Safety and Efficacy of a Thymidine Kinase Negative Equine Herpesvirus-1 Vaccine in Young Horses

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ABSTRACT

A drug induced equine herpesvirus-1 (EHV-1) mutant lacking thymidine kinase inducing activity was developed and evaluated as a vaccine. The safety and effectiveness of the vaccine to protect against experimentally induced EHV-1 respiratory disease were evaluated in weanling horses free of EHV-1 neutralizing antibody. The vaccine was safe when administered either intramuscularly or intravenously, and EHV-1 was not shed intranasally during the 12 days following administration. Intranasal challenge with virulent EHV-1 was used to evaluate vaccine efficacy. Following challenge, there was a significantly ($p < 0.05$) greater increase in peak body temperatures and duration of nasal virus shedding in the nonvaccinates, and a significant ($p < 0.05$) increase in serum neutralizing antibody titers in the vaccinates.

RÉSUMÉ

Une souche mutante d'herpesvirus équin 1 (HVE-1) dénuée d'activité thymidine kinase a été développée et évaluée comme vaccin. La sécurité et l'efficacité du vaccin contre la forme respiratoire de HVE-1 ont été évaluées chez des poulains de cinq à sept mois d'âge n'ayant pas d'anticorps circulant neutralisant pour l'HVE-1. Le vaccin s'est démontré sécuritaire lorsqu'administré par voie intraveineuse ou intramusculaire, et HVE-1 ne fut pas isolé des muqueuses nasales dans les 12 jours ayant suivi l'inoculation du vaccin. L'efficacité du vaccin a été évaluée suivant l'administration d'une souche virulent de l'HVE-1. Lorsque comparés aux poulains vaccinés, les poulains non-vaccinés présentèrent une augmentation plus marquée ($p < 0.05$) de la température rectale et de la durée d'excrétion virale. Les poulains vaccinés montrèrent une augmentation plus marquée ($p < 0.05$) des titres d'anticorps lorsque comparés aux poulains non-vaccinés.

INTRODUCTION

Equine herpesvirus-1 (EHV-1), commonly called equine rhinopneumonitis virus, is associated with respiratory disease, abortion, perinatal foal mortality and posterior paresis. Currently available vaccines incorporate modified live viruses (MLV) or inactivated viruses (1-3). With repeated administration, these vaccines, like repeated EHV-1 infections, result in protective immunity against the respiratory disease (4). However, immunity against viral replication in the upper respiratory tract, cell-associated viremia and the abortigenic and neuropathological consequences is transitory with either repeated vaccination or natural exposure (5). The critical need for a safer and more effective vaccine is indicated by continued epizootics of EHV-1 induced respiratory disease and the sporadic occurrence of the abortigenic and neuropathological consequences despite intensified vaccination programs.

The recognition of two genetically distinct EHV-1 subtypes (1-fetal and 2-respiratory) has helped to clarify the variations seen in clinical respiratory syndromes and to explain the occurrence of the abortigenic and neurological forms of the disease (6). Although restriction endonuclease analysis has revealed significant genetic differences between the two subtypes (7), both can cause respiratory disease and it is believed that appreciable cross-protection occurs between subtypes following repeated infection (6,8).

The selective use of mutagenic agents led to the emergence of thymidine kinase negative (TK^-) mutants of herpes simplex virus (9). These mutants had reduced virulence and ability to establish latency in mice, while maintaining immunogenicity and viability (10). Thymidine kinase negative mutants for infectious bovine rhinotracheitis (IBR) and pseudorabies virus (PRV) have been shown to be stable after passage in cattle and swine, respectively (11,12). The TK^- IBR vaccine was safe in pregnant cows and prevented respiratory disease in animals challenged with virulent IBR virus (11). Safety and effectiveness of a TK^- PRV vaccine in young swine and pregnant sows has also been reported (12,13).

The purpose of this study was to evaluate the safety of a drug induced TK^- EHV-1 vaccine, and to determine its capacity to prevent or ameliorate

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the clinical expression of experimentally induced rhinopneumonitis.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The experiments conformed to the Guide to the Care and Use of Experimental Animals, Canadian Council on Animal Care. Fourteen weanling horses (five to seven months of age), free of EHV-1 serum neutralizing antibody (SNA), were used to determine safety and efficacy of the TK⁻EHV-1 vaccine. They were maintained together on pasture, and fed free choice Coastal-Bermuda hay and 2.5 kg of 14% protein grain ration per animal twice daily. Water was available *ad libitum*.

EXPERIMENTAL DESIGN

To assess vaccine safety, the horses were divided into two groups. Group I, eight horses, received 2 mL of vaccine containing 7 x 10^8 plaque-forming units (PFU) either intramuscularly (six horses) or intravenously (two horses). Group II, six horses, received 2 mL of Eagle's minimum essential medium (EMEM) (Gibco, Grand Island, New York) intramuscularly. The vaccine and placebo were administered in a double blind fashion.

To evaluate vaccine efficacy, these same horses were divided as follows: Group I, the eight horses that had been vaccinated either intramuscularly or intravenously; Group IIa, four horses that received placebo (challenge control); and Group IIb, two animals that received placebo (contact control). Animals in groups I and IIa were inoculated intranasally with virulent EHV-1 45 days after treatment. Group IIb animals were not inoculated with virulent EHV-1, and served as contact controls.

VIRUSES

The reference TK⁺EHV-1 (Army 183) subtype 1 virus (14) was propagated by intra-amniotic inoculation of a pregnant pony mare with 2 mL of a 20% suspension of EHV-1 infected equine fetal lung in EMEM. The fetus was spontaneously aborted six days after inoculation, and a 20% homogenate of EHV-1 infected fetal lung tissue in EMEM was prepared and stored in aliquots at -90°C for restriction endonuclease analysis and animal inoculation. This second passage was designated F120 and contained 1.5 x 10^6 median tissue culture infectious doses ($TCID_{50}$) mL. Isolate F1245 was from a ten month fetus that was spontaneously aborted 15 days after a pregnant mare was inoculated intranasally with a 20% fetal lung suspension (F120) containing TK⁺EHV-1 (Army 183). Bovine isolate 9BSV4, an EHV-1, subtype 1, was recovered from a nasal swab of a 26 day old calf (15). Working stocks of 9BSV4 were prepared and titrated in bovine kidney (BK) cells. Thymidine kinase positive EHV-1 (RQ) is a cell culture attenuated strain of EHV-1, subtype 1 (16), derived from a commercial vaccine. Stocks of EHV-1 (RQ) were prepared by inoculating African green monkey kidney (Vero) cells at an input multiplicity of infection of about 0.01 PFU per cell and incubating for about three days, at which time extensive cytopathic effects were observed. Plaque titrations were performed in Vero cells at 34.5°C, as previously described (17).

PREPARATION AND ANALYSES OF VIRAL DNA

Working stocks of EHV-1 (Army 183), and isolates F1245 and 9BSV4 were propagated only once in equine dermis (ED) cells for DNA isolations to minimize genetic changes in culture. The DNAs from these isolates, and from EHV-1 (RQ) and EHV-1 (H6b) infected cells were prepared by the Triton-100 method, as described (18). For restriction endonuclease analyses, viral DNAs were cleaved EcoRI and BamHI enzymes (New England Biolabs, Beverly, Massachusetts). The fragments were separated by electrophoresis on 0.6% agarose gels, stained with 0.5 μg/mL of ethidium bromide, visualized over a long-range UV-illuminator, and photographed (18).

VIRUS ISOLATION

Nasal swabs were collected on days 0 through 12 postvaccination (PV) and postchallenge (PC), and processed as previously described (19). Nasal swabs were thawed, agitated in a vortex mixer and centrifuged at 1900 x *g* for 30 min. The supernatant fluid was inoculated in 0.2 mL amounts onto Vero and BK cell cultures in triplicate and blind passaged once. Whole blood was collected in ethylenediaminetetraacetate (EDTA) tubes (Vacutainer Systems, Rutherford, New Jersey) on days 0 through 12 PV and PC. Peripheral blood leukocytes (PBL) were separated from 10 mL of EDTA blood by differential centrifugation with separation medium (Ficoll-Paque, Pharmacia, Piscataway, New Jersey) and washed twice in EMEM containing 2% fetal bovine serum (FBS). The PBL were cocultured on Vero and BK cell monolayers in triplicate. After five days incubation at 36°C, negative cultures were frozen, thawed and blind passaged once.

CELL CULTURES

Vero and BK cells were propagated in EMEM supplemented with 10% FBS, and with 200 IU/mL penicillin, 200 μg/mL streptomycin, 50 μg/mL gentamycin, and 2.5 μg/mL amphotericin B. Maintenance medium consisted of EMEM containing 2% FBS (19). Vero and RAB-9 (rabbit skin) cells for vaccine preparation were grown in EMEM supplemented with 10% FBS, 20 mM sodium bicarbonate plus 10 mM HEPES buffer, pH 7.3 (Sigma Chemical Co., St. Louis, Missouri), and 2 mM L-glutamine plus 0.005% neomycin. Bromodeoxyuridine (BrdUrd) resistant, TK⁻RAB (BU) cell mutants were cultivated in the same growth medium as the parental RAB-9 cells supplemented with BrdUrd (25 μg/mL), except for the passage preceding each experiment (20).

VACCINE

The TK⁻EHV-1 (H6b) mutant was isolated from TK⁺EHV-1 (RQ) by modifications of procedures previously described to obtain stable TK⁻mutants of vaccinia virus, herpes simplex virus, marmoset herpesvirus, bovine herpesvirus type 1, and pseudorabies virus (10-12, 17, 20). The strategy entails: (i) sequential virus passages in gradually increasing concentrations of one or more nucleoside analogs to induce mutations in the virus population and to enrich for TK⁻mutants and (ii) plaque purifications.

The EHV-1 (RQ) was propagated at low input multiplicity in Vero cells in medium containing 2.5 μg/mL, 5.0 μg/mL, 25 μg/mL and 100 μg/mL, respectively, of 1-B-D arabinofuranosylthymine (araT), and for one passage in BrdUrd (5 μg/mL) and then plaque purified in medium containing 100 μg/mL araT. The virus, designated "plaque H" was propagated in medium containing 100 μg/mL araT, 20 μg/mL BrdUrd, and plaque purified a second time in medium containing 20 μg/mL BrdUrd. The virus designated "plaque H6" was passaged three more times in 20 μg/mL BrdUrd. The virus designated "plaque H6b" was then propagated for one more passage in 20 μg/mL BrdUrd. The vaccine virus prepared in the absence of BrdUrd had a titer of about 3.5×10^8 PFU/mL.

ANIMAL INOCULATION

The horses were infected with TK⁺EHV-1 (Army 183) subtype 1, by intranasal inoculation with 2 mL of a 20% lung suspension containing 3×10^6 $TCID_{50}$. Administration of 1.0 mL per nostril was accomplished with a 3 French, 5-inch catheter (Sovereign Tomcat Catheter, Monoject Co., St. Louis, Missouri).

ASSESSMENT OF CLINICAL SIGNS

Rectal temperatures and the presence of nasal discharge were recorded for all horses ten days before and 12 days following both vaccine or placebo administration and viral challenge. Whole blood was collected in EDTA, and hemograms were performed sequentially before and after vaccine or placebo administration, and before and after viral challenge.

SERUM NEUTRALIZING ANTIBODY TESTS

Serum was collected from all horses and assayed for antibody to EHV-1, both pre- and postvaccination and before and after EHV-1 challenge. A microtiter test using Vero cell cultures and an RQ TK⁻strain of EHV-1 (subtype 1, RQ) was used to determine antibody titers (19).

STATISTICAL ANALYSIS

The Statistical Analysis System (SAS), using repeated measure analysis of variance (Proc GLM) (21), was used to compare rectal temperatures, and total PBL, neutrophil and lymphocyte counts between groups following vaccination and challenge. Total PBL, neutrophil and lymphocyte counts were evaluated using their logarithmic values.

Duration of viral shedding and nasal discharge, and serum neutralizing antibody (SNA) titers to EHV-1 were evaluated between groups on SAS using nonparametric tests. The median two-sample test was used to compare duration of viral shedding, and the Wilcoxon two-sample test was used to compare duration of nasal discharge and SNA titers. Serum neutralizing antibody titers to EHV-1 were evaluated between groups using a Wilcoxon (nonparametric) test. The level of significance for all analyses was $p < 0.05$.

Group IIb (contact controls) was not included in the statistical analysis between groups because of the indirect method of virus exposure.

RESULTS

The vaccine virus was an araT-resistant, BrdUrd-resistant, 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG) resistant EHV-1(H6b) mutant lacking TK inducing activity (17). Absence of TK inducing activity by EHV-1(H6b) was demonstrated by enzyme assays, and confirmed by autoradiography on glass slides and by thymidine plaque autoradiography (17). The TK⁻phenotype was stable. Reversion to TK⁺ did not occur even when EHV-1 (H6b) was propagated in TK⁺ selective, hypoxanthine-aminopterin-thymidine media (17).

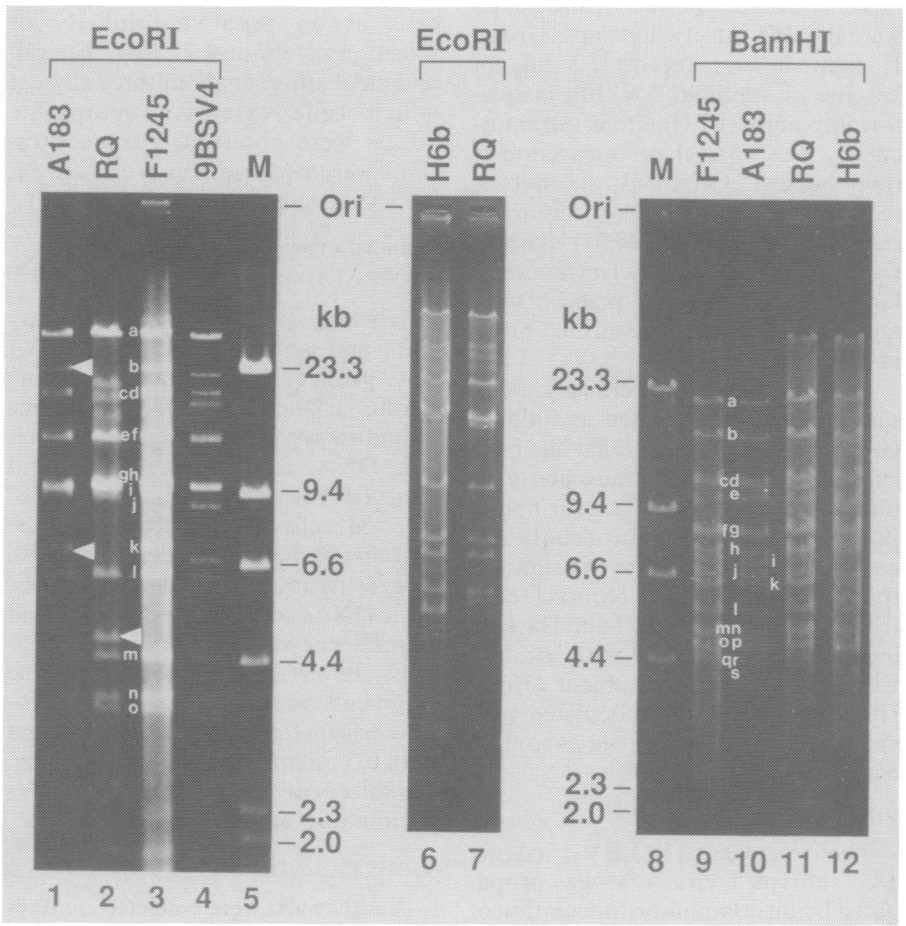

Fig. 1. Agrose gel electrophoresis analyses of DNAs from EHV-1 (Army 183), EHV-1 (F1245), EHV-1 (9BSV4), EHV-1 (RQ) and EHV-1 (H6b) after cleavage with restriction endonucleases EcoRI and BamHI. Marker (M) phage lambda fragments are shown in lanes 5 and 8. The arrows of lane 1 and 2 point to the EcoRI-b and EcoRI-k fragments of EHV-1 (Army 183) and the shortened EcoRI-k fragment of EHV-1 (RQ). Small letters adjacent to EcoRI fragments of EHV-1 (F1245) and the BamHI fragments of EHV-1 (F1245) and EHV-1 (Army 183) correspond to the nomenclature of Whalley *et al* (22). Ori is origin of electrophoresis. Direction of electrophoresis is from top to bottom.

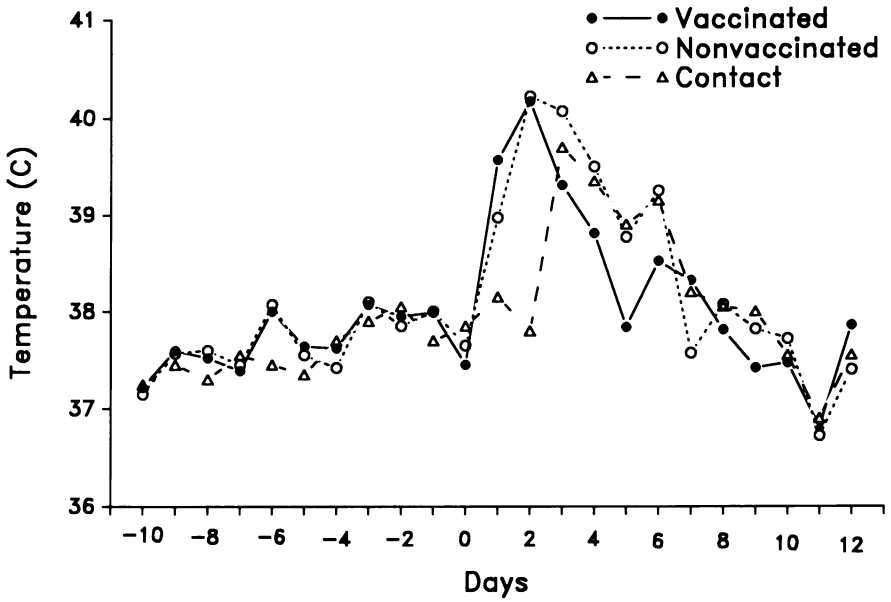

Fig. 2. Mean morning rectal temperatures of horses vaccinated with thymidine kinase negative EHV-1 (Group I), nonvaccinated controls (Group IIa), and nonvaccinated contact controls (Group IIb). Groups I and IIa were experimentally challenged intranasally with EHV-1 on day 0, and group IIb was exposed to groups I and IIa on day 0.

Restriction endonuclease fingerprinting analyses demonstrated that the restriction pattern of F1245 virus recovered from the aborted fetus was indistinguishable from that of the reference EHV-1 (Army 183) subtype 1 strain, as expected. The restriction patterns of the attenuated EHV-1 (RQ) strain and the EHV-1 (9BSV4) bovine isolate were similar, but not identical to that of the EHV-1 (Army 183) virus. The EcoRI-b, EcoRI-k and BamHI-d fragments of the EHV-1 (RQ) strain were approximately 5 kb, 2 kb and 1 kb smaller than the corresponding fragments of the EHV-1 (Army 183) strain (Fig. 1). Also, the BamHI-qrs multimolar band (about 4.2 kb) was not visible. The EcoRI-b, EcoRI-d, and the EcoRI-k fragments of EHV-1 (9BSV4) were likewise smaller than those of EHV-1 (Army 183). In addition, a 9 kb fragment, possibly representing a terminal EcoRI-j fragment of reduced size, was present in the EHV1-1 (9BSV4) pattern, but not in the EHV-1 (Army 183) pattern. The altered DNA fragments mapped in the terminally repeated and unique short regions of the EHV-1 genome (22), indicating that there were deletion mutations in these regions of the EHV-1 (RQ) and EHV-1 (9BSV4) genomes. Passage of the TK^+EHV-1 (RQ) strain in Vero cells in media with araT and BrdUrd to isolate the TK^-EHV-1 (H6b) mutant further altered the restriction endonuclease pattern of EHV-1 (RQ). Specifically, the BamHI-d fragment was missing and the EcoRI-o and BamHI-j fragments, which are located about 0.05-0.13 map units on the EHV-1 genome, were smaller in TK^-EHV-1 (H6b) than in the TK^+EHV-1 (RQ) strain (Fig.1). Thus, besides inactivation of the TK gene function, the sizes of restriction endonuclease fragments were altered following treatment with the BrdUrd mutagen and the araT and BrdUrd selection of the EHV-1 (H6b) strain.

Body temperatures of the experimental horses following vaccine or placebo administration showed no significant fluctuations from baseline. No clinical evidence of respiratory disease was exhibited by either group. All blood values remained within normal limits during the 12 day monitoring period, except for a neutropenia (1962/μL) in one of the nonvaccinates on day 6 postplacebo and a slight leukocytosis (14800/μL) in one of the vaccinates on day 8 PV. All attempts at virus isolation from nasal swabs and PBL failed to yield viral growth in culture. Serum neutralizing antibody titers remained negative 21 and 35 days after intramuscular vaccination. The two horses which were vaccinated intravenously developed SNA titers of 1:4 on day 21 PV.

Significant elevations in temperature were seen in all three groups of

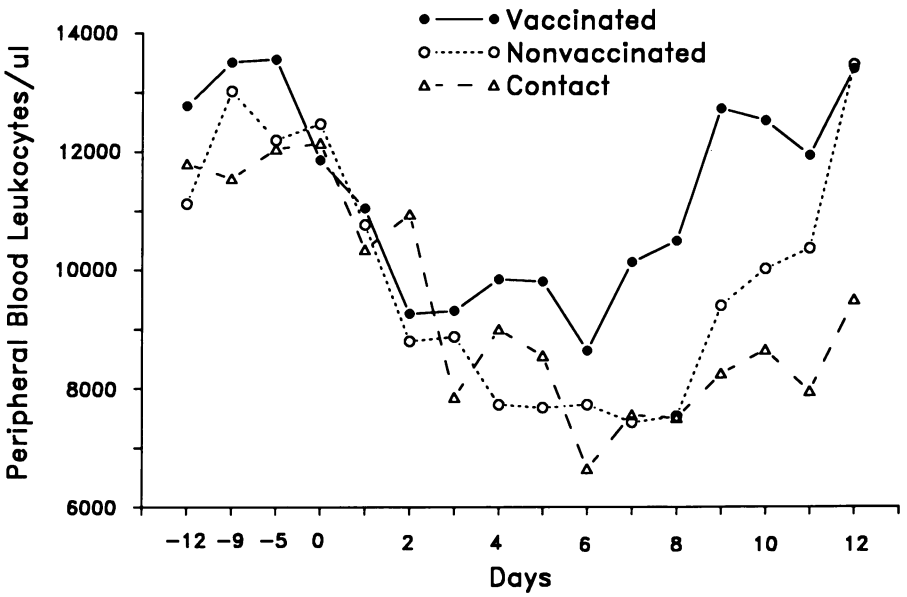

Fig. 3. Mean peripheral blood leukocyte counts of horses vaccinated with thymidine kinase negative EHV-1 (Group I), nonvaccinated controls (Group IIa), and nonvaccinated contact controls (Group IIb). Groups I and IIa were experimentally challenged intranasally with EHV-1 on day 0, and group IIb was exposed to groups I and IIa on day 0.

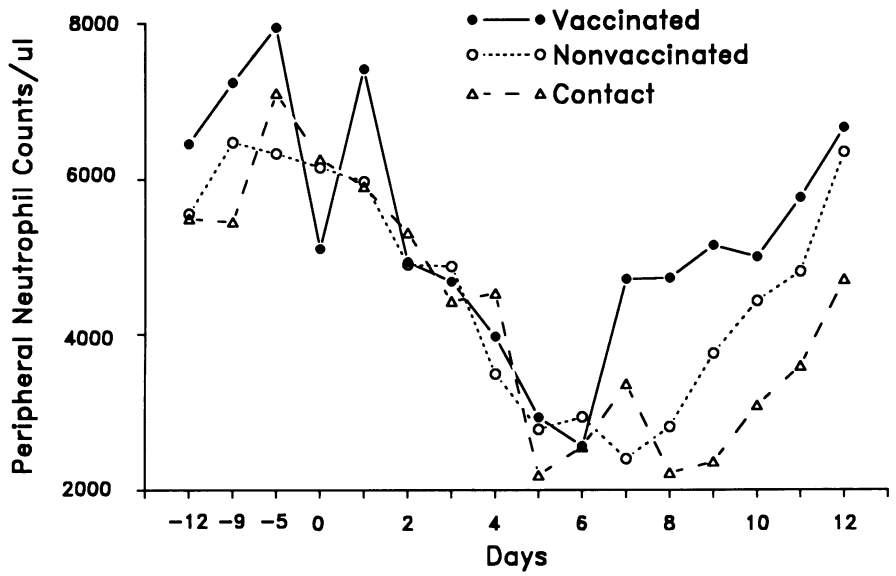

Fig. 4. Mean peripheral neutrophil counts of horses vaccinated with thymidine kinase negative EHV-1 (Group I), nonvaccinated controls (Group IIa), and nonvaccinated contact controls (Group IIb). Groups I and IIa were experimentally challenged intranasally with EHV-1 on day 0, and group IIb was exposed to groups I and IIa on day 0.

horses following viral challenge, with elevations for group IIb (contact controls) appearing two days later than groups I and IIa. Mean body temperatures for the three groups are shown in Fig. 2. Elevated temperatures (> 39° C) persisted for four days PC in group I and six days in group IIa. Mean body temperatures for group IIa were significantly higher than group I on days 4 and 6 PC. No significant differences in duration of fever were observed between groups I and IIa.

Following challenge with virulent EHV-1, all animals exhibited a precipitous decrease in PBL; however, values for vaccinated animals remained higher than nonvaccinates. Mean total PBL counts were significantly higher in group I on days 7 through 10 PC (Fig. 3) and mean total neutrophil counts were significantly higher in group I on days 7 and 8 PC (Fig. 4).

Isolations of EHV-1 from nasal swabs for all animals are shown in Table I. Duration of EHV-1 viral shedding was significantly greater in nonvaccinates (group IIa) than in vaccinates (group I). Virus was not isolated from PBL of any horse.

An anamnestic SNA response occurred in vaccinates PC (Table II). Peak SNA responses were found on day 14 in vaccinates (group I) and day 35 in nonvaccinates (groups IIa and IIb). Serum neutralizing antibody titers in group I were significantly greater than groups IIa and IIb on days 14, 21 and 35 PC.

DISCUSSION

The TK⁻EHV-1 (H6b) vaccine virus was obtained by serially passaging the Vero-adapted TK⁺EHV-1 (RQ) strain in media containing the nucleoside analogs BrdUrd and araT.

The combined use of these two drugs was done to avoid the isolation of TK mutants with reduced, but finite levels of TK activity. Mutants with low levels of TK activity may be virulent (23,24). AraT is useful for enriching for herpesvirus mutants deficient in thymidine phosphorylating activity, particularly in TK⁺ host

TABLE I. Isolation (+) of EHV-1 from nasal swabs of thymidine kinase negative EHV-1 vaccinated (Group I), nonvaccinated control (Group IIa) and nonvaccinated contact control (group IIb) horses

		Isolation (+) of EHV-1								
Group	Horse #	0	1	2	3	4	5	6	7	8
I	1[b]	-	+	+	-	-	-	-	-	-
	9[c]	-	+	+	-	-	-	-	-	-
	14[c]	-	+	+	+	+	-	-	-	-
	27[c]	-	+	+	-	-	-	-	-	-
	29[c]	-	+	+	-	-	-	-	-	-
	31[c]	-	+	+	-	-	-	-	-	-
	37[c]	-	+	-	-	-	-	-	-	-
	54[b]	-	+	-	-	-	-	-	-	-
IIa	20	-	+	+	+	+	+	+	-	-
	28	-	-	+	-	-	-	-	-	-
	30	-	+	+	+	+	+	+	+	-
	34	-	-	+	+	+	+	+	-	-
IIb	13	-	-	-	-	-	+	+	+	-
	39	-	-	-	-	-	+	+	+	+

[a]Groups I and IIa were experimentally challenged intranasally with TK⁺ EHV-1 on day 0, and group IIb was exposed to groups I and IIa on day 0
[b]Intravenous vaccination
[c]Intramuscular vaccination

TABLE II. Serum neutralizing antibody titers of horses vaccinated with thymidine kinase negative EHV-1 (Group I), nonvaccinated controls (Group IIa) and nonvaccinated contact controls (Group IIb)

		Serum neutralizing antibody titers			
	Horse	Postchallenge[a] Day			
Group	#	0	14	21	25
I	1[b]	N	64	64	16
	9[c]	N	128	32	16
	14[c]	N	32	16	32
	27[c]	N	128	128	32
	29[c]	N	64	32	16
	31[c]	N	128	64	32
	37[c]	N	128	64	32
	54[b]	N	64	64	64
IIa	20	N	4	8	16
	28	N	16	8	16
	30	N	16	32	16
	34	N	4	8	8
IIb	13	N	N	4	8
	39	N	16	32	32

[a]Groups I and IIa were experimentally challenged intranasally with EHV-1 on day 0. Antibody titers are reported as the reciprocal of the highest serum dilution which neutralized EHV-1
[b]Intravenous vaccination
[c]Intramuscular vaccination
N = Negative

cells, because araT is relatively nontoxic to mammalian cells. However, araT selection often yields mutants with high reversion frequencies and with only partial deficiencies in TK activity (25). Hence, araT selection was combined with BrdUrd selection as a more effective strategy for maximizing the probability of isolating nonreverting EHV-1 mutants with no detectable TK inducing activity.

Restriction endonuclease analyses verified that TK⁻EHV-1 (H6b), like its TK⁺EHV-1 (RQ) parent, was a subtype 1 strain, and also revealed that alterations in cleavage sites had occurred relative to the reference EHV-1 (Army 183) subtype 1 strain. These changes mainly consisted of large deletion mutations in fragments mapped in the terminally repeated and in the unique short regions of the TK⁺ EHV-1 (RQ) and TK⁻EHV-1 (H6b) genome. In addition, changes in DNA restriction fragments at 0.05-0.13 map units differentiated the TK⁻EHV-1 (H6b) from the parental TK⁺EHV-1 (RQ) strain. The observation that genome alterations, in addition to changes in the TK gene, were produced by araT and BrdUrd treatment was not unexpected. We have previously described temperature-sensitive double mutants in herpesvirus populations of TK⁻mutants isolated following BrdUrd mutagenesis and selection (20).

Studies on the DNAs of European EHV-1 vaccine strains and on EHV-1 passaged on heterologous host cells have shown that alterations localized in the U_s region of the genome, in reiterated sequences mapping at 0.92-0.95, and in sequences mapping at 0.16-0.24 are common (15,22,26). Deletions in the U_s and reiterated sequences of pseudorabies DNA are characteristic of pseudorabies vaccine strains (12,27), and also occur in infectious bovine rhinotracheitis virus DNAs at map positions 0.09-0.20 and at map positions 0.83-0.92 (28). Thus, the DNA fingerprint alterations described here for EHV-1 vaccine strains have been found in other alpha herpesviruses.

Following intramuscular and intravenous administration of the vaccine, there was no clinical evidence of local inflammation, respiratory disease, deleterious systemic responses, or hematological abnormalities. Equine herpesvirus-1 serum neutralizing antibodies were not detectable in any of the IM vaccinated horses through day 35, but were present at low levels in the IV vaccinates at day 21 PV. The lack of antibody response in the EHV-1 immunonaive vaccinates was not surprising since a significant response usually occurs only after repeated exposure to specific antigenic stimuli (6). The low immune response following vaccination with the TK⁻EHV-1 vaccine is consistent with TK⁻studies in other species (12,13).

Neutropenia, which has been reported to occur with EHV-1 infection (29,30) was observed in all of the experimental animals. The significantly lower mean neutrophil counts of the nonvaccinates suggest that the vaccine may have afforded some protection against EHV-1-induced neutropenia.

The significant reduction in nasal shedding of virus indicated decreased viral replication in the vaccinates, and was similar to results of a previous study with other TK⁻vaccines (11,12). Equine herpesvirus-1 was not recovered from the PBL PV or PC. The inability to recover virus from these cells was unexpected and not fully understood; however, low cell culture sensitivity or host response may be contributing factors.

The immunogenicity of the TK⁻EHV-1 vaccine was demonstrated by enhanced antibody responses in vaccinated animals following EHV-1 challenge.

Despite recently identified inter- and intrasubtypic variations in the EHV-1 genome, it is generally accepted that a potent subtype 1 vaccine may stimulate immunity against strains of either subtype (6). Therefore, a subtype 1 strain was selected for both the vaccine and the challenge virus.

Several reasons may explain the vaccine's inability to prevent clinical signs of respiratory disease following challenge with virulent EHV-1. A single inoculation may not have stimulated sufficient immunity, the vaccine virus may have been over attenuated prior to TK gene deletion, and the EHV-1 challenge dose may have been greater than that of a natural infection.

Further investigations using more immunogenic TK⁻EHV-1 deletion mutants and alternative vaccination regimens may be necessary to develop a superior vaccine product and optimal vaccination protocol. In addition, studies are needed to evaluate the safety of TK⁻EHV-1 vaccines in pregnant mares, and the capacity to provide protection against EHV-1 induced abortion.

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