Equine Herpesvirus Type 1 Mutant Defective in Glycoprotein E Gene as Candidate Vaccine Strain

Koji TSUJIMURA^{1)*}, Tomoki SHIOSE^{1,2)}, Takashi YAMANAKA¹⁾, Manabu NEMOTO¹⁾, Takashi KONDO¹⁾ and Tomio MATSUMURA¹⁾

¹⁾Epizootic Research Center, Equine Research Institute, Japan Racing Association, 1400-4 Shiba, Shimotsuke, Tochigi 329-0412 and

²⁾Present address: Racehorse Clinic, Ritto Training Center, Japan Racing Association, 1028 Misono, Ritto, Shiga 520-3085, Japan

(Received 24 March 2009/Accepted 1 July 2009)

ABSTRACT. An equine herpesvirus type 1 (EHV-1) mutant, ΔgE, defective in glycoprotein E (gE) was evaluated as a modified live virus (MLV) vaccine. Colostrum-deprived Thoroughbred foals inoculated intranasally (i.n.) or intramuscularly (i.m.) with ΔgE did not exhibit any clinical signs of respiratory disease except for a mild nasal discharge in 1 i.n. inoculated foal on Days 1 and 3 post-infection. In contrast, the intranasal inoculation of foals with the revertant of ΔgE resulted in biphasic pyrexia, mucopurulent nasal discharge and swelling of submandibular lymph nodes. These results indicated that gE plays an important role as regards EHV-1 virulence in horses. The ability of ΔgE to protect against wild type EHV-1 challenge infection was assessed using i.m. vaccinated foals. Foals inoculated twice i.m. with 10⁵ or 10⁶ plaque-forming units (pfu) of ΔgE at an interval of 3 weeks exhibited no clinical evidence of local inflammation, respiratory disease or deleterious systemic responses. Remarkable increases in SN antibody titer to EHV-1 were observed in all vaccinated foals after the 2nd inoculation with ΔgE. Following a wild type EHV-1 challenge infection, vaccinated foals showed milder clinical symptoms than foals vaccinated with a placebo. Specifically, 1 of 3 foals vaccinated with 10⁶ pfu of ΔgE exhibited no clinical symptoms other than a mild nasal discharge for 1 day. Additionally, the virus load of nasal shedding and viremia were reduced by vaccination. These results suggest that ΔgE would be a good candidate as an MLV vaccine.

KEY WORDS: attenuated live vaccine, EHV-1, gE.

J. Vet. Med. Sci. 71(11): 1439–1448, 2009

Equine herpesvirus type 1 (EHV-1) is a major causative agent of respiratory disease in horses and can also lead to abortion and neurological disease [1]. In particular, the recent increase in neurological EHV-1 outbreaks among horses in the U.S.A., the U.K. and Europe is of major concern to the horse industry worldwide since affected animals often have an unfavorable prognosis [4, 5, 7–9, 12]. Vaccination is considered an important control measure for EHV-1 induced diseases. In Japan, however, only 1 inactivated vaccine with limited efficacy is available. It has been proposed that the ideal EHV-1 vaccine should stimulate 3 types of immune response namely, a mucosal virus neutralizing antibody, a systemic virus neutralizing antibody, and EHV-1 specific cytotoxic T lymphocytes (CTL) [2]. Kydd *et al.* reported that an inactivated EHV-1 vaccine failed to induce CTL responses [16]. In contrast, modified live virus (MLV) vaccines usually induce adequate humoral and cellular immune responses in inoculated animals [24]. Currently, MLV vaccines available for EHV-1 contain a tissue culture-adapted RacH strain that has been attenuated by repeated passages in non-equine cells [15, 26]. Goodman *et al.* showed that the RacH-based MLV vaccine provided immunized horses with better protection against neurological disease caused by EHV-1 than an inactivated vaccine [11]. However, the original RacH was reported to cause mild biphasic pyrexia in horses [23]. Although current vaccines

contain RacH derivatives [15], there is concern about the introduction of these MLV vaccines into Japan. This is because a live vaccine designed to protect against EHV-1 should be completely safe in Japan since it would be largely used to inoculate Thoroughbred racehorses, which are delicate animals and thus difficult to maintain in excellent racing condition. Additionally, to our knowledge, there is no serodiagnostic test that can distinguish anti-RacH antibodies from anti-wild type (wt) EHV-1 antibodies.

The glycoprotein E (gE) deletion mutants of bovine herpesvirus type 1 (BHV-1) and pseudorabies virus, both of which belong to the same subfamily as EHV-1, *Alphaherpesvirinae*, have been used as MLV vaccines in the field [19, 25]. The lack of gE in these vaccines is a useful marker for differentiation between natural infection and vaccine administration using type specific enzyme-linked immunosorbent assay [30, 31]. In a previous study, we generated EHV-1 mutants with a deletion in the glycoprotein I (gI) or gE gene, and showed that these mutants were avirulent but were immunogenic in experimental animal models [29]. The mutants contained no heterogeneous DNA and therefore could be considered candidates for a MLV vaccine strain, since there is a strict safety regulation as regards the practical use of products made by genetic recombination in Japan. However, it has been reported that the gI deletion mutant of BHV-1 is less immunogenic than the gE deletion mutant [14]. Therefore, in this work, we examined the gE deletion mutant of EHV-1 (ΔgE) to ascertain whether it retains virulence and immunogenicity in horses. The results indicate that ΔgE is avirulent for colostrum-deprived Thor-

* CORRESPONDENCE TO: TSUJIMURA, T., Epizootic Research Center, Equine Research Institute, Japan Racing Association, 1400-4 Shiba, Shimotsuke, Tochigi 329-0412, Japan.
e-mail: koji_tsujimura@jra.go.jp

oughbred foals and that an intramuscular vaccination with Δ gE provided foals with partial protection against EHV-1 respiratory disease.

MATERIALS AND METHODS

Viruses and cells: The wt strain of EHV-1 used throughout this study was 89C25p. This strain was plaque-purified from a low passage field strain 89C25, which was isolated from a racehorse during an epizootic outbreak of EHV-1 respiratory disease in 1989 [22]. The generation of Δ gE, which was derived from 89C25p, and its revertant (gE-rev) has already been described [29]. The working stocks of the viruses were propagated in fetal horse kidney (FHK) cells. MDBK cells (an established bovine kidney cell line) were used for serum neutralization (SN) tests. Both FHK and MDBK cells were used for virus isolation. The cells were grown in Eagle's minimum essential medium (EMEM) to which bicarbonate buffer and antibiotics had been added, and supplemented with fetal bovine serum (FBS) as appropriate.

Animals: All experimental procedures were approved by the Animal Care Committees of the Equine Research Institute. Fifteen Thoroughbred foals between 25 and 92 days old were used for this study. All the foals were separated from their dams for 24 hr immediately after birth, during which time they were fed a commercial milk substitute, and the dam's udders were regularly stripped out to remove immunoglobulin-rich colostrum, as described by Jeffcott [13]. The foals and their dams were then reunited and kept together for approximately 3 months. During the study, each foal was housed in an individual isolation stall.

Experimental designs: To assess the virulence and immunogenicity of Δ gE and gE-rev, 6 foals (Nos. 1 to 6) were divided into 3 groups of 2 animals. Foals Nos. 1 and 2 (59 and 51 days old, respectively) and foals Nos. 3 and 4 (59 and 52 days old, respectively) were inoculated intranasally (i.n.) with 10^7 plaque-forming units (pfu) of Δ gE and 10^6 pfu of gE-rev, respectively. Under sedation with medetomidine hydrochloride, the inoculum (in 10 ml EMEM) was injected into both nares of the foals (approximately 5 ml per naris) using a nebulizer. Foals Nos. 5 and 6 (79 and 56 days old, respectively) were inoculated intramuscularly (i.m.) with 4×10^5 pfu of Δ gE (in 1 ml EMEM) by injection in the neck.

To evaluate the vaccine efficacy of Δ gE, 9 foals (Nos. 7 to 15) were divided into 3 groups of 3 animals. Vaccine groups I (foals Nos. 10, 11, and 12: 80, 57, and 39 days old, respectively) and II (foals Nos. 13, 14, and 15: 92, 70, and 28 days old, respectively) were inoculated i.m. with 10^5 and 10^6 pfu of Δ gE (in 1 ml EMEM) at an interval of 3 weeks, respectively. Similarly, a placebo group (foals Nos. 7, 8, and 9: 80, 65, and 25 days old, respectively) were inoculated with EMEM. Four weeks after the 2nd inoculation, all foals were challenged i.n. with 10^6 pfu of EHV-1 89C25p. The intranasal and intramuscular inoculations were performed using the method described above.

Clinical monitoring: Following inoculation with the

viruses, rectal temperatures were recorded each afternoon and clinical signs associated with EHV-1 infection, including nasal discharge and swelling of submandibular lymph nodes, were recorded at the time of sample collection. Temperatures $\geq 39.0^\circ\text{C}$ were considered to indicate pyrexia. Nasal discharge was graded as follows: 0, no discharge; 1, serous discharge; 2, mucopurulent discharge; 3, severe mucopurulent discharge. Swelling of the submandibular lymph nodes was graded as follows: 0, hardly palpable node; 1, slightly palpable node; 2, easily palpable node; 3, enlarged painful node.

Sample collection: For virus detection, nasal swab and peripheral blood samples were collected daily for the 1st week after inoculation with the viruses, and every other day for the 2nd week from each horse with the exception of foals Nos. 5 and 6 (See Tables 2 and 3). Following 2 inoculations with Δ gE, samples for virus isolation were not collected from vaccine groups I and II until the start of the challenge. Serum samples were collected at weekly intervals and stored at -20°C until use. Nasal samples were obtained using 1×1.5 cm absorbent cotton swabs, which were then immersed in 2.5 ml of EMEM supplemented with 2% FBS. Blood samples were collected from the jugular vein using untreated and heparinized evacuated tubes (VenojectTMII, Terumo, Tokyo, Japan).

Virus isolation: Virus isolation was conducted immediately after sample collection. Approximately 1×10^7 peripheral blood mononuclear cells (PBMCs) fractionated using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) or 0.5 ml of filtered nasal fluid samples were co-cultured with suspended FHK or MDBK cells in 25-cm² flasks. The flasks were then incubated at 37°C and observed for cytopathic effects daily for 6 days. Isolated viruses were identified by plaque morphology and/or the polymerase chain reaction (PCR) established by Lawrence *et al.* [17], which discriminates EHV-1 from a genetically- and antigenically-related virus, equine herpesvirus type 4 (EHV-4).

DNA extraction: Approximately 3×10^7 PBMCs and 0.4 ml of nasal swabs from each time point were processed using a QIAamp DNA blood kit (Qiagen Sciences, Germantown, MD), with final DNA elution volumes of 60 and 200 μl , respectively. The DNA concentration of the PBMC samples was adjusted to 50–60 ng/ μl . Five μl of each DNA sample was subjected to the PCR assays described below.

Real-time quantitative PCR: The number of EHV-1 gene copies in nasal swab and PBMC samples was determined using the real-time quantitative PCR (qPCR) established by Goodman *et al.* [10]. qPCR was performed using a 7500-FAST real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) with a reaction mixture containing TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM primers, a 250 nM probe, and 5 μl of DNA sample, with a total volume of 20 μl . The thermal cycling program consisted of 20 sec at 95°C , followed by 40 cycles at 95°C for 3 sec and at 60°C for 30 sec. The primers and probes designed for EHV-1 IR6 and equine $\beta 2$ -microglobulin ($\beta 2$ -M) genes have been described elsewhere [10].

Plasmids inserted with partial IR6 or β 2-M gene were used as a standard. All assays were run in the absolute quantitation mode, in triplicate, against the serial 10-fold dilution of standards spanning seven orders of magnitude. EHV-1 IR6 gene copies in the PBMC samples were normalized per million β 2-M gene copies.

Nested PCR for EHV-1 gE gene: Nested PCR for the EHV-1 gE gene was conducted to distinguish Δ gE from a wt EHV-1. The outer primer pair (gE-F: 5' GGTGCCGTGCGCAAACATTT3' and gE-R: 5' CCGAAGCACTCTGTGTTATCGCTG3') corresponds to nucleotide positions (nt) 133922 to 133941 and 136089 to 136066 of the EHV-1 genomic sequence published by Telford *et al.* [28], respectively (GenBank Accession No. AY665713). The inner primers (gE-FN: 5' AGTTGTGGGGGTAGATG GGGTTG3' and gE-RN: 5' AGTCGAAGGAGTCGTCAG AGTCCG3') started at nt 134172 and 135834, respectively, and resulted in 514 and 1663 bp amplification products for Δ gE and a wt EHV-1, respectively. The reaction mixture (20 μ l) consisted of 0.5 μ M of each primer, 1 $\times$ QIAGEN Fast Cycling PCR Master Mix (Qiagen GmbH, Hilden, Germany), 1 $\times$ Q-solution and 5 μ l of extracted DNA sample (for the 1st round) or 0.02 μ l of the 1st round product (for the 2nd round). The PCR was employed according to the optimized cycling protocol recommended by the manufacturer. The amplification products were analyzed by electrophoresis in 2.2% FlashGel Cassettes (Lonza, Rockland, ME, U.S.A.).

SN antibody titration: Sera diluted with EMEM (1:2.5) were inactivated at 56°C for 30 min and mixed with equal volumes of EMEM with 20% guinea pig complement to form the initial dilutions (1:5). Serial two-fold diluted sera (200 μ l) were mixed with an equal volume of virus (approximately 150 pfu per 100 μ l) and incubated at 37°C for 1 hr in the presence of 10% guinea pig complement. The samples (50 μ l/well) were then inoculated onto monolayers of MDBK cells in 24-well plates (4 wells per dilution), and incubated at 37°C in a 5% CO₂ incubator for 3 hr with agitation every 15 min to allow for virus adsorption. An overlay medium (0.5 ml of EMEM containing 1% methylcellulose) was then added to each well, and the plates were incubated at 37°C in a 5% CO₂ incubator for 3 days. Antibody titers were expressed as the highest serum dilution

that demonstrated a 50% or greater reduction in plaque numbers compared with those of control wells. A fourfold or greater increase in antibody titer was considered significant.

Statistics: Data obtained from the vaccination and challenge experiment were analyzed with statistical software (SigmaStat 3.1, Systat Software Inc., CA, U.S.A.). Rectal temperatures and log-transformed values of EHV-1 gene copies were examined with a two-way repeated measures analysis of variance and a multiple test using the Student-Newman-Keuls (SNK) method. The cumulative clinical scores in each group and the number of viremic foals in each group on each day were analyzed by a Kruskal-Wallis analysis of variance by ranks and a multiple test using the SNK method. Significance was determined at a value of $p < 0.05$. The values of the normalized EHV-1 gene copies in the PBMC samples were log-transformed with the formula $x' = \log(1 + x)$ to avoid taking the log of zero (Zero means no virus was detected by qPCR).

RESULTS

Virulence and immunogenicity of Δ gE and gE-rev: There was no febrile reaction in the foals inoculated i.n. or i.m. with Δ gE (Table 1). In addition, these foals exhibited no other clinical symptoms of respiratory disease except for a mild nasal discharge (grade 1) in foal No. 2 on Days 1 and 3 post-infection (data not shown). In contrast, an obvious elevation in rectal temperature ($\geq 39.0^\circ\text{C}$) was observed in the foals inoculated with gE-rev for 1 to 6 days from Day 1 post-infection and this was followed by a 2nd pyrexia response on Days 9 (No. 3) and 11 (No. 4) post-infection. Furthermore, a mucopurulent nasal discharge and swelling of the submandibular lymph nodes were observed in those foals (data not shown).

The results of EHV-1 detection in nasal swab and PBMC samples are shown in Tables 2 and 3, respectively. Nasal virus shedding and viremia were detected in the foals inoculated with gE-rev by both virus isolation and qPCR. In contrast, after the intranasal inoculation with Δ gE, EHV-1 was detected only in nasal swab samples by qPCR for 6 days from Day 1 post-infection. These detected viral genomes were identified as Δ gE by the nested PCR. The peak titers

Table 1. Pyrexia observed in foals inoculated with Δ gE and gE-rev

Route	Inoculum	Foal No.	Rectal temperatures of foals with pyrexia ^{a)}
I.N. ^{b)}	Δ gE	1	—
		2	—
	gE-rev	3	40.1 (1), 39.1 (9)
		4	39.5 (1), 39.4 (2), 39.6 (3), 39.0 (4), 39.6 (5), 39.4 (6), 39.0 (11)
I.M. ^{c)}	Δ gE	5	—
		6	—

a) Rectal temperatures are expressed in °C. Days post-infection are represented in parentheses. —; No pyrexia was observed.

b) Intranasal inoculation.

c) Intramuscular inoculation.

Table 2. Results of EHV-1 detection in nasal swab samples by virus isolation or by qPCR

Route	Inoculum	Foal No.		Days post-infection											
				0	1	2	3	4	5	6	7	8	10	12	14
I.N. ^{a)}	ΔgE	1	VI ^(c)	–	–	–	–	–	–	–	–	–	–	–	–
			PCR ^{d)}	–	2.8	2.2	2.2	2.5	2.4	2.1	–	–	–	–	–
	gE-rev	2	VI	–	–	–	–	–	–	–	–	–	–	–	–
			PCR	–	2.6	3.7	3.2	2.5	1.4	0.6	–	–	–	–	–
		3	VI	–	+	+	+	+	+	+	+	+	–	–	–
			PCR	–	4.4	6.0	6.1	5.0	5.4	5.3	5.2	3.2	3.1	1.7	0.7
		4	VI	–	+	+	+	+	+	+	+	–	–	–	–
			PCR	–	4.5	6.3	6.4	6.5	6.7	6.4	5.1	3.6	2.5	2.2	1.8
I.M. ^{b)}	ΔgE	5	VI	–	–	–	–	NT ^{e)}	–	NT	–	NT	NT	NT	NT
			PCR	–	–	–	–	NT	–	NT	–	NT	NT	NT	NT
	6	VI	–	–	–	–	NT	–	NT	–	NT	NT	NT	NT	NT
		PCR	–	–	–	–	NT	–	NT	–	NT	NT	NT	NT	NT

a) Intranasal inoculation.

b) Intramuscular inoculation.

c) Results of virus isolation. +/–; Positive/Negative.

d) Results of qPCR. Log-transformed values of EHV-1 IR6 gene copies are shown. –; No virus was detected.

e) NT denotes not tested.

Table 3. Results of EHV-1 detection in PBMC samples by virus isolation or by qPCR

Method	Inoculum	Foal No.		Days post-infection											
				0	1	2	3	4	5	6	7	8	10	12	14
I.N. ^{a)}	ΔgE	1	VI ^(c)	–	–	–	–	–	–	–	–	–	–	–	–
			PCR ^(d)	–	–	–	–	–	–	–	–	–	–	–	–
	gE–rev	2	VI	–	–	–	–	–	–	–	–	–	–	–	–
			PCR	–	–	–	–	–	–	–	–	–	–	–	–
		3	VI	–	–	–	–	–	+	–	–	–	+	–	–
			PCR	–	–	–	–	–	0.5	–	–	–	2.8	2.3	0.9
		4	VI	–	–	+	–	+	+	+	–	+	+	+	+
			PCR	–	–	0.5	0.5	2.2	1.9	1.5	1.7	1.6	2.6	3.4	2.0
I.M. ^{b)}	ΔgE	5	VI	–	–	–	–	NT ^(e)	–	NT	–	NT	NT	NT	NT
			PCR	–	–	–	–	NT	–	NT	–	NT	NT	NT	NT
	6	VI	–	–	–	–	NT	–	NT	–	NT	NT	NT	NT	NT
		PCR	–	–	–	–	NT	–	NT	–	NT	NT	NT	NT	NT

a) Intranasal inoculation.

b) Intramuscular inoculation.

c) Results of virus isolation. +/–; Positive/Negative.

d) Results of qPCR. Log-transformed values of normalized EHV-1 IR6 gene copies are shown. –; No virus was detected.

e) NT denotes not tested.

of the nasal EHV-1 IR6 gene copy in a logarithmic unit for the foals inoculated i.n. with Δ gE and gE-rev ranged from 2.8 to 3.7 and from 6.1 to 6.7, respectively. The intramuscular inoculation of Δ gE did not lead to the detection of any virus in any nasal swab or PBMC sample.

SN antibody titers to EHV-1 after inoculation with Δ gE or gE-rev are shown in Table 4. Significant increases in the SN antibody titer were observed in foals Nos. 3, 4, 5 and 6, which were inoculated i.n. with gE-rev and i.m. with Δ gE, within 3 weeks of inoculation. In contrast, the foals inoculated i.n. with Δ gE showed no increase in SN antibody titer during the observation period.

Clinical reactions after vaccination and challenge infection

Following the 2 intramuscular inoculations with Δ gE, there were no indications of any adverse local or systemic responses in Vaccine groups I and II, which were inoculated i.m. with 10^5 and 10^6 pfu of Δ gE, respectively, at an interval of 3 weeks. Daily mean rectal temperatures following the challenge infection of the vaccinated and placebo foals with the EHV-1 89C25p strain are shown in Fig. 1. Pyrexia was observed in the placebo group within 2 days of the challenge, and it lasted for 3 to 4 days. In contrast, although 5 of 6 vaccinated foals developed pyrexia on Day 2 post-challenge, it disappeared the next day. The remaining vaccinated foal, No. 15 in Vaccine group II, showed no febrile response throughout the observation period. Additionally,

Table 4. SN antibody titers to EHV-1 detected in foals inoculated with Δ gE or gE-rev

Route	Inoculum	Foal No.	Antibody titers at indicated weeks after inoculation			
			0	1	2	3
I.N. ^{a)}	Δ gE	1	1:10	1:5	1:5	1:5
		2	1:10	1:10	1:10	1:10
	gE-rev	3	1:20	1:20	1:80	1:160
		4	1:10	1:5	1:2560	1:2560
I.M. ^{b)}	Δ gE	5	1:10	1:5	1:20	1:40
		6	<1:5	<1:5	1:20	1:40

a) Intranasal inoculation.

b) Intramuscular inoculation.

Table 5. Results of nasal discharge grade scored for each horse after challenge infection

Group	Foal No.	Days post-challenge												Total ^{a)}	Mean ^{b)}
		0	1	2	3	4	5	6	7	8	10	12	14		
placebo	7	0	0	0	0	2	3	3	3	3	2	0	0	16	11.3 $\pm$ 4.16
	8	0	0	0	2	2	1	1	1	1	0	0	0	8	
	9	0	0	0	1	2	3	2	1	1	0	0	0	10	
Vaccine I	10	0	0	0	1	1	0	0	0	0	0	0	0	2	4.67 $\pm$ 2.31
	11	0	0	0	3	2	1	0	0	0	0	0	0	6	
	12	0	0	0	2	1	0	0	2	1	0	0	0	6	
Vaccine II	13	0	0	0	0	0	0	0	0	0	0	0	0	0	1.67 $\pm$ 2.08
	14	0	0	0	2	1	1	0	0	0	0	0	0	4	
	15	0	0	0	0	1	0	0	0	0	0	0	0	1	

Scoring of nasal discharge grade is described in MATERIALS AND METHODS.

a) Total nasal discharge grades from Day 0 to 14 for each horse.

b) Mean of total nasal discharge grades for each group: Mean $\pm$ standard deviation.

the rectal temperatures of each vaccine group from day 3 to 6 were significantly lower than those of the placebo group. After the challenge infection, a mucopurulent discharge was observed for all placebo foals and it was severe (grade 3) in foals No. 7 and 9 (Table 5). In contrast, 2 foals and 1 foal in Vaccine groups I and II, respectively, exhibited a mucopurulent discharge. The cumulative score for the nasal reaction recorded in each vaccine group was significantly lower than that recorded in the placebo group. All foals except No. 15 manifested an enlarged submandibular lymph node after the challenge. The most severe case was one of the placebo foals (No. 9), which exhibited a score of grade 3. However, the differences between the cumulative scores of the lymph node reactions of the groups were non-significant (data not shown).

Virus detection in vaccinated and placebo foals after challenge infection: On the day of the challenge, EHV-1 was not detected in nasal swab and PBMC samples taken from the vaccinated and placebo foals. The number of days on which live viruses were isolated from nasal swab samples ranged from 5 to 11 days in the placebo group and from 2 to 5 days in the vaccine groups. In contrast, qPCR detected nasal virus shedding in all foals from Day 1 post-challenge until the end of the observation period (Fig. 2). Nasal EHV-1 IR6 gene copies of each vaccine group were significantly reduced from Day 6 to 14 post-challenge compared with those of the placebo group with the exception of

Day 8 for Vaccine group I. Additionally, on Day 7 post-challenge, Vaccine group II showed significantly lower nasal virus titers than that of Vaccine group I.

Table 6 shows the numbers of viremic horses that were detected by qPCR and/or virus isolation in each group on each day after challenge infection. All placebo foals remained positive for viremia from Day 3 post-challenge to the end of the observation period. In contrast, the numbers of days, on which all 3 foals in Vaccine groups I and II exhibited viremia, were 1 and 2, respectively. Statistical analyses showed significant differences between the number of viremic horses in the vaccine and placebo groups. Furthermore normalized EHV-1 IR6 gene copies in the PBMC samples from each vaccine group were significantly reduced on Days 10 and 12 post-challenge compared with those of the placebo group (Fig. 3). Vaccine group I also showed a significant reduction on Days 5 and 7 post-challenge.

Δ gE was not detected in the nasal swab and PBMC samples collected from vaccinated foals after challenge infection by the nested PCR (data not shown).

Serological responses in foals after vaccination and challenge infection: SN antibody responses to EHV-1 after vaccination and challenge infection are shown in Table 7. One foal in Vaccine group I (No. 10) and all the foals in group II exhibited a significant increase in SN antibody titer 2 weeks after the 1st inoculation with Δ gE. In addition, all vac-

Fig. 1. Febrile responses of vaccinated and placebo foals after challenge infection. Data are shown as means and standard deviations of daily rectal body temperatures for each group ($n=3$). a: Days in which differences between vaccinated and placebo groups were significant ($p<0.05$).

Fig. 2. Results of EHV-1 detection in nasal swab samples of 3 groups by qPCR. The group means of log-transformed (base 10) values of IR6 gene copies per template DNA are shown. Error bars represent 1 standard deviation. a: Days in which differences between each vaccine and placebo groups were significant ($p<0.05$). b: Days in which differences between Vaccine groups I and II were significant ($p<0.05$).

Table 6. Number of viremic horses per group (out of 3)

Group	Days post-challenge													
	1	2	3	4	5	6	7	8	10	12	14			
placebo	2	1	3	3	3	3	3	3	3	3	3			
Vaccine I	0	1	0	3	1	2	2	2	2	0	0			
Vaccine II	1	1	0	1	2	3	3	2	1	0	1			

nated foals showed an anamnestic boost in SN antibody response 1 week after the 2nd inoculation with Δ gE. The geometric means of Vaccine groups I and II for the fold-dilution of SN antibody titers immediately before challenge infection were 80.0 and 127.0, respectively. After challenge infection with 89C25p, 5 of 6 vaccinated foals developed a significant increase in SN antibody titer 1 week earlier than the placebo foals.

Fig. 3. Results of EHV-1 detection in PBMC samples of 3 groups by qPCR. The group means of the log-transformed (base 10, with the formula $x' = \log [1+x]$) values of IR6 gene copies per million equine $\beta 2$ -M gene copies are shown. Error bars represent 1 standard deviation. a: Days in which differences between vaccinated and placebo groups were significant ($p < 0.05$).

Table 7. SN antibody titers to EHV-1 detected in foals after vaccination and challenge infection

Group	Foal No.	Antibody titers at indicated weeks after the start of vaccination										
		0 ^a)	1	2	3 ^a)	4	5	6	7 ^b (0)	8(1)	9(2)	10(3)
placebo	7	<1:5	NT ^c)	NT	<1:5	NT	NT	NT	<1:5	<1:5	1:1280	1:1280
	8	1:5	NT	NT	<1:5	NT	NT	NT	<1:5	<1:5	1:160	1:2560
	9	1:20	NT	NT	1:10	NT	NT	NT	1:10	1:5	1:640	1:2560
Vaccine I	10	<1:5	<1:5	1:40	1:40	1:320	1:320	1:160	1:160	1:2560	1:5120	1:5120
	11	1:5	1:5	1:10	1:10	1:40	1:40	1:20	1:20	1:640	1:2560	1:2560
	12	1:5	<1:5	1:5	1:10	1:160	1:160	1:160	1:160	1:640	1:5120	1:5120
Vaccine II	13	<1:5	<1:5	1:10	1:10	1:160	1:160	1:80	1:80	1:640	1:1280	1:1280
	14	<1:5	<1:5	1:80	1:80	1:640	1:320	1:320	1:160	1:2560	1:5120	1:2560
	15	1:5	1:5	1:20	1:20	1:160	1:320	1:320	1:160	1:320	1:2560	1:2560

a) Inoculation of Δ gE was conducted at an interval of 3 weeks.

b) Challenge of wt EHV-1 (89C25p) was conducted. Weeks after challenge are represented in parentheses.

c) NT denotes not tested.

DISCUSSION

In the present study, we examined the virulence of the gE deletion mutant of EHV-1, Δ gE, and its ability to protect against challenge infection with a wt EHV-1 strain in colostrum-deprived Thoroughbred foals. Ten of 15 foals had detectable SN antibodies to EHV-1 before the experiment (Tables 4 and 7). However, no clinical manifestation that suggested infections with EHV-1 and/or EHV-4, which causes a similar respiratory disease to EHV-1, was observed in the foals or their dams prior to the commencement of the study. Furthermore, at the start of the experiment, no foal had a detectable complement fixation (CF) antibody to EHV-1 (data not shown). The CF antibody, which is cross-reactive with both EHV-1 and 4, is considered to be indica-

tive of recent infection with EHV-1 and/or 4 [15]. In fact, significant increases in the CF antibody were observed in the foals within 2 weeks of inoculation with gE-rev or a wt EHV-1 (data not shown). Therefore, this suggested that the detected SN antibodies were derived from residual maternal antibodies in the dams' milk that the foals took more than 24 hr after birth. Chong *et al.* reported that completely hand-reared Welsh Mountain pony foals, which were fed bovine colostrum for the 1st 18 hr after birth and a commercial mare's milk replacer until they were weaned, showed no detectable SN antibody to EHV-1 at intervals over a 3-month isolation period [6]. Although Jeffcott has reported that a foal's small intestine is no longer permeable to macromolecules (radioiodine-labeled polyvinyl pyrrolidone; mean molecular weight 160000) at 24 hr after birth [13],

some foals might subsequently absorb immunoglobulin. However, we considered that those pre-existing SN antibodies had little effect on the outcomes of the experiments with the exception of foal No. 3, which had the highest pre-existing antibody titer (as described later).

Foals inoculated i.n. with Δ gE did not exhibit any clinical signs of respiratory disease except for a mild nasal discharge in foal No. 2 on Days 1 and 3 post-infection. In contrast, the clinical signs observed in foal No. 4, which was inoculated i.n. with gE-rev, corresponded well to those observed in foals inoculated with the parental strain, 89C25p, in our previous study [21]. Furthermore, after inoculation with gE-rev, foal No. 3 developed mild clinical manifestations but including biphasic pyrexia, even though it had a relatively high SN antibody titer at the start of the experiment. The inoculation with gE-rev resulted in nasal virus shedding and viremia, which were detected by both virus isolation and qPCR. In contrast, after intranasal inoculation with Δ gE, no live virus was isolated from any nasal swab or PBMC samples. Additionally, although virus shedding was detected in the foals inoculated i.n. with Δ gE by qPCR, the peak viral titers were remarkably lower than those observed in the foals inoculated with gE-rev. Similarly, after the intranasal inoculation of 6 foals with the avirulent EHV-1 mutant, Δ gE Δ gI-*lacZ*, both of whose gE and gI genes were deleted, nasal virus shedding was detected in only 1 foal for 1 day (on Day 2 post-infection) and viremia was not observed in a previous study [20]. In contrast, Slater *et al.* reported that a thymidine kinase-deficient mutant of EHV-1, which still had a slight residual virulence to foals, was isolated from nasal swabs obtained from inoculated foals for 5 days from Day 1 post-infection with relatively high titers [27]. These data indicate that gE plays an important role as regards EHV-1 virulence in horses. The reduced virulence of Δ gE in i.n. inoculated foals might be due to restricted virus replication *in vivo*. Additionally, the foals (Nos. 5 and 6) inoculated i.m. with Δ gE also exhibited no clinical signs, and the viruses were not detected in any nasal swab or PBMC sample by either virus isolation or qPCR. Although intramuscular inoculation with gE-rev was not evaluated in the present study due to the limited number of foals, we have previously observed that after intramuscular inoculation with 10^6 pfu of EHV-1 89C25p strain, 2 conventionally reared horses (approximately 1 year old), which had no detectable CF antibody to EHV-1, showed pyrexia, nasal virus shedding and viremia (unpublished data). These data suggest that Δ gE is avirulent in colostrum-deprived Thoroughbred foals when administered either i.n. or i.m.

The absence of any increase in SN antibody titer to EHV-1 observed in the foals inoculated i.n. with Δ gE in the present study was similar to that observed for foals inoculated with Δ gE Δ gI-*lacZ* [20]. It was reported that intranasal inoculation with Δ gE Δ gI-*lacZ* induced only weak protectivity in inoculated foals from a manifestation of respiratory symptoms after challenge infection with a virulent EHV-1 strain. In contrast, the foals inoculated i.m. with Δ gE exhibited a significant increase in SN antibody titer to EHV-1

within 3 weeks of inoculation despite the inoculated amount being 25 times smaller than that used for the foals inoculated i.n. with Δ gE. This result suggests that intramuscular inoculation with Δ gE might elicit better humoral immunity than intranasal inoculation. A similar phenomenon was reported for BHV-1 mutants [18]. More SN antibody titers were detected in calves inoculated i.m. with BHV-1 mutants deficient in the UL49 homolog, UL49.5 homolog and dUTPase genes than in i.n. inoculated animals [18]. Furthermore, intramuscular inoculation with those mutants elicited better protection against a wt BHV-1 challenge than intranasal inoculation. It should also be noted that routine intranasal administration to Thoroughbred racehorses would not be easy, as they would usually have to be closely restrained during the treatment. Therefore, we examined the efficacy of intramuscular vaccination with Δ gE in protecting against a wt EHV-1 challenge. Following 2 intramuscular inoculations with Δ gE at an interval of 3 weeks, there was no clinical evidence of local inflammation, respiratory disease or deleterious systemic responses. Remarkable increases in SN antibody titer to EHV-1 were observed in all vaccinated foals after the 2nd inoculation with Δ gE. Additionally, the SN antibody titers in 5 of 6 vaccinated foals remained at more than 1:80 until the onset of challenge infection (4 weeks after the 2nd inoculation with Δ gE). The most notable effect of the vaccination was a reduction in clinical signs, including pyrexia and mucopurulent nasal discharge, following challenge with a wt EHV-1 strain. Specifically, 1 of 3 foals vaccinated with 10^6 pfu of Δ gE exhibited no clinical symptoms other than a mild nasal discharge for 1 day. Furthermore, we observed a significant reduction in the amount of EHV-1 shedding in the nasal mucus of the vaccinated foals. The ability to suppress initial virus replication in the upper respiratory tract is an important feature of EHV-1 vaccines as it would interrupt virus transmission to new hosts [2]. Although the challenge infection with a wt EHV-1 resulted in viremia irrespective of vaccination status, there were fewer viremic foals in each vaccine group on each day than in the placebo group. Additionally, the virus loads in the PBMC samples of the vaccinated foals were significantly reduced. PBMC infection is thought to be central to EHV-1 pathogenesis since the virus disseminates to the reproductive tract and central nervous system via cell-associated viremia, which results in abortion and neurological disease, respectively [1]. Therefore, this observation indicates the advantage in the use of Δ gE in immunoprophylaxis.

EHV-1 mutant devoid of gE was avirulent in colostrum-deprived Thoroughbred foals when administered either i.n. or i.m. The attenuation seemed to be related to the impaired ability of Δ gE to replicate in a host animal. In a previous study, we showed that gE contributed to the ability of EHV-1 to spread directly from cell to cell *in vitro*, but was not involved in the process of virus maturation and release or in virus attachment and penetration [29]. The cell-to-cell spread of EHV-1 is possibly crucial as regards its replication in the mucous membrane of the respiratory tract and/or the

subsequent infection of PBMCs. However, further investigation is needed to reveal the role of gE in the *in vivo* growth of EHV-1.

Intramuscular inoculation with Δ gE at a titer of 10^5 or 10^6 pfu conferred a degree of protection against the manifestation of respiratory symptoms induced by EHV-1 infection in colostrum-deprived Thoroughbred foals. The virus load in nasal mucosa and PBMCs was also reduced by vaccination. Future studies will concentrate on the vaccine efficacy of Δ gE in horses previously infected with EHV-1 and/or EHV-4 since most field horses have a history of natural infection with those viruses [22]. It is still unclear whether Δ gE will be more effective than the present inactivated vaccine in the protection against EHV-1 related disease. The immune responses that the ideal EHV-1 vaccine should stimulate are a mucosal virus neutralizing antibody, a systemic virus neutralizing antibody and EHV-1 specific CTL [2]. Breathnach *et al.* reported that intramuscular administration with an inactivated EHV-1 vaccine not only stimulated serum antibody production but also primed the postchallenge mucosal antibody response [3]. Although it was confirmed that intramuscular administration with Δ gE induced high titers of SN antibody, its ability to stimulate the mucosal immune compartment was not investigated in the present study. Additionally, induction of the specific CTL response, which is considered advantage of a MLV vaccine over an inactivated vaccine, was also not evaluated. In the future studies, these 2 types of immune reactions after intramuscular administration with Δ gE should be investigated to compare its vaccine efficacy with the present inactivated vaccine properly.

It is considered that a MLV vaccine designed to protect Thoroughbred racehorse population against EHV-1 should be safe and reduce not only clinical signs of the disease but also virus transmission. Additionally, there is a strict safety regulation as regards the practical use of products made by genetic recombination in Japan. Therefore, the results presented in this study suggest that Δ gE, which contains no heterogeneous DNA [29], would be a good candidate as an MLV vaccine.

ACKNOWLEDGMENTS. We thank Akira Kokubun and Junko Tsujimura for invaluable technical assistance. We would like to express our gratitude to all the staff members who kept the foals in good condition.

REFERENCES

- Allen, G. P., Kydd, J. H., Slater, J. D. and Smith, K. C. 2004. Equid herpesvirus 1 and equid herpesvirus 4 infections. pp. 829–859. *In: Infectious Diseases of Livestock* (Coetzer, J. A. W. and Tustin, R. C. eds.), Oxford University Press, Cape Town.
- Allen, G. P., Kydd, J. H., Slater, J. D. and Smith, K. C. 1999. Advances in understanding of the pathogenesis, epidemiology and immunological control of equine herpesvirus abortion. pp. 129–146. *In: Equine Infectious Diseases VIII* (Wernery, U., Wade, J. F., Mumford, J. A. and Kaaden, O. R. eds.), R&W Publisher, Newmarket.
- Breathnach, C. C., Yeargan, M. R., Sheoran, A. S. and Allen, G. P. 2001. The mucosal humoral immune response of the horse to infective challenge and vaccination with equine herpesvirus-1 antigens. *Equine Vet. J.* **33**: 651–657.
- Borchers, K., Thein, R. and Sterner-Kock, A. 2006. Pathogenesis of equine herpesvirus-associated neurological disease: a revised explanation. *Equine Vet. J.* **38**: 283–287.
- Cardwell, J., Smith, K., Newton, R., Blunden, T., Bestbier, M. and Whitwell, K. 2003. EHV paralytic disease in the south of England. *Vet. Rec.* **152**: 441–442.
- Chong, Y. C., Duffus, W. P., Field, H. J., Gray, D. A., Awan, A. R., O'Brien, M. A. and Lunn, D. P. 1991. The raising of equine colostrum-deprived foals; maintenance and assessment of specific pathogen (EHV-1/4) free status. *Equine Vet. J.* **23**: 111–115.
- Gilkerson, J. R. and Barrett, E. J. 2008. Equine herpesvirus neurological disease. *Equine Vet. J.* **40**: 102–103.
- Goehring, L. S., van Winden, S. C., van Maanen, C. and Sloet van Oldruitenborgh-Oosterbaan, M. M. 2006. Equine herpesvirus type 1-associated myeloencephalopathy in The Netherlands: a four-year retrospective study (1999–2003). *J. Vet. Intern. Med.* **20**: 601–607.
- Goehring, L. S., van Maanen, C. and Sloet van Oldruitenborgh-Oosterbaan, M. M. 2005. Neurological syndromes among horses in The Netherlands. A 5 year retrospective survey (1999–2004). *Vet. Q.* **27**: 11–20.
- Goodman, L. B., Loregian, A., Perkins, G. A., Nugent, J., Buckles, E. L., Mercorelli, B., Kydd, J. H., Palù, G., Smith, K. C., Osterrieder, N. and Davis-Poynter, N. 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. *PLoS Pathog.* **3**: 1583–1592.
- Goodman, L. B., Wagner, B., Flaminio, M. J., Sussman, K. H., Metzger, S. M., Holland, R. and Osterrieder, N. 2006. Comparison of the efficacy of inactivated combination and modified-live virus vaccines against challenge infection with neuropathogenic equine herpesvirus type 1 (EHV-1). *Vaccine* **24**: 3636–3645.
- Henninger, R. W., Reed, S. M., Saville, W. J., Allen, G. P., Hass, G. F., Kohn, C. W. and Sofaly, C. 2007. Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian center. *J. Vet. Intern. Med.* **21**: 157–165.
- Jeffcot, L. B. 1973. The mechanism of transfer of maternal immunity to the foal. pp. 419–435. *In: Proceedings of the 3rd International Conference on Equine Infectious Diseases* (Bryans, J. T. and Gerber, H. eds.), S. Karger AG, Basel.
- Kaashoek, M. J., Rijsewijk, F. A., Ruuls, R. C., Keil, G. M., Thiry, E., Pastoret, P. P. and van Oirschot, J. T. 1998. Virulence, immunogenicity and reactivation of bovine herpesvirus 1 mutants with a deletion in the gC, gG, gI, gE, or in both the gI and gE gene. *Vaccine* **16**: 802–809.
- Kydd, J. H., Townsend, H. G. and Hannant, D. 2006. The equine immune response to equine herpesvirus-1: the virus and its vaccines. *Vet. Immunol. Immunopathol.* **15**: 15–30.
- Kydd, J. H., Watrang, E. and Hannant, D. 2003. Pre-infection frequencies of equine herpesvirus-1 specific, cytotoxic T lymphocytes correlate with protection against abortion following experimental infection of pregnant mares. *Vet. Immunol. Immunopathol.* **96**: 207–217.
- Lawrence, G. L., Gilkerson, J., Love, D. N., Sabine, M. and Whalley, J. M. 1994. Rapid, single-step differentiation of equid herpesviruses 1 and 4 from clinical material using the polymerase chain reaction and virus-specific primers. *J. Virol. Methods* **47**: 59–72.

18. Liang, X., Chow, B. and Babiuk, L. A. 1997. Study of immunogenicity and virulence of bovine herpesvirus 1 mutants deficient in the UL49 homolog, UL49.5 homolog and dUTPase genes in cattle. *Vaccine* **15**: 1057–1064.
19. Mars, M. H., de Jong, M. C., Franken, P. and van Oirschot, J. T. 2001. Efficacy of a live glycoprotein E-negative bovine herpesvirus 1 vaccine in cattle in the field. *Vaccine* **19**: 1924–1930.
20. Matsumura, T., Kondo, T., Sugita, S., Damiani, A. M., O'Callaghan, D. J. and Imagawa, H. 1998. An equine herpesvirus type 1 recombinant with a deletion in the gE and gI genes is avirulent in young horses. *Virology* **242**: 68–79.
21. Matsumura, T., O'Callaghan, D. J., Kondo, T. and Kamada, M. 1996. Lack of virulence of the murine fibroblast adapted strain, Kentucky A (KyA), of equine herpesvirus type 1 (EHV-1) in young horses. *Vet. Microbiol.* **48**: 353–365.
22. Matsumura, T., Sugiura, T., Imagawa, H., Fukunaga, Y. and Kamada, M. 1992. Epizootiological aspects of type 1 and type 4 equine herpesvirus infections among horse populations. *J. Vet. Med. Sci.* **54**: 207–211.
23. Mayer, A. 1970. Vaccination of horses against equine herpesvirus 1 infection. pp. 41–45. *In*: Proceedings of the Second International Conference on Equine Infectious Diseases. (Bryans, J. T. and Gerber, H. eds.), S. Karger AG, Basel.
24. Minke, J. M., Audonnet, J. C. and Fischer, L. 2004. Equine viral vaccines: the past, present and future. *Vet. Res.* **35**: 425–443.
25. Müller, T., Bätza, H. J., Schlüter, H., Conraths, F. J. and Mettenleiter, T. C. 2003. Eradication of Aujeszky's disease in Germany. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **50**: 207–213.
26. Pette, J. 1966. Development of a living virus vaccine against rhinopneumonitis of horses. pp. 112–116. *In*: Proceedings of the First International Conference on Equine Infectious Diseases (Bryans, J. T. ed.), The Grayson Foundation, Lexington.
27. Slater, J. D., Gibson, J. S. and Field, H. J. 1993. Pathogenicity of a thymidine kinase-deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. *J. Gen. Virol.* **74**: 819–828.
28. Telford, E. A., Watson, M. S., McBride, K. and Davison, A. J. 1992. The DNA sequence of equine herpesvirus-1. *Virology* **189**: 304–316.
29. Tsujimura, K., Yamanaka, T., Kondo, T., Fukushi, H. and Matsumura, T. 2006. Pathogenicity and immunogenicity of equine herpesvirus type 1 mutants defective in either gI or gE gene in murine and hamster models. *J. Vet. Med. Sci.* **68**: 1029–1038.
30. van Oirschot, J. T., Kaashoek, M. J., Maris-Veldhuis, M. A., Weerdmeester, K. and Rijsewijk, F. A. 1997. An enzyme-linked immunosorbent assay to detect antibodies against glycoprotein gE of bovine herpesvirus 1 allows differentiation between infected and vaccinated cattle. *J. Virol. Methods* **67**: 23–34.
31. van Oirschot, J. T., Houwers, D. J., Rziha, H. J. and Moonen, P. J. 1988. Development of an ELISA for detection of antibodies to glycoprotein I of Aujeszky's disease virus: a method for the serological differentiation between infected and vaccinated pigs. *J. Virol. Methods* **22**: 191–206.