Higher resistance of porcine trigeminal ganglion neurons towards pseudorabies virus-induced cell death compared with other porcine cell types in vitro

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Trigeminal ganglion (TG) neurons are important target cells for many alphaherpesviruses, constituting major sites for latency/reactivation events. Here, the in vitro kinetics of productive infection of the swine alphaherpesvirus pseudorabies virus (PRV) and resulting cell death in primary porcine TG neurons were determined, and these were compared with similar kinetics in many other porcine cell types. Confocal microscopy showed that all TG neurons expressed late genes such as viral glycoproteins, and that these glycoproteins were processed through the Golgi and reached the cell surface as in other cell types, albeit with a delay of ± 2–6 h. However, TG neurons were much more resistant towards PRV-induced cell death compared with all other porcine cell types tested (non-neuronal TG cells, superior cervical ganglion neurons, epithelial kidney cells, arterial endothelial cells, dermal fibroblasts and cells derived from a porcine swine kidney cell line). About half of the TG neurons survived up to 96 h post-inoculation (end of experiment), whereas all other cell types almost completely succumbed within 2 days post-inoculation. In addition, infection with a strongly pro-apoptotic PRV strain that misses the anti-apoptotic US3 protein did not lead to substantial apoptosis in TG neurons, even at 72 h post-inoculation. Thus, primary porcine TG neurons can be infected with PRV in vitro, and are remarkably more resistant to PRV-induced cell death compared with other porcine cell types, suggesting a cell type-specific resistance to alphaherpesvirus-induced cell death that may have important implications for different aspects of the virus life cycle, including latency/reactivation events.

INTRODUCTION

Alphaherpesviruses, such as pseudorabies virus (PRV) and herpes simplex virus type 1 (HSV-1) have developed an intriguing relationship with the nervous system (reviewed by Preston, 2000; Tomishima et al., 2001). They have the ability to infect and establish lifelong latent infections in sensory neurons innervating the primary site of replication. Both for PRV and HSV-1, primary infection takes place at the mucosal epithelium of the upper respiratory tract and trigeminal ganglion (TG) neurons innervating the epithelium are the predominant site of latency for both viruses. Entry into the nervous system occurs when the viral envelope fuses with the axolemma at or near the axon terminus. After fusion, the capsid, and possibly part of the tegument, is moved towards the neuronal cell body by means of microtubule-associated fast axonal transport (Bearer et al., 2000; Smith et al., 2004). Once the capsid has been delivered near the nuclear membrane, viral DNA enters the nucleus.

Viral DNA delivery to the nucleus of TG neurons may lead to either of two possible infection patterns. Either a full replication cycle is initiated, which occurs in a cascade-like manner: expression of immediate-early and early genes gives rise to viral DNA replication, followed by expression of late genes at the final stages of the replication-cascade. Alternatively, a latent infection can be established at the very early stages of the virus replication cycle, before the onset of DNA replication. After establishment of latency, stress-related stimuli may lead to virus reactivation and initiation of a full virus replication cycle. Both during primary infection and reactivation, full alphaherpesvirus replication is thought to result in rapid cell death (reviewed by Preston, 2000).

However, under physiological conditions, TG neurons should be able to postpone alphaherpesvirus-induced cell death following a full virus replication cycle long enough to allow newly produced virus to travel long distances towards axon termini. Furthermore, based on several indirect data, it has already been suggested that TG and other neurons may survive (limited) virus replication
during primary infection or reactivation, and enter or resume latency afterwards (Aleman et al., 2001; Geiger et al., 1995; Perng et al., 2000; Simmons & Tschanke, 1992). Taken together, this may suggest that TG neurons might have a higher resistance towards alphaherpesvirus-induced cell death compared with other cell types. However, the kinetic time-course of an alphaherpesvirus infection and the possible resulting cell death in TG neurons of its natural host has, to date, never been studied.

Therefore, the aims of this study were to examine the interaction between PRV, a swine alphaherpesvirus, and primary TG neurons of its natural host in vitro. More specifically we determined (i) whether a PRV infection in porcine TG neurons in vitro generally results in a typical full virus replication as assessed by evaluating the kinetics of expression and processing of viral glycoproteins and comparing these with similar kinetic studies in different other porcine cell types and, if so, (ii) whether TG neurons can resist cell death induced by PRV to some extent.

**METHODS**

**Viruses and cell culture.** PRV strains 89V87, NiA3 and US3 null NiA3 were used, and were described previously (Baskerville et al., 1973; Nauwynck & Pensaert, 1992; van Zijl et al., 1990).

**Cells**

Swine kidney (SK) and swine testicle (ST) cells. Cells were seeded on glass coverslips and grown to confluence in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 0·3 mg t-glutamine ml⁻¹, 100 U penicillin ml⁻¹, 0·1 mg streptomycin ml⁻¹ and 0·1 mg kanamycin ml⁻¹ (basic culture medium).

Superior cervical ganglion (SCG) cells. Primary neuronal cultures were prepared from SCG from porcine fetuses collected at the slaughterhouse (gestation stage between 6·5 and 10 weeks) as adapted from Wang et al. (1995).

**TG cells.** Primary neuronal cultures were prepared from TG from 4 to 10-week-old piglets. TG were dissected and longitudinally cut into two pieces before starting enzymic digestion with 0·2% collagenase A (Roche). Every 30 min, dissociated cells were harvested by centrifugation at 200 g for 5 min and the collagenase solution was re-used for further digestion of TG. After full digestion of the ganglia, the collected cell suspension was centrifuged at 200 g for 10 min. The pellet was resuspended in culture medium (basic culture medium without glutamine and supplemented with 30 mg nerve growth factor ml⁻¹) and cells were plated on poly-D-L-ornithine- and laminin-coated glass coverslips. One day after seeding, cells were washed extensively to remove non-adherent cells and fresh culture medium was added. Culture medium was replaced every 3–4 days.

**Epithelial kidney cells.** Primary porcine epithelial cells were isolated from kidneys from 4 to 10-week-old piglets. Briefly, after removal of the capsule and the upper layer of the cortex, tissue originating from the mid part of the cortex was collected and cut into small pieces. Repetitive incubation of the tissue pieces with 2·5 mg trypsin ml⁻¹ resulted in a single-cell suspension, which was centrifuged at 400 g for 10 min. Cells were resuspended and seeded in basic culture medium supplemented with 0·5% lactalbumin hydrolysate. All experiments were performed on first passage cells, seeded on glass coverslips and grown to confluence. Purity of epithelial cells was analysed by fluorescent staining with the anticytokeratin 7 monoclonal antibody (Dako), a marker for simple ductal epithelia found in kidneys. Approximately 90% of the cells expressed cytokeratin 7.

**Dermal fibroblasts.** Primary cultures of dermal fibroblasts were established from skin of porcine fetuses collected at the slaughterhouse (gestation stage between 6·5 and 10 weeks). Briefly, dermal tissue was cut into small pieces and subjected to repetitive trypsinization (2·5 mg trypsin ml⁻¹). The resulting single-cell suspension was centrifuged at 400 g for 10 min and cells were resuspended and seeded in basic culture medium. All experiments were performed on first passage cells, seeded on glass coverslips and grown to confluence.

**Arterial endothelial cells.** Primary porcine endothelial cells were isolated as described previously by Van de Walle et al. (2003).

**Inoculation of cells.** Cells were infected with virus at an m.o.i. of 10 TCID₅₀ in culture medium. After incubation for 1 h at 37 °C, the inoculum was aspirated and fresh culture medium was added.

**Antibodies and reagents.** Porcine FITC-labelled polyclonal anti-PRV antibodies were used as described previously (Nauwynck & Pensaert, 1995). Porcine biotinylated polyclonal anti-PRV antibodies were prepared from anti-PRV hyperimmune (HI) serum according to the manufacturer’s instructions (Amersham). The mouse IgG₂a monoclonal antibody against PRV gB (1C11) was produced at the laboratory (Nauwynck & Pensaert, 1995). Mouse IgG₁ monoclonal antibodies, anti-neurofilament-68 and anti-Map2 (a+b), were purchased from Sigma and the rabbit polyclonal antibodies directed against active caspase-3 were obtained from R&D Systems. The mouse IgG₁ monoclonal antibody directed against gianthin was kindly provided by Dr. H.-P. Hauri (Biozentrum, University of Basel; Linstedt & Hauri, 1993).

The following secondary antibodies were used. FITC-conjugated goat anti-mouse, FITC-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies, Texas red-conjugated streptavidin, Alexa Fluor 350-conjugated streptavidin (Molecular Probes) and isotype-specific FITC-conjugated rat anti-mouse IgG₁ and biotinylated rat anti-mouse IgG₂a antibodies (Serotec).

DNA fragmentation was detected based on the TUNEL reaction by using the In Situ Cell Death Detection Kit (fluorescein) obtained from Roche.

**Immunofluorescent staining procedures.** After being washed in PBS, cells were fixed with either 4% paraformaldehyde for 10 min at room temperature followed by permeabilization with 0·2% Triton X-100 in RPMI 1640 (Gibco-BRL) for 3 min at room temperature or with methanol for 20 min at −20 °C for intracellular staining. For cell surface staining, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Antibodies were always diluted in PBS: primary antibody dilutions ranging from 1:30 to 1:100, FITC-conjugated secondary antibody dilutions 1:100, Texas red-conjugated and Alexa Fluor-conjugated secondary antibody dilutions 1:50 and the isotype-specific biotinylated rat anti-mouse IgG₂a antibody dilution 1:100. Cells were incubated with antibody for 1 h at 37 °C, washes were performed with PBS for 5 min at room temperature. The detection of fragmented DNA with the TUNEL reaction was performed according to the manufacturer’s instruction (Roche). Non-fixed cells were assessed for viability using ethidium-monoazide-bromide (EMA) (Molecular Probes) as described previously (Riedy et al., 1991) and fixed afterwards as described above. Cells were mounted and analysed by fluorescence and
confocal microscopy. In case of the viability assay, 150 or 200 cells were scored for each coverslip. Experiments were repeated three times and results are presented as mean percentages with corresponding standard deviations.

**Infectious centre assays.** Infectious centre assays were performed essentially as described previously (Van de Walle et al., 2003) using different sources of infectious virus.

**Cell-free virus.** PRV 89V87 infectious virus particles (10^5 or 10^7) were neutralized using 20% PRV-neutralizing porcine HI serum (serum neutralization titre 768) (Nauwynck & Pensaert, 1995) in MEM for 40 min on ice and subsequently 2 x 10^5 and 2 x 10^6 neutralized virus particles, respectively, were added to a monolayer of coverslip-grown ST cells.

**Infected SK cells.** SK cells were inoculated with PRV 89V87 at an m.o.i. of 10 and cultivated in suspension as described previously (Favoreel et al., 2004). At 96 h post-inoculation (p.i.), cells were washed once and 10^6 SK cells were incubated in 20% HI serum as described above. Next, 5 x 10^6 SK cells were added to a monolayer of coverslip-grown ST cells followed by gentle centrifugation for 2 min at 44 g.

**Infected TG neurons and non-neuronal cells.** TG cultures were inoculated with PRV 89V87 at an m.o.i. of 10 and at 96 h p.i. detached using PBS without Ca^2+ and Mg^2+ containing 0.5 mM EDTA. Next, cells were washed once and 10^6 TG cells were incubated in 20% HI serum as described above. Then, approximately 200 individual TG neurons or 3000 non-neuronal TG cells were manually picked, under phase-contrast microscopy, and transferred to a monolayer of coverslip-grown ST cells, followed by centrifugation for 2 min at 44 g.

At 20 h post-addition of cells or virus particles to the ST monolayer, cells were fixed with 4% paraformaldehyde followed by methanol fixation as described above. Plaques were visualized using FITC-labelled anti-PRV antibodies, and when required a neuronal marker staining was performed as described above. All plaques on each coverslip were counted. Experiments were repeated three times and results are presented as mean values with corresponding standard deviations.

**Confocal microscopy.** Confocal images were acquired using a Leica TCS SP2 confocal system (Leica Microsystems) or a Bio-Rad MRC-1024 confocal laser scanning microscope system (Bio-Rad) linked to a Nikon diaphot 300 inverted microscope (Nikon).

## RESULTS

**Description of primary neuronal cultures derived from TG**

Cell suspensions derived from TG of piglets were used to establish a TG neuronal cell culture. One day after seeding, most non-adhering cells were removed by washing. This resulted in a culture consisting of large adherent neuronal cell bodies with beginning process growth surrounded by non-neuronal cells, which eventually formed a monolayer. Although TG neurons were easily recognizable from the non-neuronal surrounding cells by their size (Fig. 1a), we used antibodies directed against Map2 or neurofilament-68 (Fig. 1b) in all our experiments not only for identification of neurons, but also to score their condition, since about 50% of seeded TG neurons were shown to be non-viable, typically characterized by no axonal growth and a shrivelled nucleus (Fig. 1c), while healthy neurons displayed axonal growth and a distinct nucleus (Fig. 1b). Non-viable neurons were not included in the experiments.

**Expression of viral glycoproteins in PRV-infected TG neurons**

In the first assay we determined how many of the TG neurons in our culture expressed viral glycoproteins, a marker for late viral gene expression, after PRV infection at an m.o.i. of 10, and compared the kinetics of this expression with similar kinetics in primary SCG neurons, non-neuronal primary TG cells surrounding the TG neurons (further referred to as ‘non-neuronal TG cells’) and SK cells. Polyclonal PRV-specific antibodies, which show strong reactivity against three major PRV glycoproteins (gB, gD and gE, not shown), were used.

Intracellular viral glycoprotein expression in PRV-infected TG neurons (Fig. 2a and b) was first observed at 6 h p.i. (in 22±4 ± 1·9% of TG neurons) and >90% of TG neurons were positive at 12 h p.i., which was delayed by 2–4 h compared to the kinetic time-course of detection of intracellular viral glycoproteins in the other porcine cell types. The kinetic delay most likely reflects a delay at the very early stages of virus infection (e.g. virus entry, delivery of genome to the nucleus), since a similar delay was observed when analysing the expression of the immediate-early protein IE180 and the early protein US3 (data not shown).

At late stages of infection, newly synthesized viral alphaherpesvirus envelope glycoproteins, most of which display late gene kinetics, are processed and incorporated in the Golgi and generally travel to the cell surface. To confirm that this typical pattern of late stages of infection is present in PRV-infected TG neurons, we examined whether viral glycoproteins were observed in the Golgi and on the cell surface of TG neurons. Double labelling of viral glycoproteins and the cis-Golgi marker giantin in PRV-infected TG neurons showed (i) that TG neurons contain an unusually...
complex Golgi structure (which was also observed in uninfected cells, not shown) and (ii) that the majority of intracellular viral glycoproteins recognized by the polyclonal antibody is juxtaposed to or co-localizes with the cis-Golgi marker (Fig. 3). This suggests that in PRV-infected TG neurons, viral glycoproteins display a normal processing through the Golgi apparatus. Cell surface expression of viral glycoproteins could also be observed in PRV-infected TG neurons (Fig. 4). Initial cell surface viral glycoprotein expression was detected at 9 h p.i. in 34.5 ± 4.3% of the TG neurons and by 21 h p.i. 91.7 ± 2.8% of these neurons expressed viral glycoproteins on their plasma membrane (Fig. 4a and b) which, as for the kinetics on intracellular expression of viral glycoproteins, is somewhat delayed compared with other porcine cell types. Intracellular Golgi-like and cell surface expression were also observed when using a gB-specific monoclonal antibody instead of the polyclonal antibodies (data not shown).

From these results, it was concluded that all porcine TG neurons were susceptible to PRV infection in vitro, that they expressed viral glycoproteins and processed these glycoproteins through the Golgi and to the cell surface, albeit with a slight delay compared with these in other porcine cell types.

Cell survival following inoculation with PRV

The data presented in the former paragraph show that, at least in vitro, only marginal differences exist in the kinetics of PRV late protein expression and processing in porcine TG neurons versus other porcine cell types, which may lead to the speculation that only marginal differences are to be expected in the subsequent cell death. However, a significant number of data indirectly indicate that TG neurons, unlike other cell types, may possibly survive an ongoing alphaherpesvirus infection to some extent (Aleman et al., 2001; Geiger et al., 1995; Hood et al., 2003; Simmons & Tscharke, 1992). To clarify this issue further, we examined whether, in our in vitro assay, TG neurons are more resistant to PRV-induced cell death compared to a broad range of other primary and immortalized porcine cell types. Therefore, primary porcine TG and SCG neurons, primary epithelial kidney cells, primary arterial endothelial cells, primary non-neuronal TG cells, primary dermal fibroblasts and immortalized SK cells were inoculated with PRV 89V87 and, at different times p.i., assessed for viability as described in Methods.

Fig. 5(a) demonstrates that the percentage of dead SCG neurons increased rapidly during infection resulting in 79.7 ± 1.2% dead PRV-infected SCG neurons at 16 h p.i., the final time point measured. No further time points could be included because of detachment of cells. Dermal fibroblasts, arterial endothelial cells and non-neuronal TG cells showed a comparable increase in dead cells at early...
incubated with genistein, which has been shown to be neurotoxic to primary rat cortical neurons in a dose-dependent manner (Linford et al., 2001). Addition of 200 genistein μM to TG cultures at 24 and 48 h p.i. increased the percentage of dead TG neurons significantly (Fig. 5a). In conclusion, a significant fraction of porcine TG neurons exhibited unusually high resistance to PRV-induced cell death in comparison with a broad range of other porcine cell types including SCG neurons.

Surviving TG neurons are still able to transmit infectious virus

To determine whether the observed surviving TG neurons (96 h p.i.) are still able to transmit infectious virus, an infectious centre assay was performed (Van De Walle et al., 2003). Control SK cells and TG neuronal test cultures were infected with PRV for 96 h p.i., before cells were collected. SK or TG cells (10^6) were then incubated for 40 min with 20% PRV-specific HI serum to neutralize extracellular virus. As a control, 10^6 or 10^7 PRV infectious virus particles were treated with 20% PRV-specific HI serum for 40 min. Afterwards, cells or infectious virus particles were put on top of an ST monolayer, further incubated at 37 °C for 20 h and fixed and stained for viral antigens. The addition of up to 2 × 10^7 and 2 × 10^5 neutralized infectious virus particles, 50,000 SK cells (96 h p.i.) or 3000 non-neuronal TG cells (96 h p.i.), resulted in no or only very few plaques (Table 1). In contrast, the addition of only ±200 TG neurons to ST cells produced 91.6 ± 35.9 plaques. Fig. 6 shows that SK cells (96 h p.i.) are unable to induce plaque formation upon co-cultivation with ST cells, whereas TG neurons (96 h p.i.) can induce plaque formation.

To assess the sensitivity of our viability assay, as a positive control, PRV-infected TG neuronal cell cultures were incubated with genistein, which has been shown to be...

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Fig. 5. (a) Kinetics showing the percentage of dead cells in PRV-infected TG neurons (black), SCG neurons (grey), non-neuronal TG cells (green), epithelial kidney cells (light blue), arterial endothelial cells (blue), dermal fibroblasts (red) and SK cells (yellow). Data represent mean ± standard deviation of triplicate assays. As a positive control, at 24 and 48 h p.i., TG neuronal cultures were incubated with 200 genistein μM and assessed for viability (●). (b) Confocal sections through the centre of the nucleus of a dead SCG neuron (i) at 16 h p.i. and a living (ii) and dead (iii) TG neuron at 36 h p.i. Neuronal cultures were stained for neuronal markers (FITC, green) with anti-Map2 (a + b) for SCG neurons and anti-neurofilament for TG neurons, for viral antigens (Alexa Fluor 350, blue) and for viability (EMA, red). Arrows indicate the neuronal nucleus. Bar, 20 μm.

Table 1. Plaque formation following addition of neutralized virus or infected cells to an ST monolayer

<table>
<thead>
<tr>
<th>Virus or cells pre-incubated with PRV-neutralizing antibodies</th>
<th>Approximate no. virus particles or cells put on ST monolayer</th>
<th>No. plaques*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRV 89V87</td>
<td>2 × 10⁷</td>
<td>1.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁶</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>SK cells (96 h p.i.)</td>
<td>5 × 10⁴</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>TG cells (96 h p.i.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-neuronal</td>
<td>3 × 10³</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td>Neurons</td>
<td>2 × 10²</td>
<td>91.6 ± 35.9</td>
</tr>
</tbody>
</table>

*Data represents mean ± standard deviation of triplicate assays.
proportion of TG neurons at 96 h p.i. are still able to act as a source of infectious virus.

A PRV strain without the anti-apoptotic US3 protein does not induce substantial apoptosis in TG neurons

Over the last few years, it has been demonstrated that both HSV serotypes and bovine herpesvirus 1 can counteract apoptosis via viral-encoded anti-apoptotic genes in a cell type-specific way (Lovato et al., 2003 and references therein). Additionally, such anti-apoptotic activity of specific alphaherpesvirus genes has been suggested to promote the survival of infected TG neurons and has even been suggested to possibly allow re-establishment of latency (Lovato et al., 2003; Perng et al., 2000). The US3 protein kinase, which is conserved among all alphaherpesviruses, appears to be one of the most potent anti-apoptotic HSV-encoded proteins. Recently, we showed that the PRV US3 protein kinase is able to suppress virus-induced apoptosis in ST cells (Geenen et al., 2005), and US3 is the only anti-apoptotic protein identified in PRV thus far. To assess whether PRV US3 has a role in the remarkable resistance of TG neurons towards PRV-induced cell death by protecting them from virus-induced apoptosis, TG neuronal cultures were inoculated with either wild-type (WT) or US3 null PRV at an m.o.i. of 10. At 24, 48 or 72 h p.i., cells were fixed and apoptotic cell death was determined and quantified using anti-active caspase-3 antibodies (Fig. 7a and b) or the TUNEL reaction (Fig. 7c and d).

These results indicate that neither WT nor the strongly pro-apoptotic US3 null PRV is able to induce substantial apoptotic cell death in TG neurons.

DISCUSSION

TG neurons are the predominant site of latency and reactivation events for several alphaherpesviruses. Therefore, the interplay between the virus and this specific cell type may be of special importance. As mentioned in the introduction, several studies have, based on indirect evidence, suggested that TG neurons, in contrast to other cell types, may sustain a certain amount of alphaherpesvirus replication during primary infection or reactivation, allowing enough time for the virus to be transported from the cell body to the axon termini, and perhaps even allowing the virus to enter or resume latency afterwards (Perng et al., 2000). However, neuronal cell death during infection of TG neurons by a homologous alphaherpesvirus with respect to the kinetic time-course of viral infection has, to date, not been studied.

Here we show in vitro (i) that primary porcine TG neurons display a similar, albeit slightly delayed, pattern of viral late gene product expression upon infection with PRV, but (ii) that these cells are much more resistant towards PRV-induced cell death in comparison with a broad range of other porcine cell types (immortalized and primary epithelial kidney cells, primary arterial endothelial cells, primary non-neuronal TG cells, primary dermal fibroblasts, monocytes and primary SCG neurons).

We found that at 12 h p.i. at an m.o.i. of 10 over 90% of the PRV-infected TG neurons displayed microscopically detectable levels of intracellular expression of viral glycoproteins, indicating that all TG neurons in our assay were permissive to PRV infection. Further, we showed that viral glycoproteins were processed through the Golgi and reached the cell surface, a typical pattern of late stages of infection, although with a slight, but consistent delay (± 2–6 h) compared with other porcine cell types. Since neurons have limited concentrations of cellular transcription factors (Rajcani & Durmanova, 2000), it is likely that an extended...
time is needed for PRV-infected neurons to initiate viral transcription at early stages of infection, which could account for differences in expression kinetics between non-neuronal and neuronal cells. A possible explanation for the slower kinetics in TG versus SCG neurons may perhaps be the specific complexity of TG neurons, exemplified by the large size of the cell body (± 65 μm for TG neurons versus ± 15 μm for SCG neurons) and very complex structure of the Golgi-apparatus we have observed (Fig. 3), which possibly may delay different aspects of the virus life cycle, including genome delivery at the nucleus and processing of newly formed viral glycoproteins.

It has been generally accepted that alphaherpesvirus replication ultimately results in cell death. However, indirect indications exist that alphaherpesvirus-infected neurons may survive replication to some extent, which could provide the time for virus spread to axon termini and perhaps even allow (re-)establishment of latency after virus replication has initiated (Aleman et al., 2001; Geiger et al., 1995; Sawtell, 1997; Simmons & Tscharke, 1992). To clarify this issue further, we examined the kinetic time-course of cell death upon infection of different porcine cells with PRV. We showed that 48.9% of PRV-infected TG neurons survived up to 4 days after infection (end of experiment), whereas in all other porcine cell types tested the vast majority of cells succumbed to PRV infection within 2 days. Moreover, we demonstrated that these surviving TG neurons (96 h p.i.) were still able to transmit infectious virus to other cells. This may suggests that the unusual resistance of TG neurons towards PRV-induced cell death may result in extended time of virus transmission in vivo, thereby promoting virus spread. Clearly, more research is required to test this hypothesis.

The mechanism(s) of resistance of a significant part of TG neurons towards PRV-induced cell death in vitro is still speculative, but may perhaps depend on specific viral or cellular anti-apoptotic proteins. To date, only one viral protein of PRV has been demonstrated to display anti-apoptotic activity: the US3 protein kinase (Geenen et al., 2005). To evaluate the possible importance of PRV US3 in the resistance of TG neurons towards PRV-induced cell death, in vitro cultures of TG cells (consisting of neuronal and non-neuronal cells) were infected with WT or US3 null PRV. We found that WT PRV induced apoptotic
cell death in the majority of non-neuronal TG cells at late stages of infection (≥ 48 h p.i.), whereas infection with a US3 null virus resulted in much more frequent and earlier apoptosis in these cells (24 h p.i.). However, neither WT nor US3 null PRV induced substantial apoptosis in neuronal TG cells at any of the time points tested (24, 48 and 72 h p.i.). Thus, TG neurons show a marked resistance towards PRV-induced apoptosis, and do not depend on the viral anti-apoptotic US3 protein for this resistance. It will be interesting to explore whether the PRV orthologues of the other anti-apoptotic proteins described in HSV [e.g. ICP4, ICP27, ICP22, US5, US6 and especially latency-associated transcripts (LATs), reviewed by Aubert & Blaho, 2001] display anti-apoptotic activity, and, if so, whether they are involved in the resistance of TG neurons towards PRV-induced cell death. On the other hand, TG neuron-specific cellular anti-apoptotic proteins may account for the unusual resistance of these cells to PRV-induced cell death. Interestingly, Brn-3a, a member of the IV-POU family of transcription factors, is highly expressed in sensory neurons. Brn-3a has been shown to protect sensory neurons (TG neurons and dorsal root ganglion neurons), but not sympathetic neurons (SCG) from apoptotic cell death by activating the expression of two anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-xL (Ensor et al., 2001; Smith et al., 1998a, b, 2001). This is in agreement with our findings that TG neurons, but not SCG neurons, show resistance to PRV-induced cell death. Future research will show whether this higher expression of Brn-3a in sensory versus sympathetic neurons may explain the resistance of TG neurons towards PRV-induced cell death. Although speculative, such possible involvement of Brn-3a in protecting TG neurons from PRV-induced cell death may perhaps also aid to explain why only about half of the TG neurons in our in vitro study survive PRV infection. In this context, it is interesting that Yang et al. (2000) showed that all neuronal populations within the TG appear to be capable of supporting productive infection with HSV-1 as assayed by immunofluorescent staining using polyclonal antiserum to HSV, but that some neuronal phenotypes are more permissive for establishment of latent infection with LAT expression than others. They found that a large percentage of latently infected sensory neurons expressed TrkA, the high affinity nerve growth factor-receptor. Since it has been demonstrated that the expression of TrkA is regulated by Brn-3a (Ma et al., 2003), it will be interesting to explore whether the subpopulation of surviving TG neurons express high levels of TrkA.

Although speculative, the prolonged survival of PRV-infected TG neurons combined with the somewhat delayed kinetics of viral late protein expression and processing we observe in TG neurons may perhaps provide an idea on the possibility of latency (re-)establishment after limited virus replication in vivo. The slower viral protein expression kinetics and extended survival perhaps supply the time credit needed for innate and/or adaptive immune responses to develop and suppress virus replication thereby paving the way for the establishment of latency. Indeed, it has been shown that elements of the innate and adaptive immune response e.g. interferons (IFN), CD8+ T cells, tumour necrosis factor-α, and antibodies are capable of suppressing alphaherpesvirus replication in neurons and that some of these components, like IFN-γ, are directly linked to inhibition of virus-induced neuronal cell death and induction of a quiescent/latent state of infection (Cantin et al., 1995; Geiger et al., 1995, 1997; Khanna et al., 2003; Liu et al., 2001; Oakes & Lausch, 1984; Sainz & Halford, 2002; Schijns et al., 1991; Shimeld et al., 1997; Simmons & Tscharke, 1992). Using our homologous in vitro system of swine alphaherpesvirus infection in swine TG cultures that we describe here, we are planning experiments to clarify these issues further.

In conclusion, this study shows that using a unique homologous in vitro model based on the swine PRV and a primary neuronal culture derived from the porcine TG, viral glycoprotein expression, processing and cell surface transport could be observed in all TG neurons upon infection by PRV and that these TG neurons were remarkably more resistant to PRV-induced cell death compared with other porcine cell types.

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