Defective entry of herpes simplex virus types 1 and 2 into porcine cells and lack of infection in infant pigs indicate species tropism

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We have determined if a defect at entry of the human pathogen herpes simplex virus type 1 (HSV-1) into cultured porcine cells extends to HSV-2 and if the poor susceptibility of porcine cells for these viruses is indicative of in vivo species tropism. HSV-1 replicates poorly in swine testis (ST) and other porcine cells which lack a functional non-heparan sulphate receptor(s) required for virus entry. By several criteria, ST cells resist infection by either HSV-1 or HSV-2. Infection can be restored if normal entry is bypassed by PEG-mediated virion-cell membrane fusion. Neither HSV serotype infects, replicates or produces clinical symptoms in infant pigs. No virus was isolated from any of multiple sites and seroconversion did not occur. The in vitro defect in porcine cells blocking HSV entry correlates with, and is likely to be at least partly responsible for, in vivo resistance of pigs to infection.

Herpes simplex virus (HSV) and pseudorabies virus (PRV) are related alphaherpesviruses that have a broad cell host range, many collinear genes and similar replicative cycles and clinical outcome in their natural hosts (Roizman & Sears, 1990; Wittman & Rziha, 1988). HSV-1 and -2 are human pathogens that cause clinical disease involving mucocutaneous surfaces, the central nervous system (CNS) and occasionally visceral organs (Corey, 1991). PRV is a natural pathogen of swine that causes a contagious, epidemic disease characterized by CNS and upper respiratory tract infection (Wittman & Rziha, 1988). PRV is also neurotropic and disease in infant pigs results in high mortality. PRV does not cause clinical infection in humans and infection has not been proven virologically or serologically in suspected cases (Hussell et al., 1963; Jenzsch & Apostoloff, 1970; Mbravak et al., 1987).

The outcome of virus infection is determined by the interactions between the virus and host cell constituents. The cellular or molecular basis for the broad host range and the natural mammalian hosts for these herpesviruses is not understood. Recent studies indicate that determinants of tropism occur in the cell surface receptors used for HSV-1 entry into porcine cells (Subramanian et al., 1994) and at replication post-entry for PRV into some human cells (G. Subramanian et al., unpublished results). The major obstacles to understanding HSV infection, pathogenesis and tropism have been the unavailability of a cell line that lacks the surface component(s) required for virus entry and the lack of a non-susceptible animal model for HSV infection. In this report we show that the poor susceptibility of cultured porcine cells extends to HSV-2, also because of a defect in entry. Furthermore, the in vitro restriction to HSV correlates with lack of HSV replication and no clinical infection of infant pigs in vivo, indicating that cellular receptor(s) contribute to HSV species tropism.

Yields of infectious HSV-1 and HSV-2 from swine testis (ST) cells were three to four orders of magnitude lower over time than from human embryonic lung (HEL) cells (Fig. 1a). Exposure to either HSV-1 or HSV-2 produced extensive CPE by 36 h post-infection in HEL cells but minimal CPE in ST cells (data not shown). PRV-infected ST cells also showed extensive CPE and produced approximately 10^8 p.f.u., comparable to the high virus yields observed from HSV-infected HEL cells. Even at a 300-fold higher HSV input (Fig. 1a), few foci of infection were observed in ST cells. Virus yields increased, but titres were still two orders of magnitude below the yields from low input virus into human cells.

Infectious cell centres of ST and HEL cells were compared to determine the number of cells that became infected with HSV (Fig. 1b). The number of HSV-1- or HSV-2-infected ST cell centres was about two orders of magnitude below those for HEL cells (Fig. 1b). For HSV-1, 0.16% of cells in ST monolayers were infected, compared to 36.3% of HEL cells. For HSV-2, 0.4% of
Short communication

Fig. 1. Infectious yields and infectious centres of HSV-1 and HSV-2. (a) Confluent cell monolayers of HEL (open symbols) or ST (closed symbols) were infected with 0.01 (solid line) or 3.0 p.f.u./cell (dashed line) of HSV-1 (squares) or HSV-2 (circles) for 90 min at 37 °C. After removal of inoculum, cells were overlaid and harvested at the indicated times post-infection. Virus yields as total infectious units were titred in duplicate on Vero cell monolayers. (b) Effects of PEG on HSV infectious centres were determined. HEL (narrow striped bars) or ST (broad striped bars) cells at 1 x 10^6 were infected with HSV-1 (dark bars) or HSV-2 (light bars) at 2-0 p.f.u./cell for 90 min at 37 °C. After removal of inoculum, monolayers were exposed to PEG or washed with PBS (control). Residual virus was inactivated with 1 M-citrate buffer pH 3 for 2 min. After 3 h infection, the cell were trypsinized and infected cells titrated on Vero monolayers using a methylcellulose overlay.

ST and 52.5% of HEL cells were infected. PEG-mediated membrane fusion increased infected ST cell centres to levels similar to those from human cells and indicated that ST cells were defective in HSV entry (Fig. 1b). There was no significant increase in HSV infectious centres for susceptible HEL cells. Also, transfection of virus DNA into porcine cells produces high levels of infectious virus (Subramanian et al., 1994). Thus ST cells could support virus replication if entry was permitted. Consistent with lack of entry and no expression of virus genes, PAGE analyses showed no synthesis of virus proteins or shut-off of cellular proteins after HSV-1 or HSV-2 exposure to ST cells (data not shown).

Cells exposed to both HSV serotypes were examined for the presence of input or newly synthesized virus DNA by Southern hybridization (Fig. 2). At 40 p.f.u./cell, when high virus DNA levels were detected in susceptible HEL cells, input HSV DNA could not be detected in ST cell nuclei (Fig. 2a). Newly synthesized HSV DNA was found after 24 h in HEL cells infected with either virus serotype (Fig. 2b; lanes 2, 3, 9, 10). For poorly susceptible ST cells, at low virus input and under stringent hybridization and wash conditions, no HSV-1 or HSV-2 DNA was detected (Fig. 2b; lanes 5, 12, 13). Small amounts of HSV-1 DNA (less than that using 0.01 p.f.u. into HEL cells; Fig. 2b; lane 3) were found in ST cells with 300-fold more input virus (3 x 10^6 p.f.u./cell; Fig. 2b; lane 6). The presence of a few susceptible cells in heterogeneous ST cell lines would account for this level of newly synthesized DNA (Subramanian et al., 1994; A. Perez et al., unpublished results).

We examined HSV infectivity in infant pigs and primary cells isolated from lung tissue to determine the relevance to virus tropism in vivo of the entry defect in cultured porcine cell lines. Primary porcine lung cells also produced at least three orders of magnitude lower HSV-1 and HSV-2 yields than control HEL cells (data not shown). To explore infectivity in vivo, 3-day-old pigs were inoculated intranasally with HSV-1, HSV-2 or PRV and examined over 3 weeks for evidence of clinical infection. Piglets less than 2 weeks of age are most susceptible to infection by PRV (Wittman & Rziha, 1988). By the natural route of infection through intranasal mucosal surfaces, which also allows neuronal invasion and spread, less than 10^3 p.f.u. of virus results in 100% mortality in piglets (Wittman & Rziha, 1988). Three-day-old piglets from four litters were inoculated intranasally with HSV-1 (1 x 10^6 p.f.u.) or HSV-2 (6 x 10^6 p.f.u.; Table 1). Piglets inoculated with 10^3 p.f.u. of PRV developed fever, severe respiratory and CNS disease and died within 3 days. However, none of the piglets inoculated with HSV-1 or HSV-2 developed clinical signs of disease. Temperatures of piglets and sows remained in the normal range. From pharyngeal swabs obtained on a daily basis for 10 days, virus was obtained only at day 2 post-inoculation from one piglet inoculated with HSV-1. All subsequent samples from this animal were negative suggesting that the single isolation could be survival of input virus rather than replication.

Enzymatic or physical dispersion of tissues followed by prolonged cultivation on a susceptible cell monolayer
Fig. 2. Southern blot analyses to detect input or newly synthesized virus DNA. (a) Cells were infected with HSV-1 or HSV-2 at 40 p.f.u./cell and nuclei isolated at either 2 h or 30 min to examine input virus DNA. (b) Cells were mock-infected (M) or infected with HSV-1 or HSV-2 at 0.01 p.f.u./cell or 3 p.f.u./cell as indicated for 24 h to detect replicated DNA. After DNA was extracted and quantified, virus DNA was detected by Southern hybridization analyses. One μg total DNA was digested with BamHI and probed with 32P-labelled DNA of the HSV-1 gD gene. The filters were washed twice at 65 °C in 0.1% SSC and 0.5% SDS, and exposed to film at -70 °C. Also, 25 ng of BamHI-digested HSV-1 DNA was loaded as a control (lanes C). The large arrow indicates HSV-1 DNA restriction fragments and the two smaller arrows show HSV-2 DNA fragments. The HSV gD nucleotide sequence was used to probe for virus DNA in infected cells. A unique BamHI restriction site in gD-2 allows HSV-2 to be differentiated from HSV-1.

Table 1. Clinical response in animals inoculated with HSV-1, HSV-2 and PRV

| Virus | Daily rectal temperature\* | Clinical symptoms | Daily nasal swabs | Internal organs
<table>
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<tbody>
<tr>
<td>HSV-1</td>
<td>N (21/21)</td>
<td>None (0/21)</td>
<td>(11/18)</td>
<td>(0/6)</td>
</tr>
<tr>
<td>HSV-2</td>
<td>N (14/14)</td>
<td>None (0/14)</td>
<td>(0/11)</td>
<td>(0/4)</td>
</tr>
<tr>
<td>PRV</td>
<td>Febrile (3/3)</td>
<td>(3/3)</td>
<td>(3/3)</td>
<td>(3/3)</td>
</tr>
</tbody>
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\* N refers to rectal temperatures within normal limits (38.3-40 °C). Febrile temperatures were > 40 °C.
† A single swab from a piglet from which virus grew at day 2, but subsequent swabs were negative.
‡ The number of homogenized tissue cultures that grew virus over the number of pigs from which multiple tissues were obtained. Only lymph node homogenates were cultured for PRV-infected pigs.

is a well established method for detecting the presence of herpesvirus in tissues or cells (Ben-Porat & Kaplan, 1985; Wittman & Rziha, 1988). At 3, 5 or 7 days post-inoculation, one piglet from each litter was euthanized and virus isolation attempted from different tissues. No virus was isolated from the turbinates or pharyngeal lymph nodes (primary site of replication), olfactory bulb, cerebrum or cerebellum (CNS), lung, liver, spleen or adrenals (viscera) of euthanized animals (Table 1). The absence of clinical symptoms and isolated virus indicated that infant pigs were resistant to clinical infection by both HSV-1 and HSV-2.

Production of antibodies to infectious pathogens is diagnostic of virus replication in the animal host. Both HSV and PRV are strongly immunogenic in their natural hosts (Ben-Porat & Kaplan, 1985; Wittman & Rziha, 1988; Roizman & Sears, 1990). Infant pigs are capable of mounting an immune response by 3 days of age (Wittman & Rziha, 1988). Serum samples were obtained prior to inoculation, or at 21 days thereafter, from 15 infant piglets and two sows exposed to HSV-1, and 10 piglets and two sows exposed to HSV-2. There were no detectable levels of antibodies reactive to HSV-1 or HSV-2 by an ELISA that could detect less than 0.05 μg/ml of control HSV-reactive antibody. Indicative of the sensitivity of the ELISA, background levels of non-specific transient maternal antibodies in piglets were detectable in pre-inoculation serum and were higher at 3 days post-inoculation than at 21 days. That infant pigs do not seroconvert to HSV supports the observation of poor virus replication in primary porcine cells and lack of clinical infection or virus replication in piglets. These indicate that pigs are not susceptible to HSV infection.

The natural animal hosts of HSV and PRV are man and swine, respectively. Pathogenesis of HSV in neonates and PRV infection in infant pigs can involve virus replication in the naso/oropharyngeal region followed by invasion of the CNS by a neural pathway. Large amounts of virus are detected at sites of primary virus replication, especially in the tonsils and pharyngeal lymph nodes, and lower amounts in the CNS and other organs (Corey, 1991; Wittman & Rziha, 1988). Like HSV infection in neonates (Kohl, 1987), clinical infection by PRV in new-born piglets causes severe ulcerative tonsillitis at the site of inoculation, fever, lethargy, loss of coordination and convulsions. Despite similarities in
many clinical, physiological and biochemical characteristics, differences in interactions with host cell components are responsible for viral pathogenesis and unique species tropism. Poor PRV replication in most cultured human cells (G. Subramanian & A. O. Fuller, unpublished results) correlates with lack of clinical disease in humans (Hussell et al., 1963; Jenzsch & Apostoloff, 1970). We have recently determined that a defect in HSV entry is due to lack of a functional non-heparan sulphate (HS) receptor(s) on porcine cells in vitro (Subramanian et al., 1994). This may also be the cause of lack of HSV-1 and -2 replication in vivo in primary porcine cells and lack of infection and clinical disease in infant pigs.

Cell surface receptors can determine virus tropism and pathogenesis (Racaniello et al., 1993). Receptors required for efficient entry of HSV that are not functional on porcine cells (Subramanian et al., 1994) may determine tropism of HSV-1, HSV-2 and PRV for animals. Entry of these viruses at neutral pH occurs after multiple steps of attachment (Fuller & Lee, 1992; Karger & Mettenleiter, 1993; McClain & Fuller, 1994), initially to cell surface HS receptors (Mettenleiter, 1994; Sawitzky et al., 1990; Spear, 1993) and subsequently to an unidentified non-HS receptor(s) (Lee & Fuller, 1993; McClain & Fuller, 1994; Spear, 1993). Porcine cell lines contain functional HS receptors and their poor susceptibility for HSV is determined at the level of non-HS cell receptor(s) (Subramanian et al., 1994). Primary cells of the native animal host for PRV that are poorly susceptible to HSV are probably missing a cellular receptor required for HSV entry.

Because HSV-1 and HSV-2 are closely related, it is perhaps not surprising that poor susceptibility of porcine cells for HSV-1 extends to HSV-2. On the other hand, HSV-2 behaves more similarly to PRV in cultured cells, in that CPE is observed earlier and plaque morphologies are similar on Vero cells (Ben-Porat & Kaplan, 1985; Mettenleiter, 1994; Spear, 1993; G. Subramanian et al., unpublished results). Also, the extent of DNA similarity between PRV and HSV-2 is greater than between PRV and HSV-1. Several reports have produced different results when determining whether both HSV serotypes bind common receptors on susceptible cells (Johnson et al., 1990; Spear, 1993; Vahine et al., 1979). PRV can saturate a receptor on Vero cells that is not HS and that can be bound by HSV-1 (Lee & Fuller, 1993). That PRV efficiently enters both porcine and human cells (G. Subramanian et al., unpublished results) indicates use of multiple and some common non-HS receptors by PRV, HSV-1 and HSV-2.

HSV is the most common cause of fatal, sporadic encephalitis in infants and immunocompromised hosts and has a mortality rate of over 75% if the infection is untreated (Behrman et al., 1987; Kohl, 1987). The majority of neonatal HSV disease is caused by HSV-2. Three-day-old piglets are not susceptible to infection by either HSV serotype (Table 1). The HSV inoculum of greater than 10⁶ P.f.u. was 1000-fold in excess of the PRV inoculum that causes 100% mortality in piglets (Table 1; Wittman & Rziha, 1988). None of the animals inoculated intranasally (the most common portal of virus entry with PSV) showed evidence of clinical infection, virus replication or seroconversion. This suggests an inability of HSV to infect and replicate in pigs. Poor susceptibility of cultured cell lines and primary porcine cells is indicative of events which occur in vivo with infant pigs.

Recent reports about poliovirus entry have resulted in a transgenic mouse model to study poliovirus pathogenesis, tissue tropism and neurovirulence (Racaniello et al., 1993; Ren et al., 1990). Poliovirus infection of primates and primate cell cultures is determined at the level of the cell receptor (Mendelsohn et al., 1989). Introduction of human genomic DNA containing the poliovirus receptor (PVR) gene into non-susceptible mouse L cells resulted in susceptibility to virus infection. Transgenic mice expressing the human PVR that are susceptible to poliovirus have proven extremely useful in understanding the pathogenesis of poliomyelitis and for vaccine studies using attenuated poliovirus (Racaniello et al., 1993; Ren et al., 1990). A non-susceptible animal model for HSV has not previously been available. In one approach to identify the non-HS receptor(s) for HSV, we found that transfusion of human DNA increases porcine cell susceptibility to HSV-1 infection (A. Perez et al., unpublished results). Further investigation of the findings reported here may eventually result in infant pigs that express critical receptor(s) in a transgenic animal system for study of entry, pathogenesis and tropism determinants for herpes simplex viruses. Examination of receptor use and pathogenesis will contribute to defining differences between HSV and PRV at a molecular level that account for their unique tropisms.

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References


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