

# Equine Rhinopneumonitis Virus (Herpesvirus Type 1): Attenuation in Stable Monkey Cell Line

Charles W. Purdy, DVM, PhD; Shirley J. Ford, BS; William F. Grant, BS

## SUMMARY

An isolate of virulent equine herpesvirus (EHV) type 1 was adapted to Vero stable cell line by 13 serial passages at 37 C and 50 serial passages at 26 C. Characteristics of the attenuated EHV-1 were found to be avirulent, but immunogenic in horses if injected intramuscularly. The attenuated virus was regularly isolated from peripheral leukocytes in inoculated horses, but was not recovered from nasal turbinate tissues. A mild leukopenia was noticed. The attenuated virus produced characteristic large syncytia on primary isolation in rabbit kidney (RK<sub>13</sub>) or Vero cells at 37 C in contrast to cell rounding observed with virulent EHV-1. The syncytial marker was stable through 20 serial passages in Vero cells at 37 C.

New application of double immunodiffusion test for distinguishing between EHV-1 and EHV-2 also is described.

Three antigenically distinct types of equine herpesvirus (EHV-1, EHV-2, and EHV-3) are recognized.<sup>8,11</sup> The EHV-1 refers to the equine rhinopneumonitis (ERP) or equine abortion virus and EHV-2, the slowly growing herpesviruses, including equine cytomegaloviruses. The EHV-1 is a major cause of equine respiratory disease and abortion. Respiratory and abortigenic strains of EHV-1 have been reported.<sup>11</sup> Abortigenic strains are capable of inducing respiratory signs in addition to abortion. Viruses of type 2 commonly are found in the upper respiratory tract and peripheral leukocytes of horses in the absence of any recognized disease. The EHV-2 has been recovered from other tissues, but its clinical importance is not known. The EHV-1 appears to be an antigenically homogeneous group, but EHV-2 includes an antigenically heterogeneous group.<sup>11</sup> A 3rd equine herpesvirus (EHV-3) distinct from EHV-1 and EHV-2 is the coital exanthema virus, which is transmitted venereally. The EHV-3 has been reported to be nonabortigenic.<sup>11</sup>

The purpose in the present report is to describe the characteristics of an EHV-1 which was attenuated by serial growth in stable monkey cell line at 37 and 26 C.

Also described is a newly devised application of the double immunodiffusion test for distinguishing between EHV-1 and EHV-2.

## Materials and Methods

**Experimental Horses**—Horses weighing 204 to 385 kg and ranging in age from 1 to 4 years old were used; they were of mixed breeding from closed herds and farm raised.

**Viral Agents**—An EHV-1 prototype<sup>a</sup> was isolated in California from an aborted equine fetus. Three strains of EHV-2, including equine cytomegalovirus strain 82-A,<sup>b</sup> Karpas strain,<sup>c</sup> and LK strain,<sup>c</sup> were used. Equine adenovirus<sup>d</sup> also was used.

**Tissue Culture Technique**—Mammalian cell lines used included Vero (kidney: African green monkey, *Cercopithecus aethiops*)<sup>e</sup> at passage 129, rabbit kidney (RK<sub>13</sub>)<sup>f</sup> at passage 62, and equine epidermal cells at passage 36. Cell lines were grown in Eagle's minimum essential medium (MEM), as has been described.<sup>g</sup>

The RK<sub>13</sub> cells and Vero cells were grown in MEM supported by 10% bovine fetal serum (BFS) in growth medium and 5% BFS in maintenance medium. Equine epidermal cells<sup>h</sup> were grown in MEM containing 20% BFS and maintained in MEM with 5% BFS. Changes with fresh medium were made every 4 days.

**Viral Titrations and ERP Neutralization Tests**—The RK<sub>13</sub> cell cultures used for ERP virus titrations were prepared in 125- × 16-mm screw cap tubes. Maintenance medium was MEM with 2% BFS; each milliliter contained 50 U of penicillin and 30 μg of mystatin.<sup>i</sup> Medium changes were made after 4 days. Titrations were made in triplicate cultures utilizing 0.2-ml inoculums of each serial tenfold dilution. Incubations were done in stationary racks at 37 C. Tubes were read for cytopathic effects (CPE) at 4 and 7 or 8 days after they were inoculated. Tubes inoculated with support medium only served as controls. The median tissue culture infective dose (TCID<sub>50</sub>) endpoints were determined by the Reed and Muench method.<sup>j</sup>

Serum neutralization (SN) test, using constant virus-serum dilution technique was carried out in RK<sub>13</sub> cell culture tubes.<sup>k</sup> A German strain<sup>l</sup> of EHV-1 was diluted to 100 to 150 TCID<sub>50</sub>. Freshly prepared rabbit complement (1:40)

<sup>a</sup> Obtained from Dr. D. G. McKercher, University of California, Davis, Ca, on July 18, 1972.

<sup>b</sup> Supplied by American Type Culture Collection, Viral and Rickettsial Registry and Distribution Center, Rockville, Md, on April 18, 1973.

<sup>c</sup> Obtained from Dr. Gordon Plummer, Loyola University, Stritch School of Medicine, Maywood, Il, on May 2, 1973.

<sup>d</sup> Obtained from Dr. Charles J. York, University of California, San Diego, Ca, on Aug 29, 1973.

<sup>e</sup> Approved by the Animal and Plant Health Inspection Service, for use in production of veterinary virus vaccines on Nov 9, 1972.

<sup>f</sup> Supplied by Parke Davis Company, Joseph Campau at the River, Detroit, Mi.

<sup>g</sup> Mycostatin, E. R. Squibb and Sons, Inc, New York, NY.

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was used, and the virus-serum mixture was incubated in a water bath at 37 C for 1 hour, followed by incubation at 11 C for 24 hours.

**Preparation of Viral Antigens for Immunodiffusion Test**—Virulent EHV-1, cytomegalovirus, Karpas strain, and LK strain were propagated separately in RK<sub>13</sub> cells. Tissue culture monolayers were infected with high-titer viral ( $10^{5.7}$  or  $10^{6.7}$  TCID<sub>50</sub>/ml) suspensions and allowed to adsorb for 1 hour. Freshly prepared MEM, containing 2% BFS, was then added.

Viral isolates from horses were grown in 120-ml Brockway bottles, and standard stock antigens were grown in 1-L Brockway bottles. The ratio of tissue culture area was 1:4. Cell cultures were monitored daily. After approximately 70% showed definite CPE, cells were harvested by scraping the growth vessel, and the subsequent concentration was centrifuged at  $1,000 \times g$  for 1 hour. Cells from 1-L and 120-ml bottles were resuspended in 2- and 0.5-ml tissue culture medium, respectively; suspensions were frozen at -70 C and thawed 3 consecutive times to disrupt cells. Viral antigens were then maintained at -70 C until used. Lysates of uninfected cell lines and BFS were used as antigen controls.

**Viral Antiserums**—Monospecific EHV-2 antiserum was obtained from horses with naturally acquired antibody. Horses possessing naturally acquired EHV-2 antibody were infected intranasally with virulent EHV-1 to produce bivalent EHV-1 and EHV-2 antiserum. Serums obtained 42 days after nasal infection were the best precipitating reagents.

**Double Immunodiffusion (Ouchterlony) Test**—The double immunodiffusion test used the procedure described by Ouchterlony.<sup>7</sup> The medium contained 1% agarose buffered with tromethamine-borate-EDTA solution (pH = 8.3). Melted medium (5 ml) was dispensed into 60- × 15-mm petri plates and allowed to solidify. Six uniform holes (7 mm in diameter) were cut to form an equilateral hexagon with 11-mm sides, measuring between centers of holes. A 7-mm well was cut in the center of the hexagon. Approximately 0.3 ml of viral antigen or antiserum was used to fill a well. Plates were incubated in a humidifier at ambient temperature for 48 to 72 hours and then examined for precipitin lines (Fig 1).

**Attenuation of EHV-1**—Vero cells were monolayered in capped, 1-L Brockway bottles. Medium was removed from each bottle, and cell sheets were overlaid with 12 ml of EHV-1 isolate. After adsorption for 1 hour at 37 C, 88 ml of MEM containing 2% BFS were added. Infected cell sheet was incubated at 37 C for 24 to 72 hours; 13 serial passages were made at 37 C. Virus from the 13th passage was introduced into Brockway bottles. After adsorption for 24 hours at 26 C, incubation was made at same temperature. Fifty serial passages of the virus were made at 26 C. A pool of EHV-1 was made at the 50th passage level. Exactly 150 ml of enamine stabilizer was added to the attenuated virus pool (600 ml). Stabilized virus (1.25 ml) was equally divided into 5-ml amber vials fitted with fluted stoppers. Material in the vials was lyophilized for 24 hours. Vials were then stoppered under vacuum and stored at 4 C. Before freeze drying, the attenuated EHV-1 had a titer of  $10^{6.7}$  TCID<sub>50</sub>/ml.

**Purity Testing of the Attenuated EHV-1**—The identity of the attenuated virus was determined by neutralizing the characteristic CPE in tissue culture with known EHV-1 antiserum. Standard procedures were used for testing for the presence of possible viral contaminants. These included:

(1) immunodiffusion for testing EHV-1-attenuated virus for adenovirus<sup>4</sup> and EHV-2, and (2) chicken embryo inoculation for growth of equine encephalomyelitis group<sup>5</sup> of viruses and equine influenza viruses.<sup>3</sup> The chorioallantoic and amniotic fluids were tested for hemagglutinating and hemadsorption antigens, as previously described.<sup>3</sup> Guinea pig red blood cells were used as test cells. Immunodiffusion also was used to screen for anti-equine infectious anemia (EIA) antibody<sup>2</sup> in serums of horses injected with attenuated EHV-1.

**Immunizing Dose of Attenuated EHV-1**—The immunizing dose of attenuated EHV-1 was  $10^{6.1}$  TCID<sub>50</sub> contained in 2 ml after reconstitution of the lyophilized material. Horses were given 2 intramuscular injections 28 days apart.

**Challenge Virus (EHV-1)**—Virulent EHV-1 was grown on equine epidermal cells covered with MEM containing 5% colt serum. A horse was injected intravenously with 10 ml of the harvested virus and was given 10 ml of the virus inoculum intranasally through an aerosol gun under pressure. Four days later, 50 ml of blood were collected in heparin, and 10 ml of saline nasal wash were obtained. A 2nd horse was injected intravenously with the heparinized blood and was given intranasally the saline nasal wash. The process was repeated serially in 4 horses. Virulence of the virus increased on successive transfers, as was evidenced by the increasing severity of the disease produced in the horses. Virulent EHV-1 was isolated in RK<sub>13</sub> cell cultures from the nasal turbinates of the last horse. The virus later was grown for 30 hours in 9-L roller bottles containing equine epidermal cells. The challenge virus titer averaged  $10^{7.4}$  TCID<sub>50</sub>/ml. The aqueous suspension of the virus was frozen and stored at -60 C.

**Challenge of Horses**—A challenge dose of 10 ml of virulent EHV-1 containing  $10^{7.4}$  TCID<sub>50</sub> was administered via syringe through a 35-cm catheter into the oral pharynx and along the nasal turbinates of each horse being challenge exposed.

**Viral Isolations from Peripheral Leukocytes**—Blood (20 ml) was drawn from each horse. Aliquots (10 ml) were added to screw cap test tubes containing EDTA.<sup>10</sup> Buffy coat rich plasma was removed and centrifuged. Leukocyte pellets were washed 3 times with 3 ml of saline solution. Washed pellets were resuspended in MEM; the combined suspension was cultured for virus. Leukocytes were washed from the cell sheet 24 hours later and freshly prepared medium was added.

**Viral Isolations from Nasal Turbinates**—Cotton-tipped nasal swabs (20 cm long) were inserted into thin cardboard tubes (length = 16 cm, diameter = 12 mm) leaving 5 cm of the handle exposed. The assembled units were wrapped individually and sterilized. After a sterilized unit was inserted about 10 cm into the nostril, the swab was passed through the tube onto the nasal turbinate tissue and allowed to absorb for 1 minute. Following withdrawal of the swab into the tube, the assembly was removed from the nose. The swab was placed in a screw cap test tube containing 2 ml of MEM (2% BFS) containing 0.188 µg of gentamycin/ml, 75 U of nystatin/ml, 50 µg of neomycin/ml, and 100 U of penicillin/ml. Tubes were held on ice.

Each tube containing a swab was agitated 3 times on a mixer.<sup>h</sup> Each swab was squeezed dry and removed from the tube. The fluid was transferred to a 120-ml bottle monolayered with RK<sub>13</sub> cells and allowed to adsorb for 1

<sup>h</sup> Vortex mixer, Manufactured by Scientific Industries, Inc, Bohemia, NY, for Fisher Scientific Company, Springfield, NJ.

hour. Fresh MEM (10 ml) was placed on each cell sheet. Medium was changed every 24 hours for 4 days. Subsequently the medium was changed every 4 days. Cell sheets were subcultured after 12 to 14 days into freshly prepared tissue culture bottles and held for another 12 to 14 days. All isolation bottles and RK<sub>13</sub> cell control bottles were observed daily for CPE.

## Results

*Purity of Attenuated EHV-1*—Attenuated EHV-1 was found free of adventitive agents, including EIA virus, EHV-2, equine adenovirus, and equine encephalomyelitis and equine influenza viruses.

*Characterization of Attenuated EHV-1 in Vitro*—Virulent EHV-1 produced characteristic cell rounding on

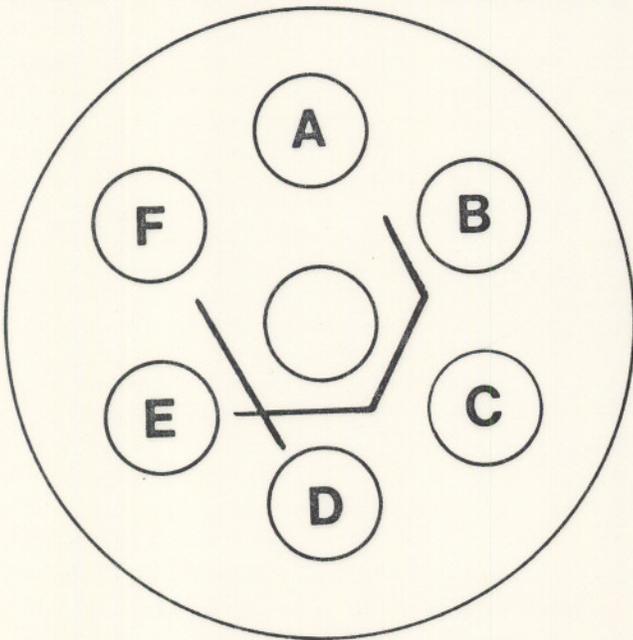
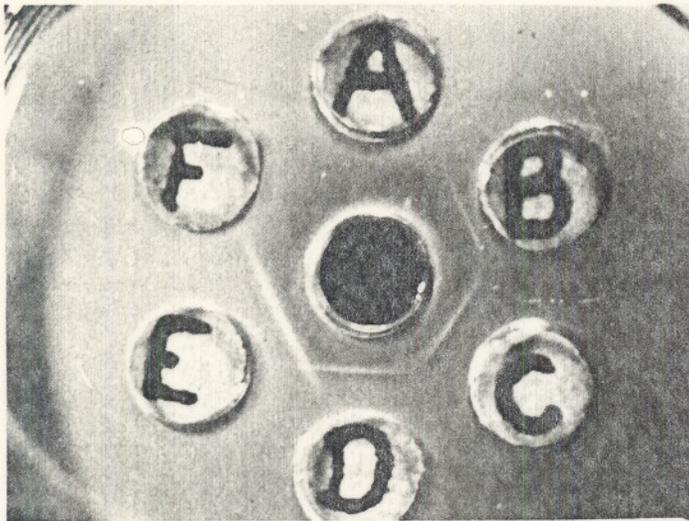


Fig 1—Photograph and drawing of differentiation of equine herpesvirus (EHV-1) and EHV-2 by double immunodiffusion. A = rabbit kidney (RK<sub>13</sub>) cell lysate and bovine fetal serum as control; B = equine cytomegalovirus (EHV-2); C = Karpas virus (EHV-2); D = LK virus (EHV-2); E = EHV-1; F = Vero cell lysate and bovine fetal serum as control; and G = bivalent anti-EHV-1 and EHV-2 horse serum.

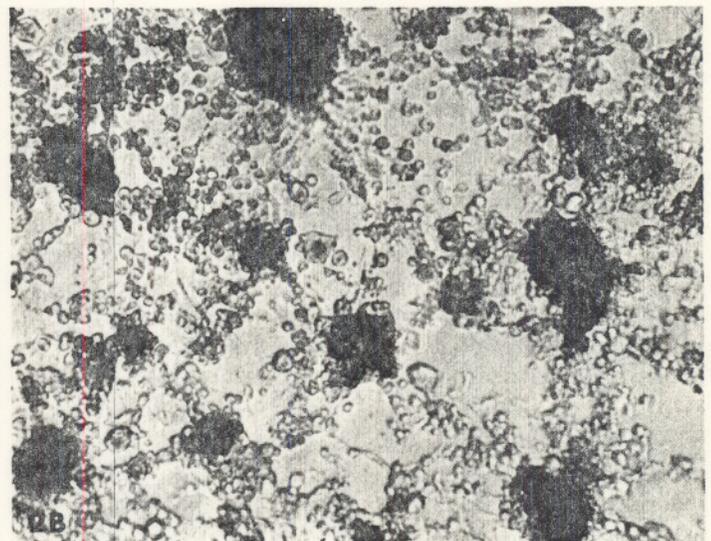
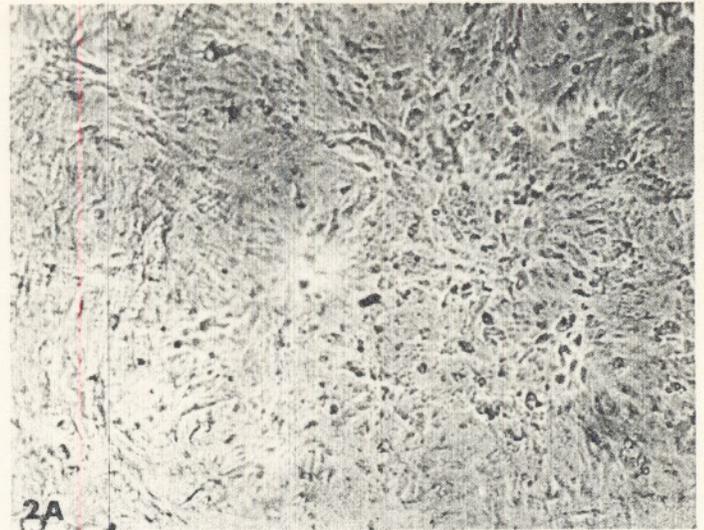


Fig 2—Vero cell cultures.

A—Uninfected normal monolayer;  $\times 267$ .

B—Growth of virulent EHV-1 with typical cell-rounding type cytopathic effect;  $\times 267$ .

C—Growth of attenuated EHV-1 with typical syncytium;  $\times 350$ .

TABLE 1—Serologic Response\* of Horses After Vaccination with Attenuated Equine Herpesvirus (EHV-1) or Challenge Exposure with Virulent EHV-1.

Horse No.	Days after 1st vaccination			
	0**	28†	56‡	84
<b>Vaccinated</b>				
52	16	256	256	1,024
53	8	1,024	128	256
54	4	256	128	256
77	0	512	128	256
104	0	8	128	128
129 (pregnant)§	0	64	.....	.....
130 (pregnant)§	0	64	.....	.....
Mean	4	312	153	384
<b>Nonvaccinated controls</b>				
75	0	0	0	2,048
79 (pregnant)¶	0	0	0	2,048
Mean	0	0	0	2,048

\* Reciprocal of serum neutralization (SN) titers. \*\* First vaccination with attenuated EHV-1. † Second vaccination with attenuated EHV-1. ‡ Challenge exposed with virulent EHV-1 28 days after 2nd vaccination; nonvaccinated controls also were challenge exposed at this time. § Horses 129 and 130 vaccinated at 4.4 and 3.4 months of gestation, respectively. Not challenge exposed. ¶ Aborted 9-month-old fetus on postchallenge exposure day 15.

primary isolation in Vero cell or RK<sub>13</sub> cell cultures at 37 C (Fig 3 A and B). The virus became well adapted to Vero cell line with 13 passages at 37 C. The CPE was lost for several passages in Vero cell line at 26 C, but became more pronounced with cell rounding until the 20th passage, when it changed to a syncytial type involving the whole cell sheet.

Attenuated EHV-1 produced large syncytia on primary isolation in Vero cell or RK<sub>13</sub> cell cultures (Fig 2C). The syncytial marker of the attenuated virus remained stable during 20 serial passages in Vero cells at 37 C.

**Response to Vaccination**—All 7 horses injected with attenuated EHV-1 remained free of local and systemic reactions. After they were injected, 4 of the 7 horses were monitored. Average rectal temperatures varied between 37.5 and 38.3 C but the mean leukocyte count dropped from 15,000 to 9,500/mm<sup>3</sup> on postinjection day (PID) 6. Attenuated EHV-1 was isolated from 20 of 25 buffy coat specimens obtained between PID 4 and 24; attempts to isolate EHV-1 from nasal turbinates failed.

Serum neutralization (SN) titers rose from an average of 1:4 prior to injection to 1:312 on PID 28 (Table 1). During the 56-PID period, 2 contact controls remained seronegative for EHV-1.

Two pregnant mares (No. 129 and 130) were seronegative against EHV-1 before given injections. They delivered normal healthy foals on PID 207 and 227. Repeated attempts to isolate EHV-1 from buffy coat specimens and from nasal turbinates obtained from the newborn foals failed.

**Response to Challenge Injection**—Five horses (No. 52, 53, 54, 77, and 104) were injected with attenuated EHV-1, and 2 controls (No. 75 and 79) were challenge exposed with virulent EHV-1 on PID 56 (Table 1). All horses given attenuated EHV-1 remained asymptomatic postchallenge exposure (PCE); average daily temperatures of 5 principals varied between 37.4 and 38.1 C over a period of 10 days PCE (Fig 3). Mean leukocyte counts remained normal for 10 days. Virulent EHV-1

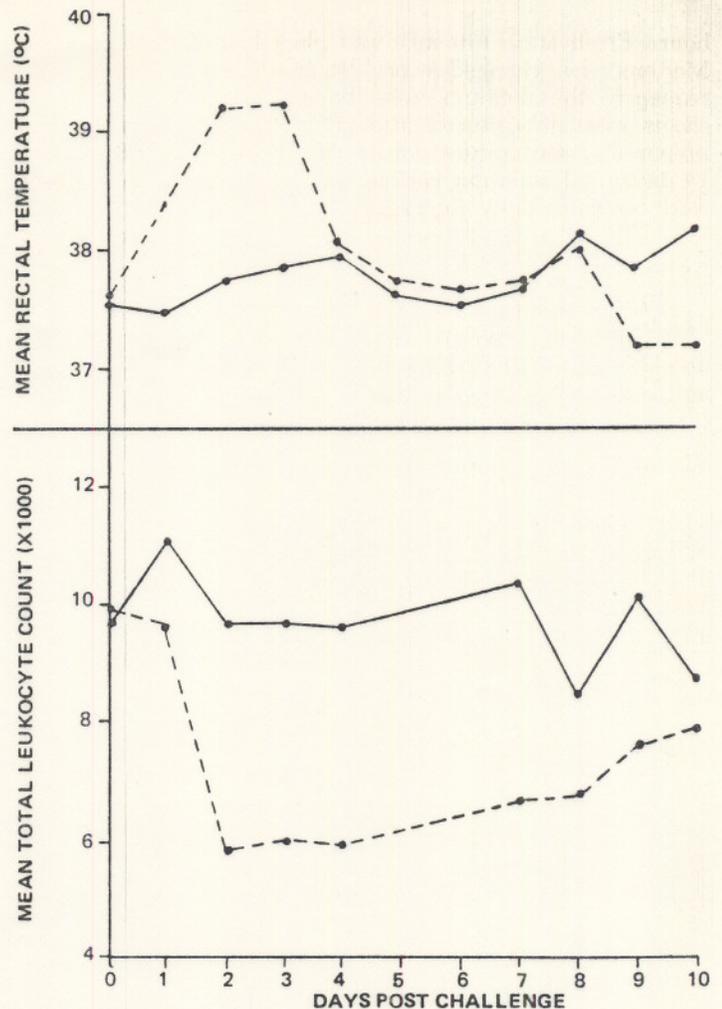


Fig 3—Mean rectal temperatures and mean total leukocyte counts after challenge exposure of horses with virulent EHV-1. ●—● = means of 5 horses which were previously injected with attenuated EHV-1 and ●- - -● = means of 2 control horses.

was not found in buffy coat specimens and was isolated on PCE days 2 and 3 from the nasal turbinates of only 1 of 5 principal horses.

Both control horses exhibited typical symptoms of upper respiratory disease PCE. A nonproductive cough was noticed in both controls. Average body temperature increased to 39.2 C and mean leukocyte count dropped to 6,000/mm<sup>3</sup> on PCE day 3 (Fig 3). Controls developed a serous nasal discharge which later became mucopurulent. The EHV-1 was regularly isolated from both buffy coat specimens and nasal swabs taken from the controls up to PCE day 10. The SN titers of controls increased from 0 prior to challenge exposure to 1:2,048 on PCE day 28. Control (No. 79) aborted a 9-month-old fetus PCE day 15. Virulent EHV-1 was isolated from lung tissues and stomach fluid of the aborted fetus.

**Identification of Viral Isolates by Double Immunodiffusion**—The EHV-1 was differentiated from EHV-2 in isolates by the double immunodiffusion test (Fig 1) employing monospecific and bivalent antiserums. The EHV-1 and EHV-2 were never found together in the same specimen. However, in 1 animal (No. 77), EHV-1 and EHV-2 were isolated on the same day from blood leukocytes and nasal turbinates, respectively.

Isolation of EHV-2 from blood leukocytes and nasal specimens was a frequent finding in experimental and control horses.

### Discussion

Virulent EHV-1 was found to produce classic upper respiratory disease and abortion in horses. After challenge exposure of controls with virulent EHV-1, the virus was isolated consistently from nasal turbinates and blood leukocytes; the challenge exposed controls exhibited fever and marked leukopenia.

In contrast, injection of horses with attenuated EHV-1 failed to elicit local or systemic responses, including fever. Results of viral isolation studies show that the attenuated virus does not replicate in nasal turbinate tissue, but most likely multiplies in the lymphoreticular endothelial system. Replication of attenuated virus would explain the slight drop in leukocyte count experienced after inoculation. Failure of attenuated EHV-1 to cause abortion in pregnant mares was in marked contrast to virulent EHV-1.

Serial passage of virulent EHV-1 in Vero cell line at low temperature was found to produce an attenuated virus void of virulence, but still immunogenic. Immunogenicity was evidenced by the serologic response of the horses PI and by their complete resistance to infection when challenge exposed with virulent EHV-1. Attenuated EHV-1 produced characteristic large syncytia on primary isolation in tissue culture at 37 C. This marker was easily distinguished from the cell-rounding CPE produced by virulent EHV-1. Rapid growth of attenuated EHV-1 in contrast to no CPE produced by virulent EHV-1 at 26 C serves as another important marker.

Retention of the syncytial marker for 20 passages in Vero cells at 37 C reflects the stability of the attenuated virus.

The common finding of EHV-2 in horses necessitated the development of the double immunodiffusion test to identify quickly tissue culture isolates of EHV-1 and EHV-2 (Fig 1).

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