Pathogenicity of a thymidine kinase-deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals

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Both intranasal (i.n.) and intracerebral (i.c.) inoculation of mice with wild-type equine herpesvirus type 1 (wt EHV-1) caused clinical signs and mortality. Virus could be recovered from target organs (turbinates, lungs and blood) for several days. By contrast, the thymidine kinase (TK)-deficient deletion mutant PR1 produced markedly less clinical disease following both i.n. and i.c. inoculation, and, in particular, no mortality occurred. PR1 did, however, establish productive infections following either route of inoculation. High titres of virus were recovered from target organs although virus did not persist for as long as wt EHV-1 and no viraemia was detected. Primary i.n. infection of mice with either wt EHV-1 or PR1 protected against subsequent challenge with wt EHV-1 5 weeks later. I.n. inoculation of specific pathogen-free (EHV-free) foals with PR1 produced results similar to those observed after infection of mice. Clinical signs were milder than for wt EHV-1 and pyrexia was short-lived or absent. PR1 could be recovered from nasal mucus at high titres but it persisted for only 5 days post-infection compared to 11 days in the case of wt EHV-1. No viraemia was detected in foals infected with PR1. On challenge with wt EHV-1, foals given a primary infection with the mutant were partially protected; but a viraemia with a TK+ EHV-1 was observed. These results demonstrate that our TK-mutant PR1 is markedly less pathogenic than wt EHV-1, despite being able to replicate in the host. The use of TK-deficient mutants of EHV-1 as potential vaccines in the horse is discussed.

Introduction

The alphaherpesvirus equine herpesvirus type 1 (EHV-1) is one of several herpesviruses known to infect horses. EHV-1 can cause abortion and neurological disease, as well as respiratory problems. It is a major pathogen of horses with considerable economic and veterinary importance (Allen & Bryans, 1986). To date, control measures have proved inadequate, and although vaccines are available, their efficacy is controversial (Burrows et al., 1984; Allen & Bryans, 1986; Burki et al., 1990).

Progress towards more effective immunoprophylaxis has been hindered by incomplete understanding of the pathogenesis and epidemiology of EHV-1. Our approach to this problem has involved the use of two animal models: colostrum-deprived specific pathogen-free (SPF), EHV-free, foals and mice. SPF foals are taken from their dams at birth and reared by hand using bovine colostrum (Chong et al., 1991). They are free from EHV infection and also from maternal antibodies. They are invaluable in the study of EHV infection, particularly the interaction between different EHVs (Gibson et al., 1992a,b). Because foal production is limited and seasonal, we have also developed a murine model for EHV-1 infection whose features show remarkable similarity to infection in the natural host (for a review, see Field et al., 1992b).

The study of thymidine kinase (TK)-deficient mutants of other herpesviruses has received considerable attention. In particular, TK is implicated in the neuropathogenicity of various alphaherpesviruses, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and suid herpesvirus type 1 (SHV-1) (Field & Wildy, 1978; Becker et al., 1984; Kit et al., 1985). Although EHV-1 is not thought to infect neurons directly, it does cause neurological problems in the horse, probably via infection of endothelial cells in the central nervous system (CNS) and subsequent ischaemic damage of nervous tissue (Patel et al., 1982). A similar sequence of events involving infection of placental blood vessels is postulated in EHV-1-induced abortion.

Previous reports of EHV-1 TK-deficient mutants suggest that they are indeed less pathogenic than the wild-type (wt) virus (Cornick et al., 1990). The behaviour of such mutants, however, has not been studied in detail. Furthermore, previous work has made use of naturally reared horses, which have the disadvantage that their EHV status is uncertain and results obtained from them are therefore difficult to interpret (Cornick et al., 1990).

Here we describe the characteristics of a drug-selected
mutant of EHV-1 that fails to induce any measurable TK activity (Fuente et al., 1992; Corrochano et al., 1993). The pathogenicity of the mutant is examined in two animal models: SPF foals and mice. Our results show that the TK-deficient mutant of EHV-1 produced a milder clinical disease in both species when compared to the parent (wt) virus. The ability of the mutant to protect against challenge with wt EHV-1 was also addressed.

**Methods**

**Virus strain.** The wt strain of EHV-1 (designated wt EHV-1) used throughout this study was Ab4 (Gibson et al., 1992a). This strain was isolated from a case of paresis and has been shown to cause paresis and abortion in experimental infections (N. Edington, personal communication). Virus working stock was prepared by infecting monolayers of RK-13 cells (see below) at low m.o.i. The working stock, passage 11, had an infectivity of 4 × 10^6 p.f.u./ml. The TK-deficient mutant of EHV-1, designated PR1, was derived from Ab4 by selection with the nucleoside analogue penciclovir (Fuente et al. 1992). The mutant was shown to contain an 879 bp deletion in the tk gene (Fig. 1; Corrochano et al., 1993). The working stock of PR1 had an infectivity of 5 × 10^7 p.f.u./ml.

**TK assay.** TK activity was determined as described by Mittal & Field (1989). Briefly, RK-13 cells were inoculated with the relevant virus strain at an m.o.i. of 0.1 to 1.0 p.f.u./cell. Cells were harvested after 48 or 72 h and cell extracts obtained following lysis with HCl. TK activity in 25 µl aliquots of the extracts from infected or uninfected cells was measured using [3H]thymidine (specific activity 20 Ci/mmol, concentration 1.6 gCi/ml).

**Tissue culture.** All tissue culture was carried out in Eagle's minimum essential medium (EMEM) to which bicarbonate buffer and antibiotics had been added, supplemented with fetal or neonatal calf serum as appropriate. A rabbit epithelial cell-line, RK-13, was used throughout. Cell cultures were maintained at 37 °C in a humidified incubator with an atmosphere of air plus 5% CO₂.

**Animals.** Female BALB/c mice, aged 3 to 4 weeks, were obtained from Bantin & Kingman Ltd. Mice were kept for 1 week before inoculation. All mice and foal infections were carried out using virus working stock suspended in EMEM, the precise dose being established by titration of surplus inoculum. Control animals received a similar cell suspension of uninfected, sonicated RK-13 cells.

**Injection of mice and foals.** Intranasal (i.n.) inoculation of mice was carried out following light anaesthesia with ether. Mice were held ventrodorsally and the inoculum (40 µl) was pipetted slowly onto the nares until all the inoculum was inspired. For intracerebral (i.c.) inoculation, mice were again anaesthetized with ether and the inoculum (20 µl) was introduced through a 25-gauge hypodermic needle into the left cerebral hemisphere. Foal inoculations were carried out while animals were restrained manually. The inoculum (in 4 ml EMEM) was then administered i.n. into both nares using a Pasteur pipette. All mice and foal infections were carried out using virus working stock suspended in EMEM, the precise dose being established by titration of surplus inoculum. Control animals received a similar cell suspension of uninfected, sonicated RK-13 cells.

**Virus isolation from mice and foals.** Mice tissues (lung, trachea, eye, CNS samples) were taken post-mortem and stored briefly on ice in 1 ml virus isolation medium (EMEM supplemented with additional antibiotics). Tissues were homogenized, sonicated at 4 °C, and then centrifuged at 3000 r.p.m. for 10 min. Supernatants were diluted in further aliquots of virus isolation medium and then plated onto RK-13 monolayers. Virus was allowed to adsorb for 60 min at 37 °C, after which medium containing 1% carboxymethylcellulose was added. When viral c.p.e. was visible, usually after 2 to 3 days, plates were stained with crystal violet and plaques counted.

Mucus samples from foals were obtained from the nasal cavity and nasopharynx by gentle suction using a foot-operated vacuum pump connected to mucus extractors (Unoplasm). Samples were diluted into 1 ml virus isolation medium, mixed thoroughly on a vortex mixer, sonicated at 4 °C and centrifuged at 3000 r.p.m. for 10 min. Supernatants were titrated as for mice tissues.

Viraemia was assessed in mice and foals by means of infectious centre assay. Blood was collected from foals by jugular venepuncture and from mice by direct cardiac puncture post-mortem using 2 mg/ml EDTA as an anticoagulant. Samples were centrifuged in microfuge tubes and the buffy coats were isolated. They were treated with distilled water to lyse the red blood cells, after which isoelectricity was restored using 10-fold normal strength PBS. White blood cells were washed to remove cell debris, cells were counted in a haemocytometer and known numbers were then plated out onto RK-13 monolayers. Thereafter, samples were treated as for virus isolation from other samples as above, except that plaques usually took longer to develop. The lower limit of this test is detection of one positive cell in 10⁴ to 10⁵ white blood cells.

No virus was recovered from any samples taken from uninfected, control mice or foals prior to experimental infection.

**Fluorescent antibody test.** Samples of white blood cells were spun down onto glass slides (cytospins), air-dried and fixed in ice-cold acetone. These samples were used for indirect immunofluorescence staining of EHV-1 antigen-containing cells as described in Awan et al. (1990). Briefly, the cells were reacted with polyvalent anti-EHV-1 hyperimmune serum raised in rabbits and then sheep anti-rabbit Ig
fluorescein isothiocyanate conjugate. Preimmune rabbit serum was used as a negative control.

Results

Characteristics of wt EHV-1 and PR1 in vitro

The growth rates of wt EHV-1 and PR1 were compared in vitro in RK-13 cells. The viruses were inoculated onto 5 cm Petri dishes at an m.o.i of 0.1. Dishes were sampled at set time points following infection. Cell-associated virus and virus free in the supernatant were measured separately. No significant difference was observed between wt EHV-1 and PR1 in the rate of virus production, release or yield/cell (data not shown). The final yield of PR1 was 10 p.f.u./cell. The TK activity of PR1 was compared to that of wt EHV-1 in RK-13 cells. In contrast to wt virus, the mutant induced no measurable TK activity (data not shown).

Intranasal inoculation with wt EHV-1 or PR1

Two groups of mice, 12 in each group, were inoculated with wt EHV-1 or PR1 at $5 \times 10^6$ p.f.u./mouse; five mice were inoculated with a suspension of uninfected RK-13 cells and kept as uninfected controls. Following infection, the group given wt EHV-1 showed typical signs of EHV-1 infection: weight loss, ruffled fur and respiratory distress, with two deaths (Fig. 2a). Only transient clinical signs were observed following PR1 infection: there were no deaths, weight loss occurred over the first 2 days post-infection (p.i.) only, and thereafter the mice appeared normal. No clinical signs were observed in the control mice.

Three mice in each infection group were killed on days 1, 3, 5 and 8 p.i. and virus titres in turbinates and lungs were measured by plaque assay. Mice infected with wt EHV-1 or PR1 showed similar high titres in both turbinates and lungs over the first 3 days p.i. (Fig. 2b, c). Thereafter, no virus was detected in tissues from mice infected with PR1; by contrast, mice given wt EHV-1 continued to show the presence of replicating virus for 8 days p.i.

Infectious centre assays were used to detect viraeemic cells. Following wt EHV-1 infection, mice were viraeemic on several days, at titres of $> 1/10^9$ white blood cells; no viraeemia was detected following infection with PR1.

Intracerebral inoculation of mice with wt EHV-1 and PR1

Dose-dependent mortality was observed following i.c. inoculation of mice (10 in each group) with wt EHV-1 (Fig. 3a). Thus mice given $4 \times 10^6$ p.f.u. of EHV-1 began to die within 3 days p.i. (Fig. 3a). When mice were given $10^7$ p.f.u./mouse by i.c. route, all mice died within 3 days p.i. (Fig. 3a).

Fig. 2. I.n. inoculation of mice with wt EHV-1 or PR1. (a) Percentage weight change after inoculation with wt EHV-1 (●) or PR1 (×). Virus titres in (b) the turbinates and (c) lungs. All mice were given $5 \times 10^6$ p.f.u./mouse i.n. of either wt EHV-1 (black bars) or PR1 (hatched bars); there were 12 mice in each group and two mice given wt EHV-1 died. Each point represents the mean±s.d. and each bar represents the mean±s.d. (n = 3); arrows indicate that no virus was isolated.
to show neurological disease after 18 to 24 h, with symptoms including weight loss, huddling, ataxia and tremors. Deaths started to occur on day 1 p.i. and by 72 h all mice had died. By contrast, mice inoculated with $4 \times 10^3$ p.f.u. showed no clinical signs and none died. Control mice given i.c. injections of uninfected RK-13 cells or medium, or virus at similar titres but via an intravenous route, also remained clinically normal and no virus could be detected in target organs.

To show that wt EHV-1 could establish productive infection in the CNS following i.c. inoculation, virus titres were measured in various organs (cerebrum, cerebellum, olfactory bulb, brainstem, eye, turbinate, lung and blood). Results are illustrated for an inoculum of $4 \times 10^4$ p.f.u./mouse (Fig. 3b). Infective virus was found in the CNS for 5 days p.i. (after which all mice had died). Virus was also present in peripheral tissues, with particularly high titres present in turbinates and lower titres in lungs. No viraemia was detected. The spread to turbinate and lung appeared to be a direct result of the inoculation technique: dye inoculated i.c. at similar volumes was found in turbinate and lung within 30 min.

The effect of i.c. inoculation of PR1 was also examined. In this experiment, two groups of 10 mice were inoculated i.c. with either PR1 or wt EHV-1, both at titres of $4 \times 10^5$ p.f.u./mouse. As before, following infection with wt EHV-1, mice became ill, showing neurological signs and mortality (Fig. 4a). All were dead by 96 h p.i. By contrast, the group given PR1 i.c. showed minimal clinical signs (ruffled fur and huddling) on days 1 and 2 p.i. and thereafter were normal. No mice in this group died (Fig. 4a).

Virus titres in various organs following i.c. inoculation with PR1 are shown in Fig. 4(b). High titres of virus were observed initially on day 1 p.i., but at a lower titre compared to those seen with wt EHV-1 (Fig. 3b). Similar to the situation following i.n. infection, PR1 did not persist as long, however, and no virus was recovered after day 2 p.i.
Ability of PR1 or wt EHV-1 to protect against challenge with wt EHV-1

Mice were divided into three groups of 15 and given an i.n. inoculation of wt EHV-1 or PR1 (both at a dose of 5 x 10^6 p.f.u./mouse) or a suspension of uninfected RK-13 cells. Clinical signs were similar to those described above: severe disease with wt EHV-1 including three deaths, minimal disease and no mortality with PR1 and no clinical signs with uninfected RK-13 cells. Five weeks after primary inoculation, all surviving mice were challenged with an i.n. infection of wt EHV-1 (6 x 10^6 p.f.u./mouse).

Mice given primary inoculation of wt EHV-1 or PR1 developed only mild clinical signs on challenge. No weight loss occurred and by day 2 p.i. mice were clinically normal (Fig. 5a). By contrast, mice previously given RK-13 inocula all developed severe disease on challenge with live virus and showed marked weight loss, respiratory signs and mortality (three deaths) (Fig. 5a).

Virus titres following challenge are shown in Fig. 5(b, c). In mice given primary inoculations with wt EHV-1 or PR1, virus was observed on day 1 p.i. only. By contrast, mice given a primary inoculation of RK-13 cells had high titres of virus in both turbinates and lungs for 5 days p.i. On challenge, viraemia (titre > 1/10^6 white blood cells) was observed in this group of mice only, and not in the mice given primary inoculations with wt EHV-1 or PR1.

Intranasal inoculation of SPF foals: primary infection with PR1 and challenge with wt EHV-1

The difference in pathogenicity of PR1 and wt EHV-1 in mice encouraged us to investigate the effects of PR1 in SPF foals. These animals are free from EHV infection and maternally acquired antibody at the start of the experiment and their responses to EHV are very predictable. This allows the use of small numbers of animals to test different inocula or experimental protocols.

Four SPF foals were divided into two groups. Two animals (F11 and F13) were inoculated i.n. with wt EHV-1 (10^7 p.f.u./foal) and two (F9 and F10) received PR1 (10^7 p.f.u./foal). The response of the two animals given wt EHV-1 was similar to that described previously (Gibson et al., 1992a). The foals showed typical clinical signs of EHV-1 infection, comprising depression, inappetence, nasal and ocular discharge, enlarged sub-turbinates and (c) lungs, after inoculation with PR1 (hatched bars), wt EHV-1 (grey bars) or RK-13 cells (black bars). Bars represent mean ± s.d. (n = 3); arrows indicate that no virus was isolated.
Table 1. Clinical signs in four SPF foals following primary inoculation with either wt EHV-1 or PR1

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*Clinical signs on mucous membranes: 0, normal (pink); +, red; + +, red, serous; + + +, red, mucopurulent, granular.
†Clinical signs in ocular and nasal discharge: 0, normal (none); +, serous; + +, mucopurulent; + + +, purulent.
‡Clinical signs in lymph nodes: 0, normal (not palpable; the nodes are not palpable in uninfected SPF foals, in contrast to naturally reared horses); +, palpable; + +, very enlarged.
§ND, Observations not made.

mandibular lymph nodes and elevated rectal temperature (Table 1; Fig. 6a). By contrast, clinical signs in the foals F9 and F10 given PR1 were markedly less severe (Table 1). An elevated rectal temperature was noted in one (F10) of the two foals on day 1 only (Fig. 6a).

Virus shedding in nasal mucus was monitored in both groups (Fig. 6b). In the first 5 days following infection, all foals excreted virus at high titres. Thereafter no virus was recovered from foals F9 and F10 infected with PR1 whereas the animals given wt EHV-1 (F11 and F13) continued to excrete virus for 11 days. Furthermore, viraemia (1/10⁵ cells) was detected in the foals given wt EHV-1 on several days p.i. No viraemia was apparent by infectious centre assay in the animals given PR1, although cells positive by the fluorescent antibody test against EHV-1 antigens were seen in cytopsins of the buffy coat.

At 103 days after primary infection with PR1, the two foals F9 and F10 were given a secondary i.n. inoculation with wt EHV-1, at a titre of 10⁷ p.f.u./foal. In contrast to a primary EHV-1 infection, no clinical signs were observed, and this included the absence of pyrexia. Virus excretion was markedly reduced in comparison with a primary inoculation of wt EHV-1 (Fig. 6b). In one foal
Pathogenicity of EHV-1 TK-deficient mutant

Fig. 6. i.n. inoculation of SPF foals with wt EHV-1 or PR1. Four foals were given primary i.n. inoculation with either wt EHV-1 [F11 (+) and F13 (×)] or PR1 [F9 (□) and F10 (■)] at 10^7 p.f.u./foal. (a) Rectal temperature. (b) Virus titre (log_{10} p.f.u./sample) in nasal mucus following primary inoculation. (c) Virus titre (log_{10} p.f.u./sample) in nasal mucus taken from foals F9, F10 and F13 following challenge with an i.n. inoculation of wt EHV-1 (10^7 p.f.u./foal) 103 (F9 and F10) or 84 (F13) days after the primary inoculation with PR1 or wt EHV-1.

(F10), virus was recovered from nasal mucus on day 1 p.i. only; in the other (F9), excretion was also observed at later time points (Fig. 6c). Both foals became viraemic (1/10^6 cells) and the virus was typed as TK+ EHV-1. For comparison, foal F13 was also re-infected with wt EHV-1 (titre 10^7 p.f.u. i.n.). Again, no clinical signs were observed; virus was detected in nasal mucus on day 1 p.i. only, at very low titre (10^1 p.f.u./sample; Fig. 6c); and no viraemia was observed (Gibson et al., 1992a).

Discussion

In this paper, we report the pathogenicity of a TK-deficient mutant of EHV-1 and its ability to protect against secondary infection with wt EHV-1 in two infection models, mice and SPF foals. The tk gene is implicated in neuropathology of various alphaherpesviruses, including HSV-1 and SHV-1 and has a role in latency and reactivation (Field & Wildy, 1978; Becker et al., 1984; Kit, 1990). These attributes have led to TK-deficient mutants being proposed as potential agents of immunoprophylaxis or as a basis for the construction of recombinant vaccines expressing alternative antigens (Kit et al., 1985, 1986; Cole et al., 1990; Post et al., 1990).

The mutant described in this paper was produced by selection for resistance to the nucleoside analogue penciclovir (Fuente et al., 1992). Penciclovir is phosphorylated selectively by herpesvirus TK and its triphosphate interferes with herpesvirus DNA polymerase (Vere-Hodge, 1992). The compound is not mutagenic and thus growth of virus in the presence of penciclovir would not be expected to select for mutations in sites other than the tk gene or DNA polymerase gene. Three mutants were obtained in this way: PR1, PR2 and PR3. Two of these, PR2 and PR3, appear to have arisen by point mutations in the tk gene (Corrochano et al., 1993). The third, PR1, however, has been shown to contain an 879 bp deletion in the tk gene (Fig. 1; Corrochano et al., 1993) and is the subject of this paper. In vitro, the PR1 mutant induces no measurable TK activity, as predicted. Its growth characteristics, including yield/cell and also release of virus into supernatant, were very similar to the parent wt EHV-1 in actively dividing tissue culture.

Following i.n. inoculation of mice, PR1 produced markedly reduced clinical signs compared to wt EHV-1, and no mortality was observed. Virus replication did occur, however, and high titres of virus were found in lungs and turbinates for 3 days p.i. (Fig. 2). It is possible, but unlikely, that this represents residual inoculum. Thus the virus is present at relatively high titres and, in other experiments, for example with mice inoculated i.n. with EHV-4, virus disappears much faster. After day 3 p.i., in contrast to wt EHV-1, PR1 was rapidly cleared. In addition, whereas viraemia was detected following wt EHV-1 infection, none was apparent following infection with PR1.

Less marked differences between wt virus and mutant were noted previously with PR3, one of the other TK-deficient mutants derived from Ab4 (Fuente et al., 1992). Following i.n. inoculation into mice, PR3, in contrast to
PR1, produced a similar level of clinical disease compared to wt EHV-1. Although mortality was reduced, it remained significant. Similarly, replication of PR3 was higher than that observed for PR1, and furthermore PR3 was able to establish viraemia (Fuente et al., 1992).

The reason for the disparate behaviour of the two mutants PR1 and PR3 remains unclear. PR3 seems to have arisen by point mutation from wt EHV-1 and reversion to wt phenotype is a possibility. Virus recovered from the lungs of infected mice, however, retained resistance to penciclovir and also lacked TK activity (Fuente et al., 1992). Both these observations suggest that the mutant remained TK-deficient. A second possibility is that the pathogenicity conferred by the tk gene does not reside in the ability of TK to phosphorylate thymidine but in some other property of the protein. A third and related reason could be that deletion within the tk gene interferes with other functions of the virus genome. For instance, the promoter of UL35 may reside within this region of the genome (Robertson & Whalley, 1988; Telford et al., 1992); UL35, however, encodes an unknown protein, but one which is essential for growth in tissue culture, a characteristic unaffected in PR1 compared to wt EHV-1. Fourth, PR1 may contain other mutations outside the tk gene, for example in the DNA polymerase. To address these questions, we are currently constructing a recombinant virus from PR1 that expresses TK activity.

The ability of primary i.n. inoculation with wt EHV-1 or PR1 to protect against subsequent challenge with wt EHV-1 was also studied. Five weeks after primary infection, mice were protected against secondary i.n. inoculation with wt EHV-1. Minimal clinical signs were observed and no deaths occurred (Fig. 5a). Virus was more rapidly cleared in comparison to unprimed control animals (Fig. 5b, c) and no viraemia was detected.

We have shown previously that mice given a primary i.n. inoculation of wt EHV-1 remain partially protected from challenge 5 months later (Field et al., 1992a). Reduction in virus titres, however, was less than that observed with the challenge described here after 5 weeks, and mice did become viraemic. These findings can be compared to observations in the natural host, which appears to retain limited protection against re-infection, but for a short duration (Allen & Bryans, 1986). Recently, we have obtained evidence that the murine immune response to EHV-1, like that of the natural host, comprises both antibody and T cell responses, but that cell-mediated immunity predominates in the control or otherwise of infection (Azmi & Field, 1993).

Although EHV-1 is not thought to infect neurons, we have shown previously that i.c. inoculation of mice with wt EHV-1 causes rapid death and that the antiviral agent (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine was able to protect the inoculated mice partially (Gibson et al., 1992c). The effect of i.c. inoculation was dose-dependent: a dose of $4 \times 10^3$ p.f.u./mouse is rapidly fatal, whereas $4 \times 10^5$ p.f.u./mouse or high titres of heat-inactivated virus ($4 \times 10^5$ p.f.u./mouse) result in no mortality (Fig. 3), substantiating the conclusion that the effect is virus-specific. It is not clear, however, how i.c. inoculation of virus produces its clinical effect. Limited neuropathology, including mild encephalitis and meningoencephalitis, was observed following i.c. inoculation, but its extent was very localized. No data have been obtained to indicate widespread productive infection of neurons or glia, contrary to the situation following i.c. inoculation of neonatal mice (Patel et al., 1982) or following infection with HSV-1 (Anderson & Field, 1983). PR1, in contrast to wt EHV-1, was markedly less pathogenic following inoculation by this route. No deaths occurred and clinical signs were minimal by day 1 p.i. (Fig. 4). We speculate that this observation may provide important clues to understanding the neuropathology of wt EHV-1 in horses. In this context, although we have not observed gross neurological deficits in SPF foals following infection with wt EHV-1, subtle neuropathology is evident. Thus SPF foals infected with wt EHV-1 develop choriorretinal lesions and neurohistopathological damage, as shown by mononuclear cell cuffs and optic nerve Wallerian degeneration (Slater et al., 1992). No such changes were detected in the two foals inoculated with PR1 (see below).

In our final experiment, we compared the effects of i.n. inoculation of PR1 and wt EHV-1 in SPF foals. These animals are specifically free from EHV and also maternal antibodies. Their response to EHV-1 has been shown to be very predictable, allowing us to make valid observations using a small number of foals (Gibson et al., 1992a, b). Our results show that primary i.n. inoculation with PR1 is less pathogenic than that with wt EHV-1. Clinical signs were milder; virus was shed in nasal mucus at high titres but only for 5 days p.i. (Fig. 6). Excretion of wt EHV-1 continues for longer and is notably biphasic (Gibson et al., 1992a). In addition, no viraemia was detected by infectious centre assay following infection with PR1, in contrast to that with wt EHV-1. Buffy coat cells bearing EHV-1 antigen, however, were demonstrated in cytospins by the fluorescent antibody test. It is possible, therefore, that PR1 can gain access to peripheral blood cells but is unable to carry out a productive infection, a theory that will be investigated further using the PCR technique.

The serological responses of the SPF foals inoculated i.n. with PR1 were also measured. In contrast to wt EHV-1 infection, titres of virus neutralization and complement fixation antibodies were very low (D. Tewari, J. S. Gibson, T. O’Neill, D. Hannant, G. P.
Our results show that despite the clinical protection and amelioration of virus shedding, superinfection with wt EHV-1 after primary inoculation with PR1 resulted in viraemia. Infection of white blood cells is thought to be central to EHV-1 pathogenesis and, therefore, this observation has obvious consequences in the use of TK-deficient mutants in immunoprophylaxis. The criteria for vaccine efficacy must be very carefully chosen. Thus although previous reports of TK-deficient mutants of EHV-1 have shown that the mutant can be administered to pregnant mares without ill effect and stimulate a serological response (D. Tewari, J. S. Gibson, T. O'Neill, D. Hannant, G. P. Allen & H. J. Field, unpublished results), they did not establish any protective ability against infection with wt EHV-1 (Cornick et al., 1990). Our observations suggest that protection, at least following PR1 infection, may not be complete.

In conclusion, the results contained in this paper demonstrate that PR1, administered via i.n. or i.c. inoculation, is less pathogenic than wt EHV-1 in both mice and SPF foals. Infection does provide a degree of protection against superinfection with wt EHV-1. Protection was not complete, however, and the evaluation of TK-deficient mutants of EHV-1 for immunoprophylaxis requires further work.

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