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Potential of Equine Herpesvirus 1 as a Vector for Immunization

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Key problems using viral vectors for vaccination and gene therapy are antivector immunity, low transduction efficiencies, acute toxicity, and limited capacity to package foreign genetic information. It could be demonstrated that animal and human cells were efficiently transduced with equine herpesvirus 1 (EHV-1) reconstituted from viral DNA maintained and manipulated in *Escherichia coli*. Between 13 and 23% of primary human $CD3^+$, $CD4^+$, $CD8^+$, $CD11b^+$, and $CD19^+$ cells and more than 70% of $CD4^+$ MT4 cells or various human tumor cell lines (MeWo, Huh7, HeLa, 293T, or H1299) could be transduced with one infectious unit of EHV-1 per cell. After intranasal instillation of EHV-1 into mice, efficient transgene expression in lungs was detectable. Successful immunization using EHV-1 was shown after delivery of the human immunodeficiency virus type 1 $Pr55^{gag}$ precursor by the induction of a Gag-specific $CD8^+$ immune response in mice. Because EHV-1 was not neutralized by human sera containing high titers of antibodies directed against human herpesviruses 1 to 5, it is concluded that this animal herpesvirus has enormous potential as a vaccine vector, because it is able to efficiently transduce a variety of animal and human cells, has high DNA packaging capacity, and can conveniently be maintained and manipulated in prokaryotic cells.

Equine herpesvirus 1 (EHV-1), an alphaherpesvirus, causes infections in equines. Usually the disease induced by the virus is mild, and approximately 90% of equines worldwide harbor the agent, whose persistence in animals is lifelong. One of the peculiarities of EHV-1 is its tropism for blood mononuclear cells, which are the primary site for establishment of EHV-1 latency. Neuronal tissues, including the trigeminal ganglion and the brain, are only rarely infected by the agent. Neurological symptoms after infection of equines with certain EHV-1 strains are caused by infection of endothelial cells and subsequent hypoxia and neuronal degradation and are consistent with myeloencephalopathy and not myeloencephalitis (40, 62).

Herpesviridae enter target cells by fusion of the viral envelope with the plasma membrane at neutral pH after attachment of virions to cell surface glycosaminoglycans (47, 51). Glycoproteins are crucially involved in these early stages of infection, and 11 glycoproteins in the prototype member of the *Alphaherpesvirinae* subfamily, *Herpes simplex virus type 1* (HSV-1), have been identified. The herpesvirus glycoproteins involved in attachment, receptor binding, and fusion (i.e., gB, gC, gD, and the gH-gL complex) also appear to largely determine the mostly very restricted host tropism of *Alphaherpesvirinae*, i.e., the inability of animal viruses to naturally infect species other than those they have coevolved with (23). While the first contact and heparin-sensitive attachment of the studied alphaherpesviruses, including EHV-1, is mainly mediated by gC, receptor binding of HSV-1, pseudorabies virus, and bovine herpesvirus 1 (BHV-1) is via interaction of gD with cellular receptors that belong to the tumor necrosis factor and immunoglobulin (Ig) superfamilies (16, 17, 33, 41, 61, 63). A recent study has shown that EHV-1 enters cells in the absence of all known alphaherpesvirus receptors, although the mechanism of entry, i.e., fusion of viral envelopes with the cellular membrane, is identical to that of HSV-1, pseudorabies virus and BHV-1 (14).

Several vectors for potential use in human gene therapy or immunizations have been described. Among those are RNA and DNA viruses. The most commonly used vectors are replication-competent or -deficient poxviruses, retroviruses, rhabdoviruses, adenoviruses, adeno-associated viruses (AAV), and human herpesviruses, including HSV-1, Epstein-Barr virus (EBV), and human cytomegalovirus (HCMV) (3, 9, 18, 21, 22, 34, 35, 45, 50, 53, 58). One of the problems associated with RNA viruses, adenoviruses, or AAV are their limited capacity to package foreign DNA, which is normally restricted to DNA of less than 5 kbp in length (13), although gutted adenovirus vectors can harbor up to 14 kbp of foreign sequence (28). In addition, extremely high titers of adenoviruses or AAV are necessary for efficient transduction of human cells, explaining the risk for allergic or toxic reactions in patients (4, 26). While poxviruses can harbor approximately 25 kbp of foreign genetic material, human herpesviruses can package foreign DNA of more than 100 kbp in size, and the limitations caused by the low packaging capacity intrinsic to the various RNA viruses, adenoviruses, or AAV systems can be overcome by using vaccinia virus, canarypox virus, HSV-1, or HCMV (3, 35, 45). However, one disadvantage for using human HSV-1, HCMV, EBV, or vaccinia virus as vectors is the fact that many patients have already encountered infection and/or vaccination with, especially, the human herpesviruses (31, 32). Because antivector immunity and impaired efficiency of gene transfer can pose

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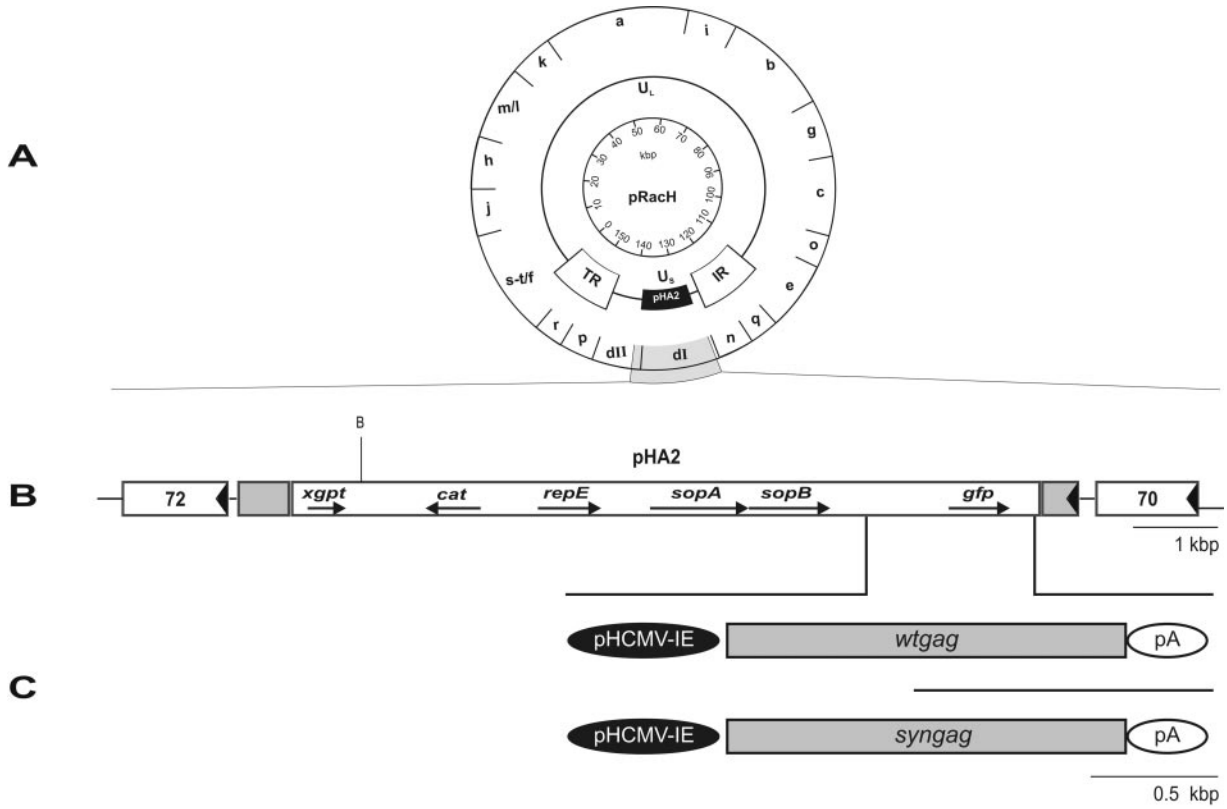

FIG. 1. (A) Schematic illustration of the EHV-1 HΔgp2 virus used in this study (48). Shown are the overall organization and BamHI map of the approximately 155-kbp HΔgp2 genome and the designation of genome regions (unique long, U_L; unique short, U_S; internal [IR] and terminal repeats [TR]). (B) Organization of the U_S region of HΔgp2 from gene 70 (gG homologue) through gene 72 (gI homologue) including the mini-F vector sequences derived from plasmid pHA2 and inserted in lieu of gene 71 encoding gp2. (C) Insertion of *wtgag* and *syngag* sequences into HΔgp2 instead of the *egfp* gene. Both genes are under the control of the HCMV-IE promoter/enhancer, and transcription termination sequences [poly(A) site] are taken from simian virus 40 (pcDNAI; Invitrogen). Scales are given in kbp.

serious problems, the development and testing of novel vector systems is one of the keys to successful development of novel and efficacious immunization schemes (46). Our studies aimed at exploring the potential of an animal virus, EHV-1, for use as an immunization vector, because it exhibited high transduction efficiencies in a wide variety of cells. Although still in the early phase of development, this nonhuman virus may prove an extremely valuable and novel tool for use as a vector in animals and humans, because (i) it has high packaging capacity, (ii) it extremely efficiently delivers foreign genes at low virus particle numbers per cell, (iii) it is not neutralized by human sera, and (iv) it can be easily tested in murine models of human disease.

MATERIALS AND METHODS

Viruses and cells. Vaccine EHV-1 strain RacH (24) or RacH virus expressing enhanced green fluorescent protein (EGFP) was grown on RK13 cells (Fig. 1). The virus expressing EGFP lacks the glycoprotein gp2 gene (gene 71) and was termed HΔgp2 (49). As a control, EGFP-expressing BHV-1 was used (55). Infectious units (IU) were determined using supernatants from infected RK13 cells collected at 24 h postinfection (p.i.). Serial 10-fold dilutions of supernatants were prepared and added to RK13 plated in six-well plates (Falcon), and virus-containing solutions were replaced with medium containing methylcellulose 2 h p.i. as described earlier (37). Human cell lines were the $CD4^+$ T-cell line MT4 (MRC ARP017), the melanoma cell line MeWo (ATCC HTB-65), the hepatocellular carcinoma cell line Huh7 (ATCC CCL-185), the kidney carcinoma cell line 293T (ATCC CRL-1573), the lung carcinoma cell line H1299 (ATCC CRL-5803), and the cervix carcinoma cell line HeLa (ATCC CCL-2). We also used Madin-Darby bovine kidney cells (MDBK) (ATCC CCL-22), Madin-Darby canine kidney cells (MDCK) (ATCC CCL-34), feline embryonic cells (FEC RIE138; Cell Line Collection Insel Riems), porcine kidney cell line PK15 (ATCC CCL-33), quail muscle QM7 cells (ATCC CRL-1962), and primary chicken embryo cells. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI medium supplemented with 10% fetal calf serum (FCS) (Gibco-BRL). Primary human, porcine, bovine, or equine peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll (Histopaque; Sigma). PBMC were washed twice in phosphate-buffered saline (PBS) and suspended in RPMI-10% FCS. PBMC were either left untreated or stimulated with concanavalin A (ConA) at a concentration of 5 μg per ml (2).

Genetic manipulations. Recombinant EHV-1 viruses expressing human immunodeficiency virus (HIV) $Pr55^{gag}$ were generated. Both authentic *gag* (*wtgag*) and a synthetic version of the $Pr55^{gag}$ open reading frame (ORF) (*syngag*) was used, in which AT-rich sequences of the authentic wild-type ORF (*wtgag*) were replaced with nucleotides, thereby employing a codon usage that occurs most frequently in highly expressed mammalian genes (12, 20). Both the *wtgag* (as a syngeneic negative control) and *syngag* sequences were inserted into recombinant HΔgp2 instead of the EGFP gene under the control of the HCMV immediate-early (HCMV-IE) promoter/enhancer by conventional homologous recombination in RK13 cells using recombinant plasmid pFL*wtgag* or pFL*syngag*, which contain the HCMV-IE promoter/enhancer (flank 1), *wtgag* or *syngag* sequences, and poly(A) sequences (flank 2) (Fig. 1). Flanks 1 and 2 were obtained by performing a standard PCR using primers 1a (5′-GGAATTCTCCCACTCGTA TCGTCGGTC-3′) and 1b (5′-GATCGGTACCTAGCGCTAGCGGATCTGA CG-3′) for flank 1. The primers bind in the mini-F (pHA2) (1) sequence and contain EcoRI and KpnI restriction enzyme sites. The PCR amplification product for flank 2 was generated using primers 2a (5′-GACTCGAGCATGCGAT CATAATCAGCCATACCA-3′) and 2b (5′-GAGTCGACAAGCTTACACAG

TABLE 1. Groups of BALB/c mice immunized by various routes[a]

Group	Antigen	No. of IU	Route[c]
1	HΔgp2-syngag-1	4×10^5	i.nas.
2	HΔgp2-syngag-1	4×10^4	i.nas.
3	HΔgp2-syngag-1	4×10^2	i.nas.
4	HΔgp2-wtgag	4×10^4	i.nas.
5	HΔgp2-syngag-1	4×10^4	i.v.
6	HΔgp2-syngag-1	4×10^4	i.m.
7	HΔgp2-syngag-1	4×10^4	i.p.
8	HΔgp2-syngag-1	4×10^4	s.c.
9	HΔgp2-syngag-1	4×10^2	s.c.
10	HΔgp2-wtgag	4×10^4	s.c.
11[b]	HΔgp2/HΔgp2-syngag-1	4×10^4	i.nas.
12	HΔgp2	4×10^4	i.nas.
13	None		

[a] Mice were immunized three times at 4-week intervals with the indicated viruses. Splenocytes from three mice were pooled, and numbers of IFN-γ-secreting $CD8^+$ cells were determined as outlined in Materials and Methods.

[b] The first immunization in this group was done with HΔgp2, the following booster immunizations with HΔgp2-syngag-1.

[c] i.nas., intranasal immunization; i.m., intramuscular immunization; s.c., subcutaneous immunization; i.v., intravenous immunization; i.p., intraperitoneal immunization.

GAGGAGTCTAACAG-3′). While primer 2a binds at position 128637 of the EHV-1 genome (54), primer 2b has its target sequence also in pHA2. Flank 2 was cloned into vector pCR2.1-TOPO (Invitrogen). Flank 1 was inserted into vector pTZ18R (Amersham) and fused with *wtgag* or *syngag* sequences released from plasmids p-wtgag and p-syngag (20) by KpnI and BamHI. Finally, flank 2 was released from pCR2.1-TOPO with XbaI and SalI and inserted behind *wtgag* and *syngag* sequences, ultimately resulting in recombinant plasmids pFL-*wtgag* and pFL-*syngag*. Recombinant EHV-1 viruses were obtained by cotransfections of HΔgp2 DNA and the pFL-wtgag or pFL-syngag into RK13 cells and picking of nonfluorescing virus plaques. Recombinant viruses were plaque purified to homogeneity exactly as described previously (49).

(Immuno)fluorescence analysis and flow cytometry. For fluorescence analysis, cells were grown in 24- or 6-well plates and subsequently infected with EGFP-expressing HΔgp2. Expression of EGFP and binding of fluorochrome-conjugated secondary antibodies were visualized using inverted fluorescence microscopes (Zeiss Axiovert and Olympus) or flow cytometry (FACScan; Becton-Dickinson), and virus-infected cells were transferred to a U-bottom 96-well microtiter plate (4×10^4 cells/well). Monoclonal antibodies directed against various human CD markers (anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD19; all antibodies from Dako) were added for 30 min on ice, and cells were washed twice with PBS. Alexa^{546} goat antimouse IgG conjugate (Molecular Probes) was added for 30 min. After two additional washes with PBS, cells were resuspended in 100 μl of PBS and analyzed by flow cytometry (at least 5,000 cells/sample) (2).

Western blotting. Approximately 10^6 RK13 cells were infected with the indicated viruses with 1 IU per cell, and cell lysates were prepared 24 h later. Samples were adjusted to 1 mg/ml protein (BCA protein assay; Pierce) and mixed with buffer containing 5% 2-mercaptoethanol (38). The samples were heated to 56°C for 2 min, and proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis. Western blotting was done exactly as described earlier using anti-gM monoclonal antibody (MAb) E2 and anti-Gag MAb 13/5 (20, 49). Antibody binding was visualized using antimouse or antirabbit IgG peroxidase conjugate (Jackson ImmunoResearch), followed by ECL detection (Pharmacia-Amersham).

Animal experiments. BALB/c or C57/black mice were inoculated by various routes (intramuscular, intravenous, intraperitoneal, subcutaneous, intranasal) with 2×10^2 to 2×10^5 IU of EHV-1 (in 20 μl) after ether anesthesia (37). Virus was prepared from supernatants of RK13 cells at 24 h p.i. after removal of cellular debris using low-speed centrifugation (10,000 × *g*, 10 min). In some experiments, mice were killed at 1 to 14 days after instillation, and lungs were removed and immediately examined in situ for expression of EGFP. Lungs were then shock-frozen in liquid nitrogen, and thin sections were prepared. Organs remained unfixed to avoid loss of the EGFP fluorescent signal. Lung sections were scanned by light and fluorescence microscopy. Pictures were taken with a digital camera (Olympus).

In immunization experiments, BALB/c mice were inoculated with virus purified as described above for a total of three times at 4-week intervals (Table 1). The most concentrated inoculum used for immunizations were also tested for the presence of HIV Gag with a p24-specific enzyme-linked immunosorbent assay to exclude the presence of immunogenic amounts of p24 in the sample (12). The highest concentration of p24 in samples containing 4×10^5 HΔgp2-syngag-1 was 6.2 ng. All other samples either contained at least 10-fold-lower amounts of p24 or undetectable levels of Gag protein. Seven days after the third immunization, spleens were recovered. To determine HIV Gag-specific $CD8^+$ T cells, a protocol for intracellular gamma interferon (IFN-γ) staining was performed. Briefly, spleens from three animals per group were homogenized, and splenocytes were purified by Ficoll gradient centrifugation. Cells (2×10^7 per ml) were resuspended in RPMI 1640 containing 10% FCS, and 100 μl was placed in one well of a 96-well flat-bottom plate (Nunc). For specific stimulation, cells were incubated for 6 h with 2 μg of peptide A9I (AMQMLKETI). Ten micrograms of brefeldin A was added to prevent secretion of IFN-γ. Unstimulated cells (RPMI) and cells stimulated with an irrelevant peptide (gp120 V3 peptide, RIQRG PGRAFVTIGKI) served as negative controls. After stimulation, cells were centrifuged at 1,200 × *g* and suspended in 100 μl PBS-1% FCS and transferred to 96-well round-bottom plates (Nunc). Fcγ III/II receptors were blocked for 10 min at 4°C using 1 μl per well rat anti-mouse CD16/CD32 (BD Pharmingen). Following blocking, 10 μl of an anti-mouse CD8a antibody conjugated with allophycocyanin (BD Pharmingen) was added and incubated at 4°C for 30 min in the dark. Cells were washed twice, resuspended in 4% paraformaldehyde-1% saponin, and washed twice with PBS-0.1% saponin. Intracellular IFN-γ was detected by incubation for 30 min at 4°C in the dark using 2 μl of an anti-IFN-γ phycoerythrin conjugate (BD Pharmingen) suspended in 100 μl PBS-0.1% saponin. After two final washes with 200 μl PBS-0.1% saponin, cells were resuspended in PBS-1% FCS and analyzed in a FACScalibur (BD). Lymphocytes were gated using forward and side scatters, and 30,000 $CD8^+$ cells were recorded, from which the number of IFN-γ-positive cells was determined. Tests were run in duplicate, and results are given as means of two experiments.

Virus neutralization assays. Virus suspensions containing 10 or 100 IU in DMEM-10% FCS were incubated for 1 to 4 h at 37°C with human antisera containing high titers of 3,601 to 5,765 units of varicella-zoster virus (VZV)-, HSV-1-, HCMV-, or EBV-specific antibodies as determined by commercially available kits (HSV-1, VZV, and HCMV, Chiron-Behring; EBV, Biotest). Controls were equine or murine sera containing anti-EHV-1 antibodies (42). The virus-antibody mixtures were added to RK13 cells and incubated for 48 to 96 h at 37°C before cytopathic effects were analyzed by light microscopy.

RESULTS

EHV-1 efficiently delivers a transgene into cells of various origins. It has previously been shown that EHV-1 can efficiently infect and replicate in cells of equine and rodent origin. Substrates for growth of EHV-1 are primary equine cells derived from nasal epithelium, lung, thyroid gland, and skin. In addition, EHV-1 strains have been shown to grow to high titers in rabbit kidney RK13 cells, mouse L-M cells, and baby hamster kidney BHK-21/C13 cells (24, 39, 40, 52). We explored the ability of an EHV-1 expressing EGFP under control of the HCMV immediate-early promoter/enhancer (Fig. 1) to infect cells of avian, bovine, porcine, canine, feline, and primate origin. EHV-1 efficiently entered cells of all these species, and between 6 and 90% of chicken embryo, quail QM7, canine MDCK, feline embryonic, and human HeLa cells expressed EGFP at 24 h p.i. with 1 IU per cell (data not shown). Similarly, the human cell lines MT4, Huh7, 293T, H1299, and MeWo, as well as bovine MDBK and porcine PK15, were shown to express the transgene at 24 h p.i. (Fig. 2). In contrast, EGFP-expressing bovine BHV-1, a close relative of EHV-1, was virtually unable to infect cells of nonbovine origin (data not shown). It must be noted that EHV-1 was unable to directly spread from cell to cell or to be released from human MT4, MeWo, 293T, or H1299 cells, as analyzed by virus titrations of supernatants of infected cells at various times p.i. and plaque formation experiments using solid-phase overlays. In contrast, cell-to-cell transmission including syncytium forma-

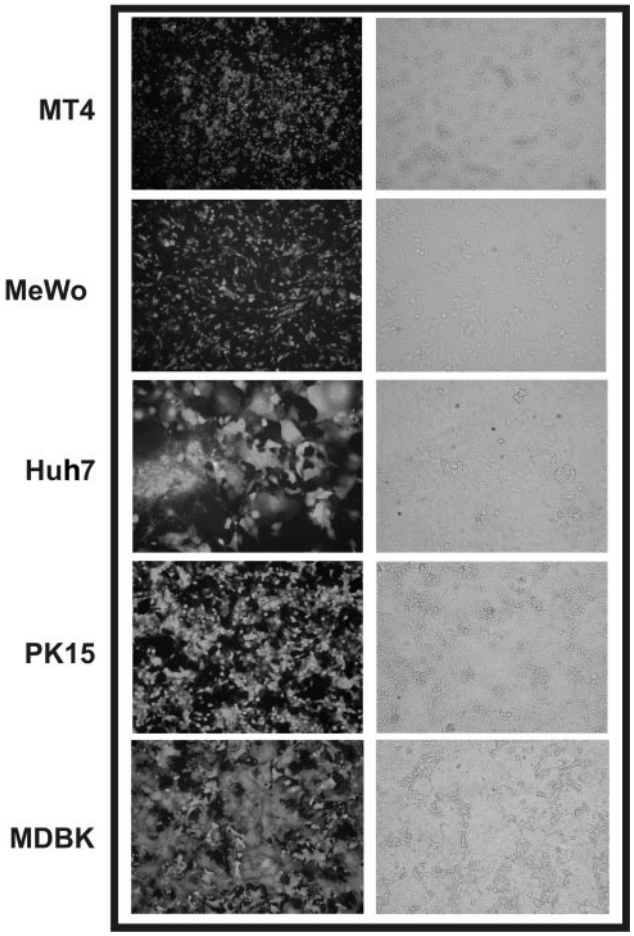

FIG. 2. Fluorescence analysis of human (MT4, MeWo, Huh7), porcine (PK15), and bovine (MDBK) cell lines after infection with HΔgp2 (1 IU per cell). EGFP expression was detected in more than 90% of the cells independently of the cell type. Formation of syncytia was observed in HΔgp2-infected Huh7 and MDBK cells.

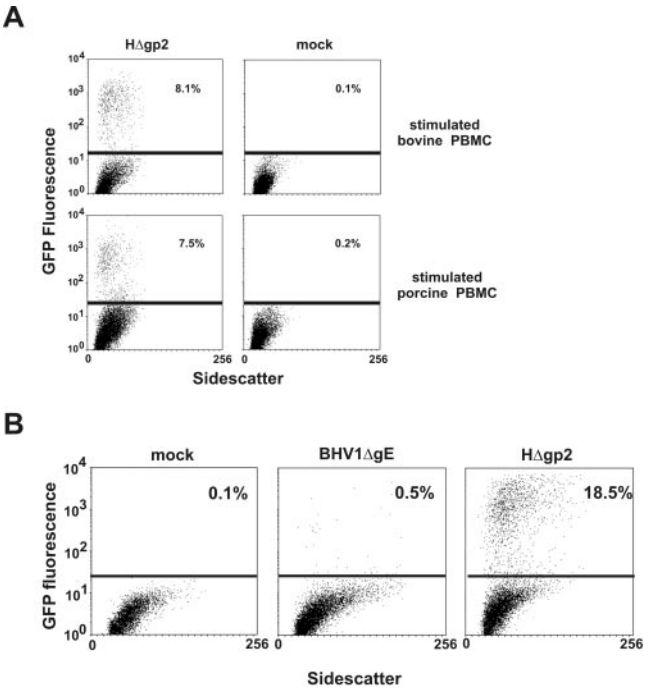

FIG. 3. (A) Flow cytometry of transduced cells. Shown is a dot plot analysis of ConA-stimulated bovine (upper panels) and porcine (lower panels) PBMC at 24 h after inoculation of cells with EHV-1 HΔgp2 (1 IU per cell). In comparison to mock-inoculated cells, expression of EGFP could be detected in 8.1% and 7.5% of stimulated bovine and porcine PBMC, respectively. (B) Flow cytometry of transduced cells. Shown is a dot plot analysis of ConA-stimulated human PBMC at 24 h after inoculation of cells with EHV-1 HΔgp2 or BHV-1ΔgE (1 IU per cell). While 18.5% of the cells were positive for EGFP expression after transduction using HΔgp2, less than 0.5% of the BHV-1ΔgE-inoculated PBMC exhibited green fluorescence.

tion and virus release was observed in HeLa cells (data not shown) or Huh7 cells (Fig. 2).

EHV-1 efficiently expresses a transgene in porcine and bovine PBMC. In the next series of experiments, we investigated the capacity of EHV-1 to enter primary cells obtained from peripheral blood of nonequine mammals. Venous blood was collected from horses, pigs, or cattle and infected with 1 IU per cell. Whereas approximately 0.5% of unstimulated PBMC of these species were expressing EGFP upon EHV-1 inoculation, approximately 8% of bovine and porcine PBMC were EGFP positive at 24 h p.i. when cells had been stimulated with ConA for 24 or 48 h (Fig. 3A). It is interesting that equine PBMC, which have been described to be a site of both lytic infection and latency of EHV-1 (62), exhibited a similar susceptibility to EHV-1 infection as was determined for PBMC isolated from the other species, and 15% were EGFP positive (data not shown). From the results we concluded that EHV-1 is able to efficiently enter PBMC of other mammals. As was the case for cultured cells, BHV-1 was not able to enter PBMC of equine or porcine origin, as reflected by the absence of EGFP-expressing cells after addition of virus to stimulated or unstimulated PBMC (data not shown).

EHV-1 efficiently transduces human PBMC. The above results on the ability of PBMC of other species to be transduced by EHV-1 were very surprising. Therefore, PBMC were collected and cultured in the absence or presence of ConA. It could be shown that PBMC of a total of four individuals could be infected with EHV-1 using 1 IU per cell. Transduction and EGFP expression were more effective if PBMC were stimulated for 24 h using ConA (Fig. 3B). As determined by flow cytometry, approximately 1% of PBMC were transduced with EHV-1 in nonstimulated cultures, and approximately 18% of PBMC were EGFP positive at 24 h after inoculation of ConA-stimulated cells. BHV-1 was not able to transduce either stimulated or nonstimulated human PBMC. From these results and those described above, we concluded that EHV-1 can efficiently transduce cell lines of various origins and that it is able to enter primary human PBMC at high efficiency.

EHV-1 mediates gene transfer into human CD3$^+$, CD4$^+$, CD8$^+$, CD11b$^+$, and CD19$^+$ cells with similar efficiency. We next sought to identify the nature of human PBMC transduced by EHV-1. Human PBMC were stimulated for 24 h with ConA or left nonstimulated before 1 IU per cell was added. Cells were incubated at 24 h p.i. with specific cell surface markers, and flow cytometry was performed. It could be demonstrated

that both $CD4^+$ and $CD8^+$ human T lymphocytes and human $CD1b^+$ cells grown out from the suspension culture (monocytes, macrophages, granulocytes) were efficiently transduced using EHV-1 as demonstrated by the appearance of double-labeled cells (detection of the respective differentiation molecules on the surface of EGFP-expressing cells), and approximately 13 to 23% of cells of each of the tested phenotypes were expressing EGFP (Fig. 4A). One explanation for the apparently lower number of transduced $CD3^+$ cells (19.4%) compared to $CD4^+$ (22.9%) and $CD8^+$ (19.9%) cells may be that individual gating procedures were applied, because cell populations showing high and low expression of the respective surface makers were identified and gates had to be adjusted independently for each of the markers (Fig. 4A). In addition, it is worthwhile to note that expression of EGFP was readily detected in approximately 11% of $CD19^+$ human cells (B lymphocytes) (data not shown). Furthermore, the $CD4^+$ MT4 cell line also could be transduced at very high efficiency (Fig. 4B). From the results we concluded that EHV-1 is able to transduce human PBMC expressing various CD molecules on their cell surface after stimulation with ConA using less than 1 IU, i.e., 100 virus particles per cell (37).

Efficient transgene expression in vivo by EHV-1. For application of the newly developed vector in vaccinations or gene therapy applications, expression of the inserted transgene(s) mediated by the vector in vivo is imperative. Therefore, expression of EGFP in vivo after intranasal instillation of 2×10^4 IU of EHV-1 into mice of various genetic backgrounds was assessed. From day 1 to day 12 after intranasal application, EGFP expression in lungs of both BALB/c and C57/black mice was readily observed. Multiple regions that exhibited high levels of autofluorescence were identified when lungs were screened under the fluorescence microscope immediately after removal from the bodies (Fig. 5A). In thin sections, high-level EGFP expression was observed in numerous cells of bronchioli and alveolae. Expression of the transgene in airway epithelia was detected until day 12 after application of the vector and confirmed the above-documented observations on native organs (Fig. 5B). It is important to stress that none of the mice having received the EGFP-expressing EHV-1 vector showed any sign of illness during the observation period, because an avirulent and genetically attenuated virus was used. These results indicated that EHV-1 is able to efficiently transduce cells of nonequine origin in vivo without causing any clinical symptoms.

The HIV $Pr55^{gag}$ precursor is efficiently expressed by HΔgp2 and induces Gag-specific IFN-γ-secreting $CD8^+$ cells. The potential of EHV-1 as a vector for delivery of a vaccine antigen was tested by the expression of the HIV $Pr55^{gag}$, and engineered vectors expressing the potent inducer of an HIV cytotoxic-T-lymphocyte (CTL) response were then inoculated into BALB/c mice. It has been shown previously that Rev- and Rev response element-independent expression of HIV Gag requires codon optimization of the ORF, such that nuclear export of the transcribed mRNA in the absence of this virus-specific mechanism becomes possible (12, 20). Therefore, recombinant EHV-1 expressing either a synthetic codon-optimized version of the $Pr55^{gag}$ ORF (*syngag*) or the authentic wild-type ORF (*wtgag*) was generated and tested. Analyses of the growth kinetics of the recombinant viruses expressing HIV

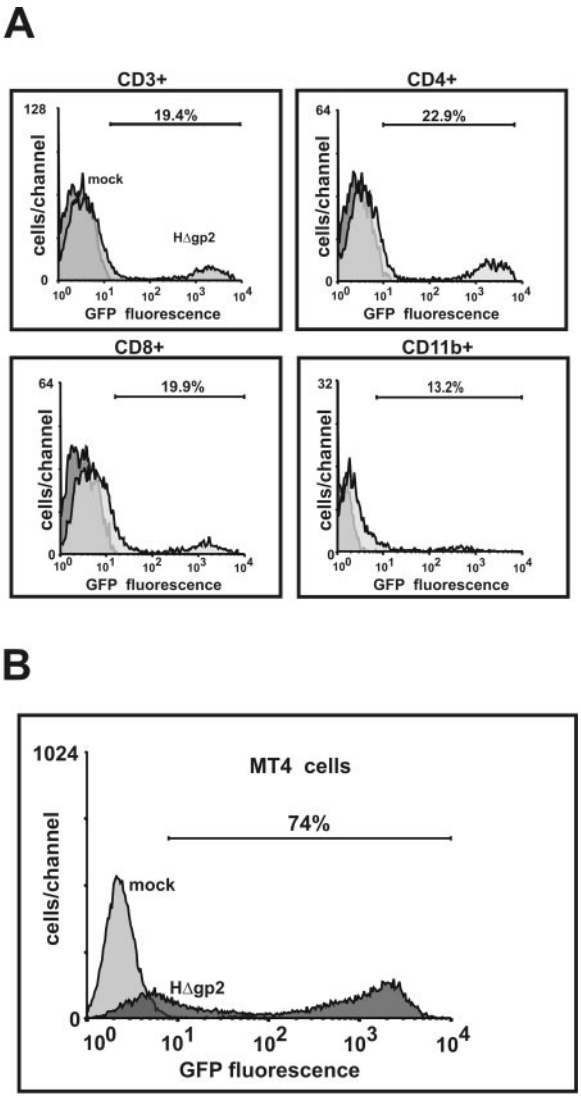

FIG. 4. (A) Flow cytometric analysis of ConA-stimulated human PBMC at 24 h after inoculation of cells with EHV-1 HΔgp2 (1 IU per cell). Following inoculation, cells were stained by indirect immunofluorescence using monoclonal antibodies specific for CD3, CD4, CD8, or CD11b and an $Alexa^{546}$-conjugated goat antimouse IgG antibody (Molecular Probes). Cell clusters positive for CD3, CD4, CD8, or CD11b were gated using the red fluorescence channel and analyzed for EGFP expression in the green fluorescence channel. Histogram analysis revealed that between 13.2% and 22.9% of the analyzed PBMC populations were positive for EGFP expression. The percentages of green-fluorescing $CD4^+$ and $CD8^+$ T cells were virtually identical. (B) Flow cytometric histogram analysis of human $CD4^+$ MT4 cells at 24 h after inoculation of cells with EHV-1 HΔgp2 (1 IU per cell). Approximately 74% of the HΔgp2-inoculated MT4 cells were positive for expression of EGFP compared to mock-transduced control cells.

$Pr55^{gag}$ revealed that no reduction in virus growth properties was observed when wild-type or synthetic HIV $Pr55^{gag}$ sequences were present (data not shown). Expression of $Pr55^{gag}$ by the mutant RacH viruses was analyzed by indirect immunofluorescence and Western blotting using MAb directed against HIV Gag (12, 20). Only the insertion of the codon-optimized *syngag* resulted in $Pr55^{gag}$ expression from two independently isolated recombinant viruses as detected with

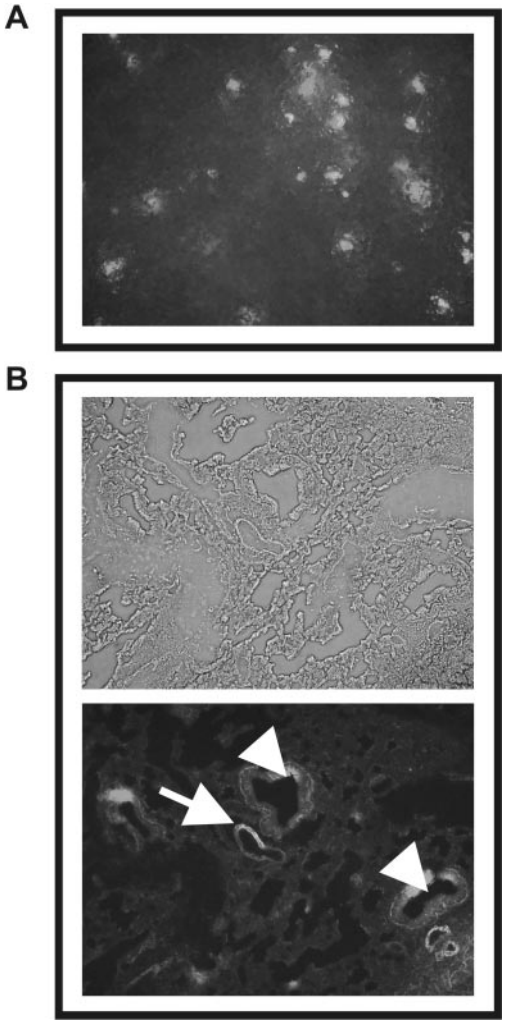

FIG. 5. EGFP expression in murine lungs at day 2 after intranasal application of 10^4 IU HΔgp2 per mouse. (A) Lungs were removed and immediately scanned in situ for EGFP expression. Magnification, ×100. (B) Thin section of a mouse lung after shock-freezing of lungs in liquid N_2. Lungs were left unfixed and unstained to maintain EGFP expression. The upper panel shows the light-microscopy image of the view shown in the lower panel under the fluorescence microscope. Note the expression of EGFP in cells of the bronchioli (arrowheads) and alveolae (arrow). Magnification, ×200.

Gag-specific MAb 13/5, whereas—as was expected—the engineered virus harboring *wtgag* sequences did not express the $Pr55^{gag}$ precursor (Fig. 6). These studies clearly demonstrated that EHV-1 can serve as a vehicle to efficiently express antigens of human viruses.

It has been shown in previous studies that HIV $Pr55^{gag}$ is a potent inductor of a $CD8^+$ CTL response (12, 59). Therefore, we examined whether the generated mutant EHV-1 viruses were able to induce such a response. It could be demonstrated that the recombinant EHV-1 expressing $Pr55^{gag}$ from the synthetic Gag ORF (HΔgp2-syngag-1) was able to induce Gag-specific IFN-γ-secreting $CD8^+$ cells (Fig. 7). When applied intranasally, an almost identical number of antigen-specific $CD8^+$ cells secreting the cytokine after stimulation with the Gag-specific A9I (12) peptide could be determined when mice

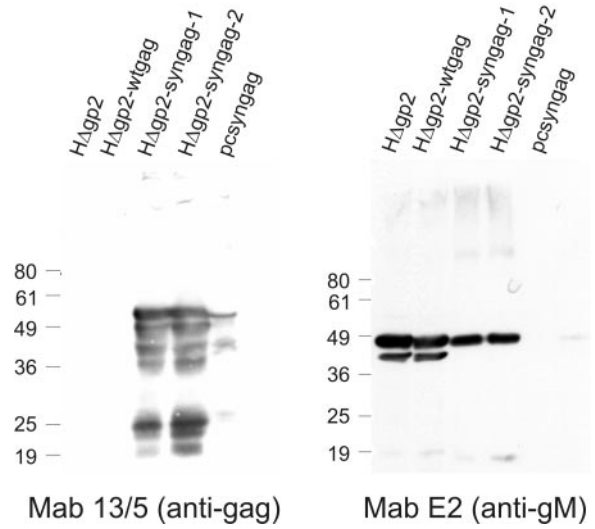

FIG. 6. Expression of *syngag* under the control of the HCMV-IE promoter/enhancer in cells infected with two independently isolated recombinant HΔgp2 viruses as detected by MAb 13/5 (left panel). No $Pr55^{gag}$ expression is detectable in an HΔgp2 virus harboring *wtgag* sequences. Transient transfection of *syngag* in the pcDNA3 vector (Invitrogen) was used as a control. The EHV-1-specific glycoprotein M (gM) MAb E2 revealed that identical protein amounts were loaded in each lane (right panel).

were immunized with 4×10^5 or 4×10^4 IU (Fig. 7, groups 1 to 3). As little as 4×10^2 IU given by the intranasal route resulted in 143 Gag-specific $CD8^+$ cells per 10^6 $CD8^+$ cells. Similarly, high frequencies of Gag-specific $CD8^+$ cells (between 150 and 490 Gag-specific $CD8^+$ cells per 10^6 cells) were observed after intravenous (group 5), intramuscular (group 6), intraperitoneal (group 7), and subcutaneous (groups 8 and 9) injection. In contrast to HΔgp2-syngag-1, the parental EHV-1

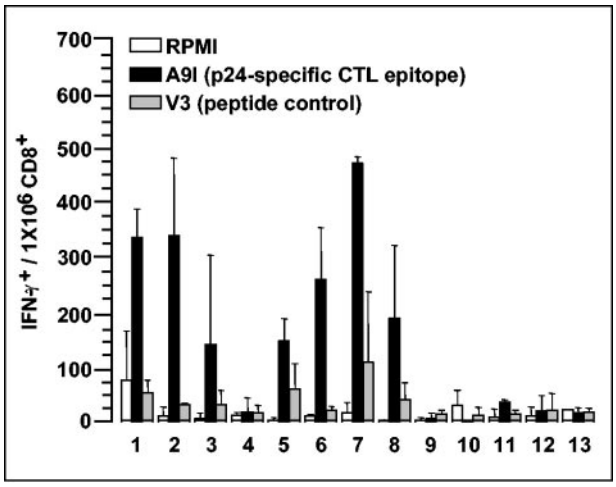

FIG. 7. Frequencies of Gag-specific $CD8^+$ cells. A total of 30,000 $CD8^+$ cells per group were recorded. The graph shows the number of IFN-γ-positive $CD8^+$ cells (normalized to 10^6 total cells) after stimulation of splenocytes with Gag-specific peptide A9I, an irrelevant V3-specific peptide, or without stimulation (RPMI). Groups and vaccination protocols are given in Table 1. Values are means of three stimulations done with the same cells.

TABLE 2. Neutralizing activity of human sera against EHV-1

Serum	Titer of antibody to strain (IU)[a]:				
	RacH (10)	RacH (100)	HΔgp2 (10)	HΔgp2 (100)	One-point concn/IFT[b]
Anti-HSV-1 A[b]	<1:4	<1:4	<1:4	<1:4	4,325
Anti-HSV-1 B[b]	<1:4	<1:4	<1:4	<1:4	4,895
Anti-HSV-1 C[b]	<1:4	<1:4	<1:4	<1:4	5,765
Anti-VZV A[b]	<1:4	<1:4	<1:4	<1:4	5,233
Anti-VZV B[b]	<1:4	<1:4	<1:4	<1:4	4,977
Anti-VZV C[b]	<1:4	<1:4	<1:4	<1:4	5,724
Anti-HCMV A[b]	<1:4	<1:4	<1:4	<1:4	3,829
Anti-HCMV B[b]	<1:4	<1:4	<1:4	<1:4	3,601
Anti-HCMV C[b]	<1:4	<1:4	<1:4	<1:4	5,103
Anti-EBV A[b]	<1:4	<1:4	<1:4	<1:4	Positive
Anti-EBV B[b]	<1:4	<1:4	<1:4	<1:4	Positive
Anti-EBV C[b]	<1:4	<1:4	<1:4	<1:4	Positive
Horse A[c]	1:256	1:64	1:256	1:128	
Mouse A[c]	1:320	1:160	1:1,280	1:320	

[a] Neutralization tests were performed using either 10 or 100 IU of the indicated viruses.
[b] Titers against human herpesviruses were determined using commercial test kits (HSV-1, VZV, HCMV, Chiron-Behring; EBV, Biotest). IFT, indirect immunofluorescence test.
[c] Sera were obtained from a horse after abortion and from an experiment in which mice were vaccinated with vaccine strain RacH and challenged with virulent EHV-1 strain RacL11.

(HΔgp2) or the virus harboring the authentic *gag* gene (HΔgp2-wtgag) was unable to induce an HIV Gag-specific $CD8^+$ response, irrespective of the route of administration (Fig. 7, groups 4, 10, 12).

EHV-1 is not neutralized by human sera containing high titers of antibodies against human herpesviruses. Finally, we addressed the question of neutralization of EHV-1 by sera containing high titers of antibodies directed against the human alphaherpesvirinae HSV-1 or VZV, as well as the human betaherpesvirus HCMV and the human gammaherpesvirus EBV. In addition, sera of three investigators routinely working with EHV-1 were tested for neutralizing activity. Sera were inactivated for 30 min at 56°C or were left untreated, and 100 or 10 IU of parental EHV-1 strain RacH or the EGFP-expressing mutant HΔgp2 virus (Fig. 1) in 100 μl was incubated with twofold dilutions (100 μl) of human sera in DMEM for 1 to 4 h at 37°C. The virus-serum mixtures were then added to RK13 cells. In none of the human sera could any neutralizing activity against EHV-1 be detected, even if the lowest serum dilutions (1:4) and only 10 IU of virus were used (Table 2). In contrast, murine or equine sera immunized once with EHV-1 strain RacH (43) readily neutralized 10 or 100 IU of both RacH and HΔgp2, exhibiting titers of 1:64 to 1:1,280 (Table 2). From these results we concluded that EHV-1 cannot be neutralized by human sera, even if they contain high titers against human herpesviruses. In addition, constant exposure of humans of both genders to EHV-1 under laboratory conditions did not result in induction of EHV-1-specific antibody titers.

DISCUSSION

In this report, we have explored the ability of EHV-1, an alphaherpesvirus, to enter and express transgenes in cells of avian and mammal origin, including humans. Besides delivering the gene encoding EGFP in vitro and in vivo, we also asked whether functional expression of an immunogen, the HIV Gag precursor, can be achieved. Both EGFP expression and a specific cellular immune response to the HIV Gag protein were demonstrated, indicating that EHV-1 may serve as an alternative gene delivery platform for vaccination purposes.

It could be shown that EHV-1 is able to enter virtually every cell type tested with high efficiency, and only one infectious unit, equaling approximately 50 to 100 particles per cell, is needed to efficiently introduce a marker gene into the cell of interest. Therefore, we have accumulated data on the capability of EHV-1 to be used as a novel vector for immunizations and gene therapy. In some cell types, EHV-1 led to the establishment of nonlytic infection, because marker gene expression could be propagated by continuous passage of infected cells without the de novo production of progeny virus as demonstrated by the absence of infectious virus in supernatants of cells harboring EHV-1 and expressing the transgene. EHV-1 is known to replicate in a variety of rodent cells in vitro; however, natural infection in vivo occurs in *Equidae* only (40). The same observation of restricted in vivo host ranges was made for a number of *Alphaherpesvirinae*, including close relatives of EHV-1, namely HSV-1, HSV-2, VZV, and BHV-1. The reason for this property of the individual members of the virus subfamily is not known; however, natural epithelial barriers at the ports of entry are thought to allow infection of the target species only (47, 51). It is worthwhile to note that EHV-1 was hardly able to infect MDCK cells grown in a monolayer but efficiently infected these cells in suspension (data not shown). This may indicate a preference of EHV-1 for infection of nonpolarized cells. The ability of EHV-1 to infect a wide variety of cells from different species and tissues was very surprising. Not only could we detect infection of various cell lines of vertebrate origin, including avian (chicken and quail), bovine, porcine, canine, feline, and human cells, we were also able to demonstrate efficient expression of a marker transgene, that of the EGFP under the control of a heterologous promoter, in primary stimulated and also unstimulated peripheral blood cells after transduction with EHV-1. Although marker gene transfer with EHV-1 was higher in ConA-stimulated as opposed to nonstimulated PBMC, transduction efficiencies of

1% in nonstimulated cultures would require at least 50-fold-higher multiplicities of infection when using, e.g., adenoviruses (19). In addition, the presented EHV-1 expression system can be used in several different ways and can easily be tested in murine model systems, because EHV-1 was shown to efficiently express the transgene in vivo.

The above results prompted us to investigate whether an immune response against a well-characterized antigen, the HIV Gag precursor, could be achieved after immunization with engineered EHV-1. In fact, a remarkable number of antigen-specific $CD8^+$ cells were detected in animals immunized with HΔgp2-syngag-1, the recombinant EHV-1 that expressed a synthetic codon-optimized version of the HIV Gag precursor. No such response was observed using an EHV-1 that expressed Gag from the authentic sequence, indicating that efficient priming of the immune system by recombinant EHV-1 vectors harboring foreign genes is possible. While direct comparisons, especially between different assays, are difficult, the number of Gag-specific $CD8^+$ cells reported using EHV-1 as a delivery vehicle appeared to be in the same range as that observed after vaccination using influenza viruses, poxviruses, or rhabdoviruses as the gene delivery vector (27, 29, 30, 36), a more efficient response may be possible if various routes for primary and booster vaccinations are used, or if prime-boost regimens using DNA and virus formulations are applied. In addition, only a weak CD8-specific response was observed in animals that had been primed intranasally with HΔgp2 virus and boosted twice with the $Pr55^{gag}$-expressing virus by the same route (Fig. 7, group 11). This finding may suggest effects of antivector immunity based on secretory IgA that appear to be at least partially responsible for anti-EHV-1 immunity in mice, especially when intervals between immunizations are short (64). If antivector immunity is indeed a factor, the numbers of Gag-specific $CD8^+$ cells determined after repeated application of HΔgp2-syngag-1, particularly by the intranasal route, may reflect only those induced by the primary immunization. Therefore, various prime-boost protocols using *syngag* DNA vaccines (12, 44) and HΔgp2-syngag-1 as the immunogens are currently under investigation. One might argue that expression of $Pr55^{gag}$ results in production of virus-like particles, which would be present in the inocula. We can also exclude that successful vaccination of mice using HΔgp2-syngag-1 was caused by a mere adjuvant effect of the vector, because only minute amounts of p24 of maximally 6 ng were present in the inocula, which is approximately 1,000-fold below the p24 amounts that have been used for successful vaccination using HIV Gag virus-like particles. As such, a specific anti-HIV CTL response to protein in the used inocula is highly unlikely (6, 7).

Besides the use of EHV-1 as an immunization vector in humans and animals, it might also be considered in the future for testing as an oncolytic vector. It has been shown here that human tumor cells, i.e., melanoma as well as kidney, cervix, and hepatocellular carcinoma cells, can be efficiently infected by EHV-1. Furthermore, it is conceivable that EHV-1 might be used for stable transduction of cells with large pieces of DNA in a variety of different cell types either for gene therapy purposes or for the in vitro application and the functional testing of murine or human BAC/PAC libraries (56-58). The high packaging capacity of EHV-1 and the fact that safe vectors can be generated by the construction of conditionally lethal mutants, which can be propagated on complementing/packaging cell lines only, are good arguments for the future use of the presented EHV-1 system as a tool for gene delivery and vaccination in humans (5, 38). Currently, we are focusing on expression of a number of immunogens in EHV-1 and on the construction of a modified vector that lacks the sole immediate-early gene, the ICP4 homologue of HSV-1. It has been shown that EHV-1 viruses lacking the ICP4 homologue are replication incompetent on noncomplementing cells and unable to express any early or late proteins de novo (15). The many advantages of replication-incompetent (herpesvirus) vectors have been described (8, 10, 11, 25, 60), and it is expected that use of the immediate-early negative EHV-1 vector further reduces the generation of an antivector immune response and may be an extremely safe vehicle for gene delivery.

The development of EHV-1 as a vector is, compared to e.g., HSV-1, still in its infancy. However, it may prove useful for immunization, because no antivector immunity in humans could be demonstrated and because EHV-1 has high DNA packaging capacity. In addition, EHV-1 can easily be manipulated using infectious genomes cloned in *Escherichia coli*. In the light of these advantages of the presented EHV-1 platform, it should be possible to exploit its properties to generate alternative and/or complementary approaches for already existing gene delivery vectors.

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