exposing the membrane to X-ray film. The exposure time was adjusted with the intensity of the signal.

Cell culture, electroporation, and recombinant virus preparation. MDBK and BEK cells were maintained as a monolayer with growth medium containing 90% DMEM, 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 10 μg/ml streptomycin. Cells were subcultured to a fresh culture vessel when growth reached 70 to 90% confluence (i.e., every 3 to 5 days) and were incubated at 37°C in a humidified atmosphere of 95% air–5% CO_2. Plasmid DNA (5 μg) in 500 μl DMEM without serum was electroporated (Equibio apparatus, 270 V, 960 μF) in cuvettes with a 4-mm gap into BEK cells from a confluent 25-cm^2 flask. Electroporated cells were returned to the flask, fed the next day, and split 1:2 when they reached confluence at 2 days postelectroporation. Cells were left to grow until CPE appeared. Recombinant viruses were propagated by infecting confluent monolayers of MDBK cells at an MOI of 0.5 $TCID_{50}$ per cell and maintaining them in MEM with 2% FBS for 2 h. The medium was then removed and replaced by fresh MEM containing 10% FBS. When approximately 90% of the cell monolayer exhibited CPE (approximately 72 h postinfection), the virus was prepared by freezing and thawing cells three times and pelleting virions through 30% sucrose, as previously described (7). Virus pellets were resuspended in cold MEM without FBS. $TCID_{50}$ were determined on MDBK cells by limiting dilution.

Western immunoblotting. Cell extracts containing 50 μg of total protein were electrophoresed through 12% SDS-polyacrylamide gels and transferred to nylon membranes by electroblotting. Membranes were incubated with monoclonal anti-BoHV-1-gD antibody (clone 1B8-F11; VRMD, Inc., Pullman, WA), probed with horseradish peroxidase-labeled anti-mouse immunoglobulin G1 (IgG1) an-

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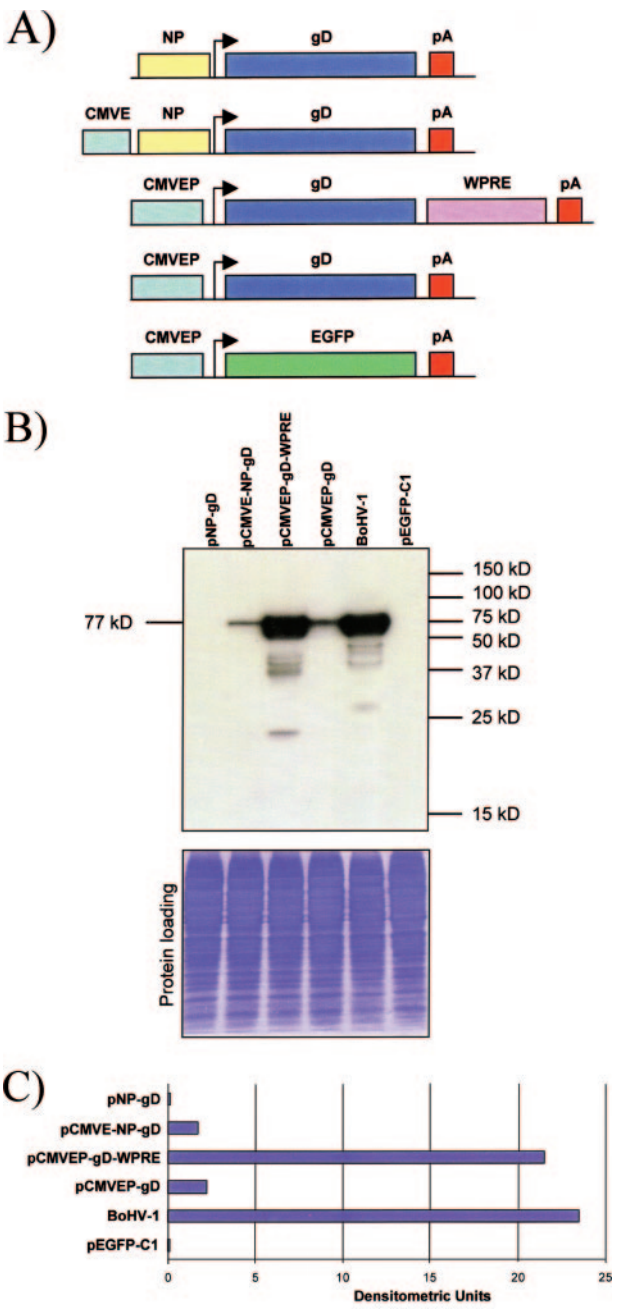

FIG. 1. Structure and evaluation of plasmid vectors expressing gD. (A) Diagram (not to scale) showing the expression cassettes included in the vectors employed throughout the study: the pgD vector, containing the gD natural promoter (NP) and the gD ORF with the BGH polyadenylation signal (pA); the pEgD vector, containing the CMV enhancer in front of the gD natural promoter (NP), the gD ORF, and the BGH polyadenylation signal (pA); the pCMVgDWPRE vector, containing the CMV enhancer promoter (CMVEP), the gD ORF followed by the WPRE, and the BGH polyadenylation signal (pA); and the pEGFP-C1 vector, used as a transfection control and negative control for gD expression, containing the CMV enhancer promoter (CMVEP), the EGFP ORF (EGFP), and the BGH polyadenylation signal (pA). (B) Western immunoblotting to detect gD in extracts of cells transfected with the constructs described above. BoHV-1-infected cells were used as a positive control. The Coomassie-stained membrane is shown as a protein loading control. (C) Bar graph displaying the relative levels of gD expression among the different constructs, based on densitometry of immunoblots. The experiment was repeated three times, and data were normalized with the signal intensity of the protein loading control. Statistical significance ($P < 0.005$) was assessed by Student's t test.

tibody (Sigma), and visualized by enhanced chemiluminescence (ECL Kit; Pierce, Rockford, IL).

Serological tests. Serum neutralization tests were performed as follows. Twenty-five microliters of each serum sample was added to the wells of 96-well plates. Twenty-five microliters of DMEM was added to each well and, for each serum tested, serial twofold dilutions were made. Positive and negative serum controls were included. Twenty-five microliters of virus suspension containing 100 $TCID_{50}$ of BoHV-1 was added to each well. After 1 h of incubation at 37°C, 50 μl of a MDBK cell suspension was added to each well and the plates were incubated for 3 days at 37°C. Expression of viral infectivity and serum neutralizing activity through CPE was detected by microscopy and crystal violet staining of the cell monolayer. The neutralization antibody titers were expressed as the reciprocal of the final dilution of serum that completely inhibited viral infectivity.

An enzyme-linked immunosorbent assay (ELISA) was carried out with a commercial kit according to the instructions of the manufacturer (ELISA IBR-IPV; Institute Pourquier); the only modification was the use, as a secondary antibody, of a goat anti-rabbit IgG (whole-molecule) peroxidase conjugate (Sigma), diluted 1:20,000.

Animal handling and care. Rabbits were cared for and used in accordance with Italian laws for animal experimentation. Rabbits were maintained at 24°C with a controlled light cycle (12 h of light, starting at 6:00 a.m.) and with food and water ad libitum. Blood samples were obtained and viral injections were performed via the auricular vein at scheduled intervals.

RESULTS

Rational design and construction of plasmid vectors expressing BoHV-1 gD. Before attempting to express BoHV-1 gD in a BoHV-4 recombinant, we first optimized a suitable expression cassette to achieve a robust expression of gD in eukaryotic cells. Several constructs were made, starting from a restriction fragment of the BoHV-1 genome containing the full gD gene, including the promoter (Fig. 1A): (i) pgD, containing the gD natural promoter, the gD ORF, and bovine growth hormone (BGH) (pA) site; (ii) pEgD, containing the HCMV immediate-early (IE) enhancer in front of the gD natural promoter followed by the gD ORF and BGH pA site; (iii) pCMVgD, containing the HCMV IE promoter-enhancer, followed by the gD ORF and BGH pA site; and (iv) pCMVgDWPRE, containing the HCMV IE promoter-enhancer, the gD ORF, and the WPRE, a 600-bp noncoding and *cis*-acting RNA sequence that acts to increase the stability and export of mRNA to the cytoplasm (6, 42), ending with the BGH pA site. Each construct was electroporated into HEK293T cells, and cell extracts were analyzed for gD expression by Western blotting 48 h postelectroporation. A plasmid expressing EGFP under the control of the CMV enhancer-promoter (pEGFP-C1) was identically transfected as a negative control for gD expression and to monitor the efficiency of transfection through the number of green cells. Cells infected with BoHV-1 were used as a positive control. As can be observed in Fig. 1B and C, the highest gD expression level was obtained from pCMVgDWPRE, which gave a level of expression comparable to cells infected with BoHV-1 and was ~9-fold higher than from pCMVgD and ~12-fold higher than from pEgD. Thus, we were able to conclude that WPRE increased the level of expression of gD from plasmids in a transient transfection assay. In contrast, no gD expression was detectable from pgD. The simple

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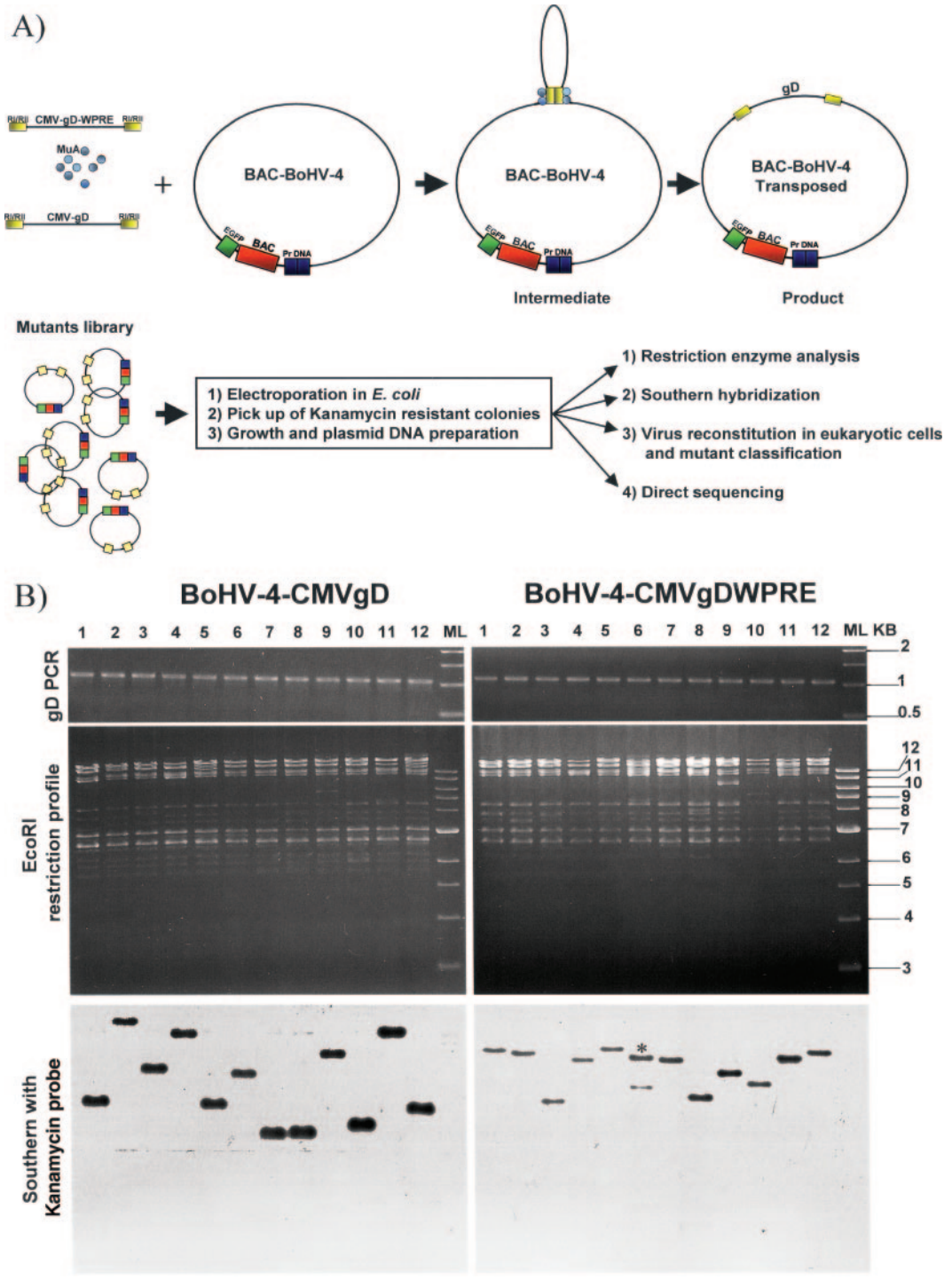

FIG. 2. Construction of BoHV-4 expressing gD. (A) Overall strategy of recombinant BAC-BoHV-4 generation. The transposon complex is assembled with BglII fragments containing RI/RII-CMVgD-Kana-RII/RI and RI/RII-CMVgD-Kana-RII/RI transposons (yellow ends) as donor DNA, BAC-BoHV-4 as a target DNA (blue boxes indicate polyrepetitive DNA, red boxes indicate the BAC plasmid backbone, and the green boxes represent the EGFP expression cassette), and MuA enzyme. The transposition reaction proceeds through an intermediate formation (looped RI/RII-CMVgD-Kana-RII/RI and RI/RII-CMVgD-Kana-RII/RI transposon complexes attached to the target BAC-BoHV-4 DNA) and a final reaction with the target DNA transposed (RI/RII-CMVgD-Kana-RII/RI and RI/RII-CMVgD-Kana-RII/RI transposons, bordered by the two yellow boxes, integrated into the target BAC-BoHV-4 DNA). A flow chart of reconstitution of the BAC-BoHV-4 recombinant is shown. The recombinant plasmids are electroporated into electrocompetent *E. coli*, the kanamycin-resistant colonies are selected on kanamycin plates, and plasmid DNA is prepared from amplified single colonies and analyzed. (B) Analysis of BAC-BoHV-4 randomly transposed clones (12 clones for RI/RII-CMVgD-Kana-RII/RI, BoHV-4-CMVgD and 12 clones for RI/RII-CMVgDWPRE-Kana-RII/RI, BoHV-4-CMVgDWPRE). (Top panel) PCR analysis demonstrating the presence of the gD ORF. (Middle panel) EcoRI restriction enzyme digestion of the transposed clones. In some of the clones, the modification of the restriction profile can be observed. (Bottom panel) Southern hybridization of the same clones, with a digoxigenin-labeled transposon-specific probe, where integration of the transposon is demonstrated in all the clones analyzed. The transposon integrated into many different restriction fragments. In the lane marked with an asterisk, the hybridization of the probe to more than one band indicates integration of the transposon into more than one site; this clone was discarded.

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CMV enhancer in front of the gD promoter was able to upregulate gD expression to a detectable level, similar to that obtained from the CMV promoter-enhancer.

MuA transposase allows random integration of heterologous expression cassettes into the BoHV-4 genome. Although the highest gD expression was achieved with pCMVgDWPRE in the transient transfection assay, we generated recombinant BoHV-4 containing the expression cassette from either pCMVgDWPRE or pCMVgD to assess whether the function of WPRE would have the same importance for gD expression in the context of the BoHV-4 genome. At the same time, we wanted to explore whether the site of integration of foreign expression cassettes into the BoHV-4 genome is crucial for the expression level. Therefore, we decided to randomly integrate the CMVgD and CMVgDWPRE expression cassettes into the BoHV-4 genome, cloned as a bacterial artificial chromosome (BAC) (14) with the Mu-Kana transposon (17). The CMVgD and CMVgDWPRE expression cassettes were excised from pCMVgD and pCMVgDWPRE, respectively, and subcloned into a pMu-Kana vector containing the Mu R-end DNA (RI/RII and RII/RI), critical for MuA enzyme binding (17, 33), and the kanamycin resistance gene under the control of a constitutively active prokaryotic promoter. The so-generated minitransposons (RI/RII-CMVgD-Kana-RII/RI and RI/RII-CMVgDWPRE-Kana-RII/RI) were excised from the vector and employed for transposition reactions, with BAC-BoHV-4 used as target DNA (Fig. 2A). Several hundred kanamycin-resistant colonies were obtained from each reaction. Twelve colonies per minitransposon were grown as overnight cultures in medium containing chloramphenicol to ensure maintenance of BAC-BoHV-4 containing a chloramphenicol resistance gene (Fig. 2B). Plasmid DNA minipreps were generated and analyzed first by PCR for the presence of the BoHV-1 gD ORF and then by restriction enzyme digestion and Southern blot hybridization (Fig. 2B). EcoRI restriction analysis was chosen because this restriction site is contained in one end of the transposon. The initial task of approximating the location of the transposon insertion in BAC-BoHV-4 was accomplished in some cases by comparing mutant restriction profiles. However, most of the restriction profiles did not show appreciable differences with untransposed BAC-BoHV-4 control DNA due to the limitations of gel electrophoresis for detecting small variations in the sizes of large fragments (Fig. 2B). However, all of the colonies tested showed the presence of the transposon when analyzed by Southern blot hybridization (Fig. 2B). The transposons appeared to be scattered among restriction fragments throughout the BAC-BoHV-4 genome. A single case of multiple insertion events was detected and discarded.

Recombinant BoHV-4 reconstitution and gD expression. Because the expression cassettes were randomly integrated into the BoHV-4 genome, in order to determine whether any Mu-Kana insertions disrupted expression of an essential gene, individual BAC-BoHV-4CMVgDWPRE and BAC-BoHV-4CMVgD mutant DNAs were electroporated onto BEK cells to regenerate the virus. Mutants capable of generating viable virus were easily detected by expression of the EGFP reporter gene carried by the BAC cassette inserted into the BoHV-4 genome (14). Hence, viable replication-competent BAC-BoHV-4 mutants gave rise to an EGFP-positive CPE (Fig. 3A) throughout the monolayer, whereas lethal knockouts were unable to spread from the initial transfected cells. Nine viable mutants were obtained for BAC-BoHV-4CMVgDWPRE and seven for BAC-BoHV-4CMVgD. Only recombinants with replication characteristics similar to untransposed clones were further characterized (clones 3 and 8 for BAC-BoHV-4CMVgDWPRE and clone 10 for BAC-BoHV-4CMVgD). Sequence analysis showed that the transposon integration site was a noncoding intergenic region for all three clones (data not shown).

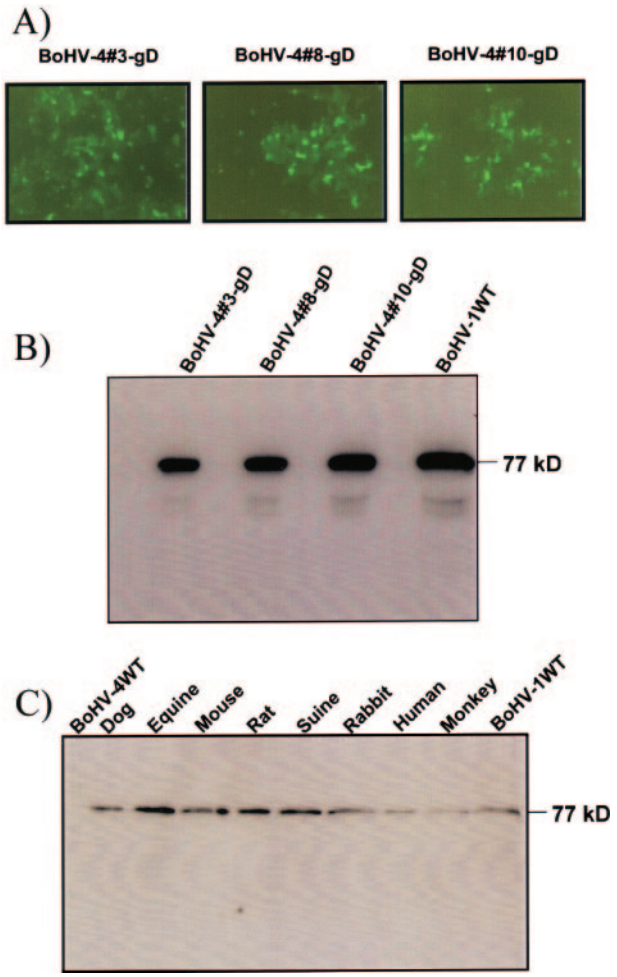

FIG. 3. (A) Representative fluorescent microscopic images (×10) of green plaques formed by viable reconstituted transposed recombinant BoHV-4 (BoHV-4#3gD, BoHV-4#8gD, and BoHV-4#10gD) after DNA electroporation into BEK cells. (B) Western immunoblotting of extracts of BEK cells infected with BoHV-4 recombinants (BoHV-4#3gD, BoHV-4#8gD, and BoHV-4#10gD) to detect gD. An abundant 77-kDa gD band is detectable, comparable to that detected in extracts of BoHV-1-infected BEK cells. (C) Representative Western immunoblotting of extracts of cell lines of different animal species infected with BoHV-4#10gD. BoHV-1-infected BEK cell extract was used as a positive control, and wild-type BoHV-4-infected BEK cell extract was used as a negative control. Similar results were obtained with clones 8 and 10 (data not shown).

To evaluate gD expression by the BoHV-4 recombinants delivering gD, BEK cells were infected with 5 $TCID_{50}$/cell of each virus; at 72 h postinfection, cell extracts were analyzed by Western immunoblotting with a monoclonal antibody against

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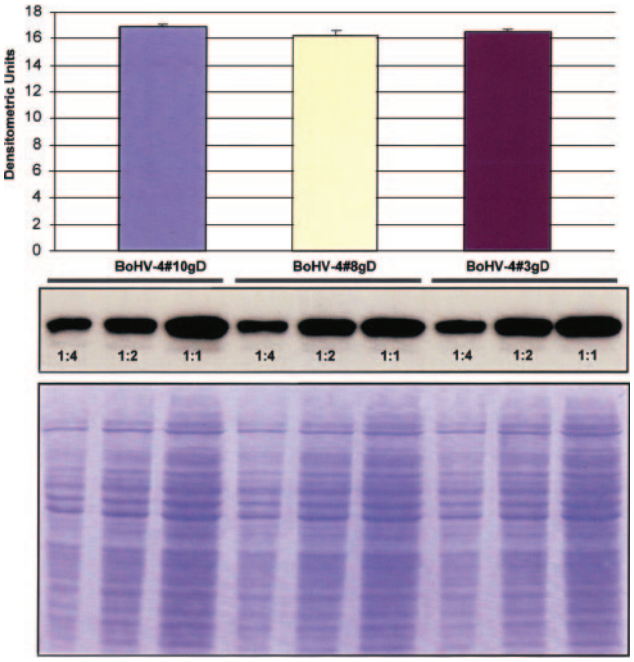

FIG. 4. Semiquantitative analysis of the gD expression level in BEK cells infected with BoHV-4#10gD, BoHV-4#10gD, and BoHV-4#10gD. Dilutions of the samples (1:1, 1:2, and 1:4), as indicated, were analyzed by Western blotting, and the gD bands were assessed densitometrically (Molecular Dynamics). The bar graph (top) displays the relative levels of gD expression among the different recombinant viruses, based on densitometry of immunoblots (middle). Values are calculated as the means of the average band densities normalized for each dilution. Error bars indicate the standard errors of the means of three experiments. The Coomassie-stained membrane is shown as a protein loading control (bottom).

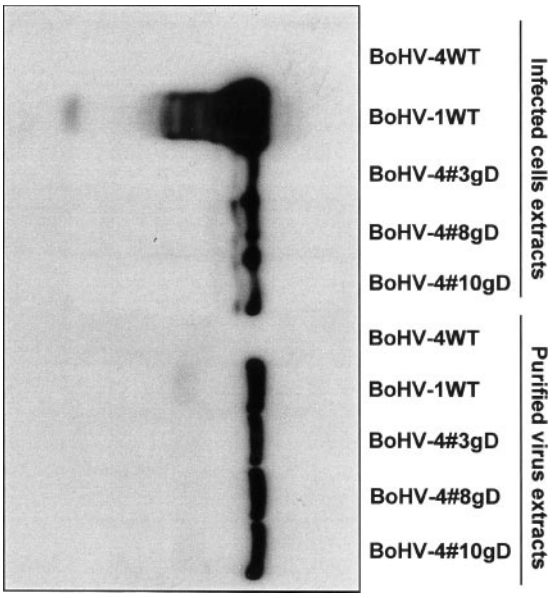

FIG. 5. Incorporation of gD into recombinant BoHV-4 particles. Extracts of virus-infected cells and extracts of virus purification through CsCl gradient centrifugation were analyzed by Western immunoblotting with an anti-gD monoclonal antibody. Positive and negative controls were performed with BoHV-1 and wild-type BoHV-4 (WT), respectively.

gD. As shown in Fig. 3B, well-detectable expression of gD was observed for all three clones tested. Then, expression was assessed in cell lines representing different animal species previously shown to be infectible by BoHV-4 (9), and expression was very well detectable for all cell lines tested (Fig. 3C).

BoHV-4 recombinants express gD at similar levels. As already mentioned, the three clones employed for the experiments deliver two types of gD expression cassettes: clone 10 lacks the WPRE element 3′ to the gD ORF, whereas clones 3 and 8 have the WPRE element. Moreover, the expression cassette is located at different points on the viral genome for each clone. To determine whether the presence of the WPRE element or the location of insertion within the BoHV-4 genome was important for the level of gD expression, a semiquantitative analysis of gD expression levels was performed. BEK cells were infected with 5 $TCID_{50}$/cell of each recombinant clone, dilutions of the cell extracts (1:1, 1:2, and 1:4), as indicated, were analyzed by Western blotting, and the bands were assessed by densitometry (Molecular Dynamics); values were calculated as the means ± standard errors of the means of the average band densities normalized for each dilution (the experiment was repeated three times), and no differences with statistical significance ($P > 0.05$) were observed among the clones as assessed by Student's t test (Fig. 4).

gD is incorporated into mature virus particles of recombinant BoHV-4. BoHV-1 gD is a typical type 1 integral membrane protein containing a signal peptide at the amino terminus and a hydrophobic transmembrane domain at the carboxyl terminus, which allows infected cells to sort gD onto the enveloped viral surface. Although incorporation into virus particles is likely not essential for generation of anti-gD antibodies, we determined whether gD expressed in recombinant BoHV-4-infected cells was incorporated into BoHV-4 virus particles. Virus preparations from the three BoHV-4 clones expressing gD were purified by CsCl gradient centrifugation and analyzed by Western immunoblotting with monoclonal antibody against gD. As expected, a strong signal for gD was detected in recombinant BoHV-4 virions but not in wild-type BoHV-4 virions (Fig. 5). The slightly faster-migrating gD precursor molecule, which is not incorporated into virions, was detected in infected cell extracts but not in virion-enriched extracts, indicating successful virion enrichment. Thus, these results indicate the incorporation of gD into BoHV-4 virus particles.

BoHV-4 expressing gD is immunogenic and elicits the production of neutralizing antibodies against BoHV-1. Because the three recombinant BoHV-4 clones expressed gD at similar levels in vitro and in accordance with Italian laws on animal experimentation, which suggest minimizing the number of animals used, a single clone of BoHV-4 expressing gD (BoHV-4CMVgD#10) was employed for in vivo studies. Before immunization procedures, BoHV-4CMVgD#10 was assessed for transgene integration site stability following serial passages (20 passages on BEK cells) and monitored by Southern blotting (data not shown).

After collection of preimmune serum, five rabbits were intravenously inoculated with 3 ml of 10^3 $TCID_{50}$/ml of BoHV-

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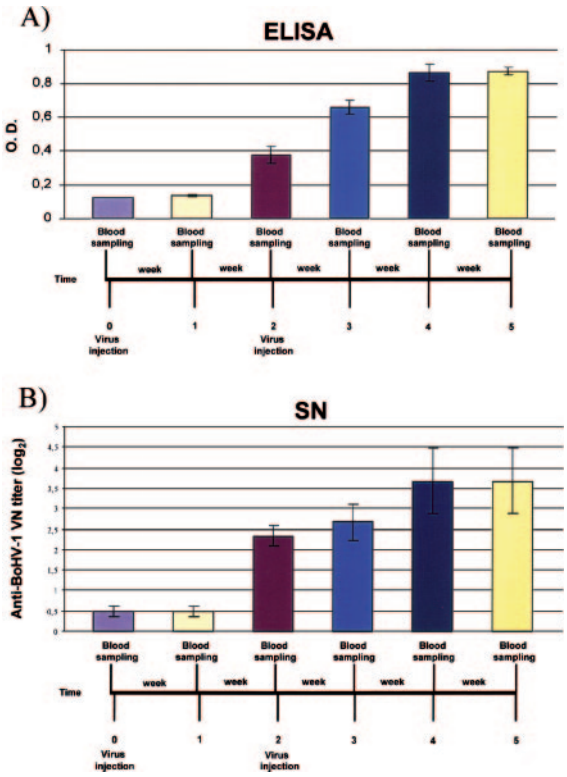

FIG. 6. Kinetics of the humoral immune responses of rabbits immunized with BoHV-4#10gD. Sera collected from rabbits before immunization and from immunized rabbits were evaluated for anti-gD antibodies by ELISA and a serum neutralization (SN) test. (A) Anti-gD antibody detected by ELISA and expressed as the optical density (O.D.) at 504 nm. (B) Serum neutralizing antibodies against BoHV-1 detected by SN and expressed as the reciprocal of the highest dilution of the serum that inhibited the development of virus-induced CPE in MDBK cells. Virus neutralization (VN) titers of >2 ($\log_2$) were considered to be positive. In both panels, each value represents the mean response of five rabbits ± the standard error of the mean.

4CMVgD#10. An identical inoculation was performed 2 weeks later. Blood samples were collected weekly from all animals for the assessment of anti-gD antibodies. Furthermore, body temperature and clinical sign development were monitored daily. None of the animals developed fever or other clinical signs during the time of observation (5 weeks). All animals developed an antibody response against BoHV-1, detectable the second week after the first viral inoculation and reaching the plateau phase at the fourth week post-viral inoculation (Fig. 6). Because the antibody titer was observable 2 weeks following the first viral inoculation and the blood samples were collected before the second viral inoculation, it is possible to conclude that a single inoculation of BoHV-4gD#10 was able to elicit a humoral immune response against BoHV-1, as demonstrated by ELISA (Fig. 6A). To determine whether the antibodies produced had neutralizing activity against BoHV-1, serum neutralization tests were performed with the same sera. Serum-neutralizing antibodies appeared with kinetics similar to antibodies detectable by ELISA (Fig. 6B). Because BoHV-4gD#10 elicits the production of BoHV-1 neutralizing antibodies and gD is incorporated into the recombinant virions, we performed serum neutralization tests with BoHV-1-positive sera and sera from BoHV-4gD#10-immunized rabbits to determine whether BoHV-4gD#10 acquired serum neutralizable properties. None of the sera were able to neutralize BoHV-4gD#10.

DISCUSSION

Live marked (gE-negative) attenuated BoHV-1 vaccine is one of the most effective means of inducing protective immunity. It has been shown to be more protective than subunit and killed vaccines, likely because it replicates in the recipient and induces a broad range of immune responses to the expressed gene products (19). However, the risk of reversion of the attenuated virus to virulence through recombination with a field virus makes this vaccine potentially dangerous and hazardous for a vaccination and eradication program. DNA vaccines, like viral vectored vaccines, are capable of raising both humoral and cellular responses, but, unlike live virus vaccines, they are extremely stable and versatile for manufacture and storage. Extensive investigation in this direction has used BoHV-1 gD formulated as a DNA vaccine (38), and many strategies have been used to enhance the potency. These include but are not limited to (i) better promoters/enhancers for increasing gene expression; (ii) sequences encoding immunomodulatory molecules, such as cytokines; (iii) direct targeting of the DNA vaccine to antigen-presenting cells or direct intradermal delivery of the DNA construct; and (iv) sequences encoding the antigen fused to molecules that facilitate antigen spreading and cross-penetration (38). The main limitation of DNA-based vaccines remains their low relative efficacy, requiring multiple boosts with high doses (up to 500 μg of plasmid DNA per injection) to generate responses comparable to those achieved from an attenuated virus vaccination.

The use of viruses as vectors for the delivery of heterologous antigens needs careful consideration because the immune system has evolved a sophisticated array of mechanisms to both detect and eliminate invading viruses. A viral vector also delivers the antigen directly into the host cell, which allows for high-level intracellular expression. Hence, the viral vector acts as an adjuvant and as a delivery system. An effective viral vector should present the expressed antigen as an immune target and should remain in the host long enough to stimulate an effective response. Because the vaccine's viral vector-associated risk is a major concern in viral vector development, attenuation is regarded as a desirable feature of a viral vector and efforts are directed toward the development of highly attenuated strains (16, 20, 23, 40). However, in the case of BoHV-4, the virus naturally exhibits limited or no pathogenicity in both natural and experimental hosts (35). Therefore, further attenuation by disruption of genes could be neither necessary nor desirable; further attenuation might limit the ability of the virus to adequately stimulate the immune response. Another feature of BoHV-4 making it an attractive potential vaccine vector is the lack of evidence for oncogenicity. Other gammaherpesviruses, such as Epstein–Barr virus, herpesvirus saimiri, human herpesvirus 8, and murine gammaherpesvirus-68, have been associated with growth-transforming ability (1, 4, 5, 18, 21, 27, 36). In contrast, no evidence for growth transformation by BoHV-4 has been obtained. Compared to other gammaherpesviruses, BoHV-4 contains few ho-

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mologs of cellular genes expected to alter the growth properties of cells (41). Although homologs of cellular genes carried by BOHV-4 have been shown to protect cells from apoptosis and BoHV-4 infection has been shown to inhibit apoptosis in some cell lines, in others, including some carcinoma cell lines, BoHV-4 infection triggers apoptosis instead (15).

Like other herpesviruses, BoHV-4 establishes persistent infections in its natural host and in an experimental host, the rabbit (10, 22, 24, 25, 30), suggesting that a BoHV-4 vaccine vector would be particularly useful for long-lasting expression of the heterologous antigen. Although BoHV-4 has been demonstrated in many tissues during persistent infection by PCR, in situ hybridization, or recovery of virus after explant culture, the accumulated evidence suggests that one site of persistence in both natural and experimental hosts is cells of the monocyte/macrophage lineage (10, 12, 22, 28, 29, 31). This is probably the most attractive BoHV-4 characteristic in terms of vaccine vector development. Because macrophages are both professional antigen-presenting cells and natural targets of BoHV-4, the delivered heterologous antigen should be directly processed and presented to the immune system, ultimately leading to an amplification of the immune response after the homing of macrophages persistently infected with BoHV-4 to the lymphoid organs, such as the spleen and lymph nodes.

One of the major concerns about vaccine vector development is preexisting antivector immunity in the host organism. In the case of BoHV-4, this should not be a problem, because BoHV-4 naturally does not elicit the production of serum neutralizing antibodies; even after introducing in BoHV-4 a heterologous protein such as BoHV-1 gD, known to be responsible for BoHV-1 serum neutralizing antibody production, no serum neutralizing activity against BoHV-4 was observed. In contrast, a good titer of serum neutralizing antibodies against BoHV-1 was obtained. The lack of neutralizing activity of immune sera against BoHV-4 expressing BoHV-1 gD may be explained by the fact that BoHV-4 has a different pattern of cell penetration; moreover, BoHV-4 is a gammaherpesvirus, while BoHV-1 is an alphaherpesvirus. Thus, serum neutralizing antibodies against gD do not interfere with BoHV-4 expressing gD cell attachment and penetration. Although the efficacy of a BoHV-4-vectored vaccine for BoHV-1 needs to be corroborated by an in vivo challenge study with a pathogenic BoHV-1 strain in the natural host, the bovine, the results we have obtained with rabbits are strongly encouraging.

ACKNOWLEDGMENTS

We thank V. L. van Santen for editing the manuscript and Mathias Ackermann for helpful discussions.

This work was financially supported by the Italian Ministry of University and Scientific Research (Italian National Grant MIUR, PRIN 2005, 2005078885).

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