Role of viral proteins and concanavalin A in in vitro replication of pseudorabies virus in porcine peripheral blood mononuclear cells

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We examined the capability of pseudorabies virus (PRV) to replicate in vitro in porcine peripheral blood mononuclear cells (PBMC) and characterized the phenotype of infected cells. In addition, we investigated whether inactivation of various PRV proteins or the expression of a foreign gene affected this replication. Finally, we studied the replication of PRV strains in concanavalin A (Con A)-stimulated lymphocytes. The replication of PRV mutants with inactivated glycoproteins gE or gG, thymidine kinase (TK), ribonucleotide reductase (RR) or US3-encoded protein kinase (PK), and the replication of PRV vector strains expressing the envelope glycoprotein E1 of hog cholera virus (HCV) were studied. By adherence of PBMC to plastic, monocytes and lymphocytes were largely separated. Infected monocytes were analysed with an immunostaining monolayer assay and infected lymphocytes were analysed with immunofluorescence staining and flow cytometry. We found that the wild-type NIA-3 virus replicated in both lymphocyte and monocyte cultures. NIA-3 infected relatively more monocytes (> 90%) than non-adherent B cells (46–65%) and T cells (17–28%); approximately equal numbers of CD4+ and CD8+ T cells were infected. Although E1 is probably involved in adsorption of HCV to host cells, the expression of E1 by PRV vector strains did not change the level of replication. Inactivation of TK and RR, but not inactivation of gE, gG or PK, severely affected the replication in both monocytes and lymphocytes. Con A stimulation of lymphocytes restored the reduced replication of the TK mutant, but not of the RR mutant. Moreover, Con A stimulation of lymphocytes reduced the replication of the wild-type NIA-3 virus. We concluded that both viral TK and RR activity are important for efficient replication of PRV in resting lymphocytes. Furthermore, Con A-stimulated lymphocytes can restore the viral TK defect and PRV replication can also be influenced by cellular metabolism.

Introduction

Pseudorabies virus (PRV) causes an economically important disease in swine. The virus replicates first in the nasopharyngeal mucosa and can subsequently invade the central nervous system via cranial nerves or affect the respiratory tract (Dow & McFerran, 1962; Gustafson, 1986; Pol et al., 1989; Mulder et al., 1994b). In addition, Wittmann et al. (1980) suggested that PRV could spread systemically via the lymphatic and haematogenic system. However, evidence to support this hypothesis was not convincing. Recently, Page et al. (1992) isolated virus from peripheral blood mononuclear cells (PBMC) of infected pigs. Moreover, Nauwynck & Pensaert (1992) demonstrated that infected PBMC induced abortion in vaccinated sows. Several observations suggest that PRV may be immunosuppressive and can predispose pigs to secondary infections. Monocytes have been shown to be highly susceptible to PRV infection in vitro (Wang et al., 1988) and the virus subsequently replicates well (Chinsakchai & Molitor, 1992). Furthermore, alveolar macrophages are highly permissive to PRV and become functionally impaired (El-Awar & Hahn, 1987; Iglesias et al., 1989).

Previously, we inoculated pigs with non-virulent and virulent PRV vector strains expressing the envelope glycoprotein E1 of hog cholera virus (HCV). The gene
encoding E1 was inserted into the glycoprotein gG locus of both strains. In the non-virulent PRV strain the virulence genes encoding gE and thymidine kinase (TK) had been inactivated. Despite the distinct affinity of HCV for cells of the immune system (van Oirschot, 1979, 1983), an increase in the replication of the non-virulent vector PRV strain in leukocytes was not detected. Leukocytes were examined because E1 is probably involved in adsorption or penetration of HCV into host cells. Recently, it was found that E1 binds to a cellular receptor (M. M. Hulst & R. J. M. Moormann, unpublished results). No infectious virus was recovered from PBMC in vivo (Mulder et al., 1994a). Infection of PBMC by the virulent recombinant PRV strain could not be determined in vivo. Which proteins of PRV are involved in virus replication in PBMC has not yet been examined and it is still unclear whether PRV can replicate in resting lymphocytes; herpes simplex virus type 1 (HSV-1) replicates in mitogen-stimulated lymphocytes but not in resting lymphocytes (Sarmiento & Kleinerman, 1990; Bouyyad & Menezes, 1990).

In this study we examined the capability of PRV to replicate in vitro in porcine PBMC and characterized the phenotype of infected cells. In addition, we investigated whether inactivation of various PRV proteins or the expression of a foreign gene (i.e. E1) affected this replication. And finally, we investigated whether the replication of PRV strains was affected in lymphocytes stimulated with concanavalin A (Con A). By adherence of PBMC to plastic, monocytes and lymphocytes were largely separated. The phenotypes of wild-type PRV-infected lymphocyte and monocyte cultures were determined using a cytometry assay using dual colour immunofluorescence and a double immunostaining monolayer assay, respectively. The replication of the wild-type NIA-3 virus in both cell populations was compared with the replication of PRV mutants with inactivated gE, gG, TK, ribonucleotide reductase (RR) or US3-encoded protein kinase (PK), or was compared with non-virulent and virulent PRV vector strains that express E1 of HCV.

Glycoprotein gE promotes cell-to-cell spread and release of PRV from several cell types in vitro (reviewed in Jacobs, 1994); gE is important for virulence and for the invasion of PRV into the central nervous system in vivo (Jacobs et al., 1993; Mulder et al., 1994b; Kritis et al., 1994a, b). Glycoprotein gG seems to have no significant role in the virulence or immunogenicity of PRV (Thomsen et al., 1987; Kimman et al., 1992). TK and RR are important enzymes in the salvage and de novo pathways of deoxynucleotidyl synthesis, respectively (Reichard, 1988) and are therefore probably needed for efficient virus replication in resting cells. Both TK and RR are important for virulence of PRV. The US3-encoded PK phosphorylates a major phosphoprotein of 112 kDa (Zhang et al., 1990) and the replication of the PK mutant was severely affected in porcine immortalized B cells (Kimman et al., 1994).

Methods

Virus strains. The NIA-3 strain of PRV (McFerran & Dow, 1975) was used as a parent strain for the development of mutant viruses and as wild-type virus in the experiments. Mutant viruses were generated by overlap-recombination of four to five DNA fragments, together comprising the entire PRV genome, and insertion mutagenesis using an oligonucleotide with translational stop codons in all reading frames and an EcoRI site as described (de Wind et al., 1990; Kimman et al., 1992). Mutant virus strains were plaque-purified three times on SK-6 cells (Kasza et al., 1971) and their integrity was confirmed by restriction enzyme analysis of the linker insertion sites. No alterations were found in PRV sequences flanking the oligonucleotides.

The gE mutant strain (M141) has a deletion of 1729 bp in the gene encoding gE. The deletion was generated between the DraI site in the 5' end of the gE gene and the EcoRI site of an oligonucleotide mutational insert in the 3' end of the gene, deleting nucleotides 6833–8562 (N. de Wind et al., personal communication). The TK mutant (M207) contains a deletion of 19 bp in the 3' part of the UL23 gene encoding TK. The 19 bp deletion is identical to the deletion in the vaccine strain 783 (Moormann et al., 1990). The strain lacks functional TK activity and is completely non-virulent for mice. The gE–TK double mutant (M111) contains the 19 bp deletion in the UL23 TK gene and the 1729 bp deletion in the gE gene. The RR mutant (M151) contains a deletion of 250 bp in the UL39 gene that encodes the ribonucleotide reductase large subunit, RR1 (de Wind et al., 1993). The RR mutant lacks RR activity and is non-virulent for pigs and mice. The PK mutant (M118) contains an oligonucleotide insertion in the 5' side of the PK encoding-US3 gene at nucleotide position 1828, generating a translational stop (de Wind et al., 1990). The gG mutant contains an oligonucleotide insertion in the 5' side of the gene encoding gG at nucleotide position 3219, also generating a translational stop (de Wind et al., 1990). The construction and characterization of strain M205 (gE'– TK'– gG'– E1'), the non-virulent vector expressing the envelope glycoprotein E1 of HCV, strain M206 (gE'– TK'– gG'– E1'), the empty vector strain without E1, and strain M12 (gG'– E1') the virulent vector expressing E1, have been described earlier (Van Zijl et al., 1991; Mulder et al., 1994a). Expression of E1 did not change the virulence or pathogenesis of the PRV vector viruses in vivo (Mulder et al., 1994a).

Isolation of peripheral blood mononuclear cells. Blood was collected from the superior venae cavae of a group of specific pathogen-free inbred miniature pigs (SLAa), aged 2–3 years (Sachs et al., 1976), into heparin-containing vacuum tubes. PBMC were isolated by means of lymphoprep (Nycomed), washed twice with PBS and resuspended in RPMI complete medium (RPMI 1640, Dutch modification; Flow Laboratories) at a concentration of 3 x 10⁶ cells/ml. RPMI complete medium contains 10% FCS, 2 mM-glutamine, 5 x 10⁻⁶ M-2-mercaptoethanol and the antibiotics penicillin (90 U/ml), streptomycin (100 μg/ml), fungizone (4.5 μg/ml), polymyxin (25 μg/ml) and kanamycin (50 μg/ml). PBMC were seeded into Greiner six-well culture plates (3 ml/well) and incubated at 37°C in a 5% CO₂ incubator. By adherence to plastic, monocytes were largely separated from lymphocytes. Directly after separation on plastic, 5–10% of the PBMC were adherent cells and the remainder were non-adherent. After 1 h, non-adherent cells were harvested and resuspended in complete medium.
The remaining adherent cells were washed three times with complete medium. For clarity, non-adherent cells are called lymphocyte cultures and adherent cells are called monocyte cultures throughout the paper. Directly after separation, lymphocyte cultures consisted of 90–93% T cells, 5–7% B cells and 5% monocytes. Monocyte cultures consisted of 80–85% monocytes and 15–20% adherent B cells.

Additionally, 80–85% of the cells from the monocyte cultures were identified as monocytes by an acid phosphatase staining method. Cell antigens expressed by the lymphocyte cultures were analysed with immunofluorescence staining and flow cytometry and those expressed by monocyte cultures were analysed with an immunostaining monolayer assay.

**Virus replication in PBMC.** To examine virus replication, lymphocyte cultures were infected at a m.o.i. of 1 and monocyte cultures at 0.1 m.o.i. for 1 h at 37 °C. Because it has been reported that monocytes were more susceptible to PRV infection, we infected the lymphocyte cultures at a 10-fold lower m.o.i. than the lymphocyte cultures. Monocyte cultures were infected in culture plates on a shaker and lymphocyte cultures were infected in Falcon tubes on a ‘rock-and-roller’. To remove non-adsorbed virus, the cells were incubated twice for 2 min with citric acid buffer (pH 3) and washed with complete medium. Uninfected cells were given the same treatment. Lymphocyte cultures were resuspended in complete medium to the original volume and seeded into culture plates at 3 ml/well. To the monocyte cultures 3 ml complete medium was added per well. The PBMC were cultured at 37 °C in a 5% CO₂ incubator and collected after 0, 24, 48 and 72 h for virus titration. Cultured PBMC were frozen and thawed, and titrated on the swine kidney cell line SK-6 (Kasza et al., 1971). Virus titration was done as described previously (Mulder et al., 1994a). Virus titres were expressed as log10 p.f.u./ml.

**Antisera.** Flow cytometric analyses and immunoperoxidase monolayer assays were done using the following antisera: MAb 74-22-15 directed against porcine monocytes (Pescovitz et al., 1984); MAb MSA4 directed against porcine CD2 (Hammerberg & Schuring, 1986); MAb SL2 (295/33) directed against porcine CD8 (Jonjic & Koszinowski, 1984); MAb 74-12-4 directed against porcine CD4 (Pescovitz et al., 1984); MAbs 27.2.1 and 27.7.1 directed against Ig κ (Kasza et al., 1984) and Ig λ (Pescovitz et al., 1984); MAbs 27.2.1 and 27.7.1 directed against Ig κ (Pescovitz et al., 1984) and Ig λ (Kasza et al., 1984); MAbs 27.2.1 and 27.7.1 directed against porcine CD8 (Jonjic & Koszinowski, 1984); MAb 74-12-4 directed against porcine CD4 (Pescovitz et al., 1984); MAbs 27.2.1 and 27.7.1 directed against porcine CD8 (Jonjic & Koszinowski, 1984); MAb 74-12-4 directed against porcine CD4 (Pescovitz et al., 1984); MAbs 27.2.1 and 27.7.1 directed against porcine CD8 (Jonjic & Koszinowski, 1984); MAb 74-12-4 directed against porcine CD4 (Pescovitz et al., 1984); MAbs 27.2.1 and 27.7.1 directed against porcine CD8 (Jonjic & Koszinowski, 1984); MAb 74-12-4 directed against porcine CD4 (Pescovitz et al., 1984); (ii) staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Fab')₂; (iii) saturation of free binding sites with non-labelled mouse IgG (Jackson Laboratories); (iv) incubation with biotinylated MAb 45.4.1 a directed against gB of PRV; (v) staining with phycocerythrin (PE)-conjugated streptavidin (Becton Dickinson). Controls included cells stained with both fluorescent reagents without any of the two primary reagents. All incubations were done at 0 °C for 30 min. Cells were washed with PBS containing 2% FCS. Flow cytometric analysis was done on a FACScan (Becton Dickinson). A sample of 5 x 10⁶ cells of lymphocyte size and internal complexity based on forward and 90 ° scatter was analysed. A threshold was set on forward scatter (channel 280 on a linear scale) to exclude debris.

**Con A stimulation of lymphocytes.** Directly after infection of lymphocyte cultures as described above, 4 µg/ml of the T cell mitogen Con A was added. The cells were cultured for 7 days at 37 °C in a 5% CO₂ incubator. The level of virus replication was determined at 24 h intervals and samples were taken several times to determine [³H]thymidine incorporation, cell number and cell viability by trypan blue exclusion.

**Results**

**Phenotype of NIA-3-infected PBMC**

We characterized infection of PBMC by the wild-type NIA-3 virus. Infected monocyte cultures were analysed with an double immunostaining monolayer assay and infected lymphocyte cultures were analysed with flow cytometry using dual colour immunofluorescence staining. In at least two different experiments, three different pigs were analysed. Monocytes were highly susceptible to PRV infection; on average 91–96% of the monocytes were PRV-positive. In contrast, no PRV-positive adherent B cells were detected (data not shown). Despite using 10-fold more virus for infection, less PRV-positive cells were detected in the lymphocyte cultures (Fig. 1). We found that in the lymphocyte cultures relatively more B cells were infected than T cells (46–65% of non-adherent B cells compared with 17–28% of T cells). Approximately equal numbers of CD4+ T cells (23–32%) and CD8+/CD4– T cells (17–32%) were infected. In contrast to the number of infected monocytes, the number of infected lymphocytes varied among pigs. For example, in one pig almost two times as many PRV-positive lymphocytes were detected as in another pig (28% against 16%). Yet, the percentage of PRV-positive cells of individual pigs was similar in subsequent flow cytometric analyses.
The viability of the infected cells was determined by trypan blue exclusion. Compared to the uninfected controls, 72 h after infection at 10 m.o.i., 4–11% of the lymphocytes had died due to infection by the NIA-3 virus. Most cells died of PRV infection between 24 and 48 h after infection. We observed that a majority of the infected monocytes lysed or lost their adherence capacity. Therefore, the exact number of monocytes that died due to virus infection could not be determined with the immunostaining monolayer assay.

**Replication of wild-type NIA-3 virus and of TK, gE, gG, RR and PK mutants in resting PBMC**

Wild-type NIA-3 virus replicated in both monocyte cultures and lymphocyte cultures (Fig. 2a, b). The virus yield in both cell populations was low. The mean virus yield of six experiments with different pigs was 0.18 p.f.u./cell in monocyte cultures and 0.054 p.f.u./cell in lymphocyte cultures. Thus, although monocyte cultures were infected at a 10-fold lower m.o.i., these cells produced on average approximately threefold more virus than the lymphocyte cultures. As a control, the NIA-3 virus and the other tested PRV mutants were kept in cell-free culture medium for 72 h. The virus titres of the PRV strains hardly declined, indicating that NIA-3 and its derived mutant strains were for the most part stable (data not shown).

The replication of NIA-3 in PBMC derived from different pigs did not vary much. For example, the virus yield of infected lymphocyte cultures derived from three different pigs ranged from log_{10} 6.1–log_{10} 6.6 p.f.u./ml after 72 h. Furthermore, in three experiments granulocytes were infected with NIA-3 at 1 m.o.i. but no virus growth was detected in these cells.

We investigated whether inactivation of the PRV proteins TK, gE, gG, RR or PK affected the replication of PRV in resting PBMC. First, the replication of a TK mutant, a gE mutant, a gG mutant and a double gE-TK together with the percentage of cells of each particular subset present in the total lymphocyte population (in parentheses). Fluorescence with label 1: (a, b) CD2 stained by MAb MSA4; (c, d) CD4 stained by MAb 74–12–4; (e, f) CD8 stained by MAb SL2 (295/33); (g, h) Ig κ and λ light chains (B cells) stained by MAbs 27.2.1 and 27.7.1, respectively. Subsequently, all lymphocytes were stained with goat anti-mouse Ig-FITC (label 1). Fluorescence with label 2: (a–d) Glycoprotein gB of PRV was identified by biotinylated MAb 45.4.1 a. Subsequently, all lymphocytes were stained with streptavidin–PE (label 2). (i) Fluorochrome conjugates control using labels 1 and 2.
Replication of PRV mutant strains in PBMC

mutant was studied in monocyte and lymphocyte cultures. The gE mutant replicated well in both cultures and reached similar virus titres to the wild-type NIA-3 virus (Fig. 2a, b), as did the gG mutant (Fig. 3a, b). In contrast, the growth of the TK mutant and the double gE–TK mutant was severely affected in both cultures (Fig. 2a, b). The virus titres of the TK mutant and the double gE–TK mutant did not increase during 72 h after infection.

Secondly, we studied the replication of a RR mutant and of a PK mutant in resting lymphocyte cultures. In contrast to the wild-type virus NIA-3 and the PK mutant, the growth of the RR mutant was severely affected in resting monocyte cultures (data not shown) and in lymphocyte cultures (Fig. 4b). The PK mutant replicated somewhat less efficiently than the wild-type virus NIA-3. Together, these results indicated that in contrast to gE, gG and PK, both viral encoded TK and RR activity are important for efficient replication of PRV in resting porcine PBMC.

Effect of Con A on replication of wild-type NIA-3 and of TK, RR and PK mutant viruses in lymphocyte cultures

We investigated the effect of Con A stimulation of lymphocytes on the replication of the wild-type NIA-3 virus and the replication of PRV mutants with inactivated TK, RR or PK. The replication of the wild-type and mutants in untreated resting lymphocytes was compared with their replication in Con A-stimulated lymphocytes. By Con A stimulation, the number of lymphocytes increased threefold and DNA synthesis, as measured by ³H incorporation, increased strongly. At 48 h after infection the stimulation index, the incorporation of ³H into the Con A-stimulated cells divided by the incorporation of untreated cells, was more than 250. Infection with NIA-3 or the TK mutant had no significant effect on this stimulation index for DNA synthesis.

Con A stimulation of lymphocytes had two effects on the replication of the PRV strains. Firstly, the replication of wild-type NIA-3 virus (Fig. 4a) and the replication of the PK mutant (Fig. 4b) were reduced in Con A-stimulated lymphocytes, as compared to the replication in resting lymphocytes. Secondly, the reduced replication of the TK mutant in resting lymphocytes was restored in Con A-stimulated lymphocytes (Fig. 4a). However, the reduced replication of the RR mutant was not restored in Con A-stimulated lymphocytes (Fig. 4b). In contrast to the TK mutant, virus titres of the RR mutant did not increase significantly in Con A-stimulated lymphocytes.

In addition to this, we studied the effect of Con A on the replication of wild-type virus NIA-3 and the TK mutant M207 in lymphocyte cultures that were precultivated for 48 h. The effect of Con A in precultivated lymphocytes was similar to that in previous assays done with freshly isolated lymphocytes (data not shown). Also, in the precultivated lymphocytes that were treated with Con A, the replication of the NIA-3 virus was partly
Fig. 3. Replication of non-virulent and virulent PRV vector strains that express E1 of HCV in resting swine PBMC. Shown are the virulent vector strain (gG+, E1+) expressing E1 (○) and the gG mutant (□), the non-virulent vector strain (gE-, TK-, gG-, E1+) expressing E1 (▲) and the control strain (gE-, TK-, gG-; ▲) in monocyte (a) and lymphocyte (b) cultures. Monocyte cultures were infected at 0.1 m.o.i. and lymphocyte cultures were infected at 1 m.o.i. Values are the mean of three experiments and error bars indicate the SD about the mean.

inhibited compared to replication in the untreated controls. Again, the reduced replication of the TK mutant in non-treated resting lymphocyte cultures was partly restored in the Con A-stimulated lymphocyte cultures. Remarkably, NIA-3 replicated much more efficiently in precultivated lymphocytes compared to
freshly isolated lymphocytes. The NIA-3 virus yield reached maximum titres within 24 h after infection and remained at this level at later time points. Also, the replication of the TK mutant in Con A-stimulated, precultivated lymphocytes was more efficient than the replication of the TK mutant in Con A-stimulated, freshly isolated lymphocytes.

**Replication of PRV vector strains in PBMC**

We investigated whether the expression of HCV E1 affected the replication of non-virulent and virulent PRV vector viruses. Because HCV has a distinct affinity for leukocytes, and because E1 is probably involved in adsorption of HCV to host cells, the insertion of E1 into these PRV vector strains could affect the replication in PBMC *in vitro*. The virulent vector virus (gG-, El+) expressing E1 and its control strain that did not express gG replicated in both monocyte and lymphocyte cultures and reached similar virus titres to the wild-type strain (Fig. 3a, b). In contrast, the growth of the non-virulent vector virus (TK-, gE-, gG-, El+) expressing E1 and its control strain (TK-, gE-, gG-) without E1 was severely affected in both cell cultures (Fig. 3a, b). Thus, expression of glycoprotein E1 of HCV by PRV did not increase virus replication in PBMC. No significant differences in replication in PBMC were observed between the virulent vector strain (gG-, El+) expressing E1 and the gG mutant strain, or between the non-virulent vector strain (TK-, gE-, gG-, El+) expressing E1 and its control strain (TK-, gE-, gG-) without E1. These experiments again showed that growth in resting PBMC of PRV strains that lack TK activity is severely affected.

**Discussion**

In this study we assessed the capability of wild-type PRV to replicate in porcine PBMCs *in vitro* and characterized the phenotype of infected cells. We evaluated the role of several proteins of PRV in replication in PBMC. We found that wild-type virus NIA-3 replicated in both monocyte and lymphocyte cultures. Although monocyte cultures were infected at a 10-fold lower m.o.i. than lymphocyte cultures, more than 90% of all monocytes were infected at 72 h post-infection, whereas at that time 46–65% of all non-adherent B cells and 17–28% of all T cells were infected, as determined with immunostaining. These results confirmed the findings of others who reported that peripheral blood monocytes are highly permissive for PRV infection (Chinsakchai & Molitor, 1992; Wang et al., 1988). The results of this study also demonstrated that PRV can infect resting lymphocytes, resulting in low amounts of virus progeny. To prove that the virus progeny in the lymphocyte cultures did not come from the residual monocyte contamination, complement and MAbs directed against porcine monocytes were employed. Nevertheless, there was no major difference in virus progeny titres from lymphocyte cultures with and without anti-monocyte plus complement treatment. Thus, the virus progeny were indeed produced by peripheral blood lymphocytes. The latter finding was also reported by Chinsakchai & Molitor (1992). In contrast, Page et al. (1992) could not detect virus production after PRV infection of resting porcine lymphocytes; the different PRV strains that were used may explain these differences. Unlike replication of PRV, HSV-1 does not replicate in freshly isolated human PBMC (Sarmiento & Kleinerman, 1990; Bouyyad & Menezes, 1990).

The yield of progeny virus in PBMC increased slowly until 72 h after infection. The replication of wild-type PRV and other virus strains in porcine PBMC was slow compared to replication in the porcine kidney cell line SK-6 (Kasza et al., 1971) or the porcine immortalized B cell line L14 (Kimman et al., 1994). However, replication of PRV in lymphocyte cultures that were precultivated for 48 h appeared much earlier. Titres of NIA-3 virus in precultivated cells had already peaked by 24 h post-infection. Sarmiento & Kleinerman (1990) also found that precultivated human PBMC (day 3 and day 7 cultures) produced significantly more infectious progeny than freshly isolated cultures. They reported that freshly isolated lymphocytes inoculated with HSV-1 or PRV induced the expression of 62 kDa and 57 kDa proteins that were associated with the innate resistance to virus infection. In contrast to freshly isolated lymphocytes, these two proteins were not expressed in precultivated lymphocytes that supported HSV-1 infection.

We assessed the role of gE, gG, TK, RR and PK in the replication of PRV in PBMC. Inactivation of gG or gE had no effect, and inactivation of PK only slightly affected the replication of PRV in resting PBMC. In contrast, inactivation of TK and RR severely affected replication. Glycoprotein gE promotes cell-to-cell spread and release of PRV from several cell types *in vitro* (reviewed in Jacobs, 1994); *in vivo* gE is important for virulence of PRV and is involved in the invasion of PRV into the central nervous system (Jacobs et al., 1993; Mulder et al., 1994b; Kritas et al., 1994a, b). The function of gG is unknown; it appears to have no significant role in the virulence or immunogenicity of PRV (Thomsen et al., 1987; Kimman et al., 1992; Mulder et al., 1994a). The US3-encoded PK phosphorylates a major phosphoprotein of 112 kDa (Zhang
et al., 1990) and the replication of this PK mutant was severely affected in the porcine B cell line L14 (Kimman et al., 1994). Herpesvirus RR mutants are defective in the de novo pathway of deoxynucleotide biosynthesis (for review see Reichard, 1988). TK mutants are deficient in the pyrimidine deoxynucleotide salvage pathway which uses nucleotides obtained from degradation of DNA (only thymidine in PRV; Jamieson et al., 1974). PK, TK and RR are important for virulence of PRV (Kimman et al., 1992; de Wind et al., 1993).

Con A stimulation of lymphocytes had two different effects on the replication of wild-type PRV and PRV mutants. Firstly, the replication of the wild-type NIA-3 virus and the PK mutant was reduced by Con A stimulation. Secondly, the reduced replication of the TK mutant was restored by Con A stimulation. Native tetrameric Con A binds specifically to α-d-glucopyranosides and α-d-mannopyranosides, and to polysaccharides or glycoproteins containing such residues (Summer & Howell, 1936). Con A can irreversibly inactivate HSV-1 infectivity. Inhibition by Con A was completely reversed by α-methylmannoside, which is a specific inhibitor of Con A (Ziegler & Pozos, 1981; Ito & Barron, 1974; Okada & Kim, 1972; Wittels & Spear, 1990). Also in our experiments we found that addition of α-methylmannoside partly reversed the reduced replication of NIA-3 and partly reversed the enhanced replication of the TK mutant (data not shown). Probably, Con A and its dimeric derivative succinyl-Con A can cross-link viral glycoproteins and thereby inhibit virus–cell and cell–cell fusion (Hennis, 1994). We further investigated whether the supernatant of Con A-stimulated lymphocytes could reduce the replication of NIA-3 and found that it did not (data not shown). This indicated that no PRV-inhibitive substances are released by Con A-stimulated lymphocytes that could explain the reduction in PRV replication.

The mitogen Con A stimulates T cells and induces proliferation (Roit, 1987). Probably, the replication of the TK-deficient mutant was restored in Con A-stimulated lymphocytes because stimulation enhanced viral deoxyribonucleotide synthesis. In contrast, Con A-stimulated lymphocytes were not able to restore the RR defect in deoxyribonucleotide synthesis. Similarly, HSV-1 TK mutants (Jamieson et al., 1974) and RR mutants (Goldstein & Weller, 1988a, b) grow very poorly in non-dividing cells compared to dividing cells. Growth of HSV-1 TK mutants (Jamieson et al., 1974) and PRV TK mutants (McGregor et al., 1985) is normal in exponentially dividing cells. In contrast, RR mutants of both viruses replicated to approximately 10-fold lower titres in most cells (Goldstein & Weller, 1988a, b; Preston et al., 1988; Jacobson et al., 1989; Brandt et al., 1991; de Wind et al., 1993). Moreover, it was found that in Vero cells the dTTP accumulation that is induced by HSV-1 infection is abolished in an HSV-1 RR mutant but not in a TK mutant (Daikoku et al., 1991). Thus, both the in vitro and the in vivo data indicate that viral RR activity is more important for efficient DNA synthesis than viral TK activity. Finally, we investigated whether expression of E1 of HCV could change the replication in PBMC or the infectivity of the PRV vector strains in vitro. As previously reported after in vivo infection of the non-virulent (TK-, gE-, gG-, E1+) vector virus expressing E1 (Mulder et al., 1994a), no changes were detected in the in vitro replication of the non-virulent and virulent PRV vector viruses in PBMC.

In conclusion, inactivation of TK and RR, but not inactivation of gE, gG or PK, severely affected virus replication in porcine PBMC. Con A stimulation of lymphocytes restored the reduced replication of the TK mutant, but not of the RR mutant. Con A stimulation of lymphocytes reduced the replication of wild-type virus NIA-3. We concluded that both viral TK and RR activity are important for efficient replication of PRV in resting lymphocytes, but only RR activity is important for replication in dividing lymphocytes. This study demonstrates that replication of PRV in porcine peripheral blood mononuclear lymphocytes depends on cell type, viral genomic factors and cellular activation state.

References


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