Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation

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In the present study, the outcome of an inoculation of equine peripheral blood mononuclear cells (PBMC) with equine herpesvirus type 1 (EHV-1) was studied in vitro. Cytoplasmic and plasma membrane expression of viral antigens, intra- and extracellular virus titres, and plaque formation in co-culture were determined. EHV-1 replicated in monocytes, although in a highly restricted way. Viral antigens were found at maximum levels (8.7% of the monocytes) at 12 h post-infection. The infection was productive in 0.16% of the monocytes. The virus yield was $10^9$ TCID$_{50}$ per productive cell. In a population of resting lymphocytes, 0.9% of cells were infected and less than 0.05% produced infectious virus. After prestimulation with different mitogens, the number of infected lymphocytes increased four to twelve times. The susceptible lymphocytes were T-lymphocytes. In mitogen-stimulated lymphocytes, clear expression of viral antigens was found on the plasma membrane.

Equine herpesvirus type 1 (EHV-1), a member of the Alphaherpesvirinae, is a major pathogen of horses, responsible for respiratory disorders, abortion, neonatal foal disease and neurological disorders. Starting from 4–6 days after EHV-1 infection, an extensive cell-associated viraemia is detected, which lasts until 9–14 days after infection (Gibson et al., 1992). T-lymphocytes seem to be the most susceptible of the peripheral blood mononuclear cells (PBMC) (Scott et al., 1983). Viraemia is associated with, for example, T-cell lymphopenia and appearance of blasts (McCulloch et al., 1993) and may occur in the presence of virus-neutralizing antibodies (Doll & Bryans, 1963; Mumford et al., 1987). Carried by infected leukocytes, EHV-1 spreads to internal organs.

Information on the interaction between EHV-1 and leukocytes is rather scarce. Scott et al. (1983) demonstrated EHV-1 infection of PBMC by co-cultivation from 2 to 14 days after experimental inoculation of ponies. Virus was not detected by co-cultivation of disrupted leukocytes. After mitogen stimulation of the leukocytes in culture, infectious virus was detected in a higher number of cells and was also found by co-cultivation of disrupted leukocytes. Dutta & Myrup (1983) performed a similar study, but extended it with in vitro infection of leukocytes. For in vivo-infected PBMC, they obtained similar results as Scott et al. (1983). In vitro-infected, non-stimulated PBMC gave a higher number of plaques than mitogen-stimulated PBMC, in contrast to the results obtained in vivo.

The main purpose of this study is to obtain more detailed information about the replication of EHV-1 in freshly isolated, equine PBMC and to investigate the effect of mitogen stimulation on the replication kinetics of EHV-1 in lymphocytes.

PBMC were isolated by density centrifugation of heparinized blood from adult, infection-immune horses on Ficoll-Paque and afterwards separated into two sub-populations by plasma-mediated adhesion as described by Nauwynck & Pensaert (1994). Adherent cells consisted predominantly of monocytes and non-adherent cells consisted predominantly of lymphocytes. Remaining monocytes in the lymphocyte-enriched population were removed by plastic adhesion during 1 h at 37 °C. The composition of the PBMC and subpopulations was determined by flow cytometry (FACSCalibur; Becton Dickinson) using MAb HB88A and DH59B (VMRD) to stain T-lymphocytes and monocytes, respectively (Tumas et al., 1994), and a hyperimmune goat serum against horse IgM (Kirkegaard and Perry Laboratories) to stain B-lymphocytes. In the population of PBMC, the percentages of T-lymphocytes, B-lymphocytes and monocytes were 54.3±0.1%, 24.4±1.7% and 9.0±0.3%, respectively. In the non-adherent population, the percentages were 66.4±5.7%, 24.9±8.7% and 10.0±0.6%, respectively, whereas in the adherent population, 79.1±5.2% of the cells were identified as monocytes.

PBMC, monocytes and lymphocytes were inoculated immediately after isolation with the Belgian EHV-1 strain.
97P70 at an m.o.i. of 10. The strain was isolated from lungs of an aborted foetus in 1997 and was plaque-purified twice in equine embryonic lung cells. After 1 h incubation at 37 °C, extracellular virus was removed by treating the cells with a citrate buffer, pH 3 (Mettenleiter, 1989). After two washing steps, cells were resuspended in leukocyte medium [RPMI 1640, 10% foetal calf serum, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1% non-essential amino acids 100 × (GibcoBRL), 1 mM sodium pyruvate] and incubated at 37 °C in 5% CO₂. Mock-infected cells were included as a control and yielded negative data in each case. Cell viability was determined by flow cytometry, using propidium iodide (1 µg/ml) at 3 and 24 h post-inoculation (p.i.). Viability was always higher than 65% and no differences were observed between EHV-1-infected and mock-infected cells.

Cells were collected at 3, 5, 7, 9, 12 and 24 h.p.i. The percentages of infected cells were determined by indirect immunofluorescence staining on acetone-fixed cell smears using protein A-purified and biotinylated rabbit antibodies against EHV-1. Samples were analysed by fluorescence microscopy (Leica DM RBE). Viral antigens appeared from 5 h.p.i. The number of infected monocytes and PBMC peaked at 12 (8.7 ± 4.2%) and 24 h.p.i. (2.5 ± 2.4%), respectively, whereas the percentage of infected lymphocytes remained below 1% (Table 1). Medium was collected to quantify extracellular virus titres. Cells were collected and freeze–thawed twice. The cell lysate was titrated for the presence of intracellular virus. Virus titration was performed on rabbit kidney (RK13) cells. In the PBMC and lymphocytes, intracellular virus titres started to increase at 5 and 7 h p.i., respectively, and reached a maximum at 12 h p.i. (Fig. 1). In the enriched monocytes, intracellular titres increased from 3 h p.i. and reached a maximum at 7 h p.i. (Fig. 1). Extracellular titres increased only in the PBMC from 9 h p.i. (Fig. 1).

For co-cultivation, cells were seeded at 1 h p.i. on RK13 monolayers. The monolayers were overlaid with 0.94% carboxymethylcellulose (Sigma). Duplicate preparations were made after disruption of leukocytes by ultrasonication. After 7 days incubation at 37 °C in 5% CO₂, numbers of plaques were counted. The percentage of infected leukocytes at 12 h.p.i. was determined by indirect immunofluorescence staining as described above. The total numbers of plaques per 10⁴ leukocytes were 11.2 ± 0.8, 15.7 ± 7.5 and 3.8 ± 1.4 for the PBMC, monocytes and lymphocytes, respectively. The percentages of infected leukocytes at 12 h.p.i. were 1.3 ± 0.57%, 1.2 ± 0.27% and 0.73 ± 0.25%, respectively. It was calculated that plaque formation was induced by 13.7 ± 8.3% of the

### Table 1. Viral antigen expression in freshly isolated and EHV-1-inoculated leukocytes

All data are expressed as the mean value of three experiments ± SD.

<table>
<thead>
<tr>
<th>Selected leukocyte fraction</th>
<th>h.p.i.</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PBMC</td>
<td></td>
<td>0.5 ± 0.2</td>
<td>1.5 ± 0.8</td>
<td>2.1 ± 0.4</td>
<td>2.3 ± 0.1</td>
<td>2.3 ± 2.1</td>
<td>2.5 ± 2.4</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td>0.3 ± 0.3</td>
<td>3.9 ± 2.1</td>
<td>6.6 ± 2.6</td>
<td>8.1 ± 3.8</td>
<td>8.7 ± 4.2</td>
<td>7.5 ± 4.4</td>
</tr>
<tr>
<td>T- and B-lymphocytes</td>
<td></td>
<td>0</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.3</td>
<td>0.7 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>0.9 ± 0.6</td>
</tr>
</tbody>
</table>

Fig. 1. Kinetics of EHV-1 replication in freshly isolated PBMC. (a) Intracellular virus titres for the total population of PBMC and for the enriched subpopulations of monocytes and T- and B-lymphocytes. (b) Extracellular virus titres. The detection limit for these assays was 1.5 log₁₀ TCID₅₀. All data are expressed as the mean value of three experiments ± SD.
infected monocytes, \(9.2 \pm 2.8\%\) of the infected PBMC and \(5.3 \pm 0.9\%\) of the infected lymphocytes. No plaques were observed in the duplicate preparations.

PBMC and lymphocytes were mitogen-stimulated by adding pokeweed mitogen (PWM), concanavalin A (ConA), phytohaemagglutinin (PHA) or ionomycin and phorbol dibutyrate (IONO/PDB) (Sigma) to the medium. Medium was further supplemented with 10 U/ml heparin. Cellular DNA analysis was performed as described by Darzynkiewicz et al. (1984). Optimal stimulation was obtained after 48 h treatment with concentrations of 4 \(\mu g/ml\) for PWM, ConA or PHA and 0.5 \(\mu M\) and 10 nM for IONO and PDB, respectively. After 48 h of mitogen stimulation, PBMC and lymphocytes were inoculated and at 1, 12 and 24 h p.i., lymphocytes were collected. The percentages of infected lymphocytes and virus titres were determined as described above. For all mitogens used, the percentage of infected lymphocytes increased four to twelve times. PWM and IONO/PDB showed the largest effect. ConA-, PWM- and IONO/PDB-stimulated lymphocytes showed an increase in intra- and extracellular virus titres; PHA only induced a slight increase in intracellular titres (Table 2).

The identity of mitogen-stimulated and infected PBMC was determined by double labelling. PBMC were collected at different times (h) p.i. and identified by specific cell markers as described above. The percentage of infected cells was determined by indirect immunofluorescence staining using protein G-purified and biotinylated horse antibodies against EHV-1; double staining experiments were carried out twice (experiments 1 and 2). Most of the infected cells were T-lymphocytes \((0.9, 7.3 \text{ and } 3.3\%\text{ at } 24, 48 \text{ and } 72 \text{ h p.i., respectively, for experiment 1 and } 0.5, 2.6 \text{ and } 2.0\%, \text{ respectively, for experiment 2}).\) Infected monocytes were only detected at 48 h p.i. \((0.5\%).\) None of the infected cells were B-lymphocytes.

Expression of viral antigens on the plasma membrane was determined for IONO/PDB-stimulated PBMC at 48 h p.i. by indirect immunofluorescence staining, using protein G-purified and biotinylated horse antibodies against EHV-1. Non-stimulated cells were included as a control. Approximately 8% of the stimulated PBMC showed expression of viral antigens on the plasma membrane.

Our results show that in fresh, unstimulated equine PBMC, monocytes are the most important cell fraction in which EHV-1 replicates. Other alphaherpesviruses such as bovine herpesvirus type 1 (Rouse & Babiuk, 1975; Nyaga & Mc Kercher, 1980), suid herpesvirus type 1 (SHV-1) (Wang et al., 1988) and herpes simplex virus (HSV) (Plaeger-Marshall & Smith, 1978; Mintz et al., 1980) also mainly replicate in monocytes/macrophages. However, for EHV-1, replication in monocytes is clearly restricted. Less than 10% of the monocytes express viral antigens and only 0.16% induce plaques on co-culture. Lack of viral antigen expression in more than 90% of the monocytes indicates that an early block exists in the replication cycle of EHV-1. Moreover, most of the infected monocytes do not produce detectable amounts of EHV-1, which may be explained by a block at another level in the replication cycle. This finding that several blocks exist in agreement with results obtained with other alphaherpesviruses in monocytes (Albers et al., 1989; Nauwynck & Pensaert, 1994). Based upon the highest extracellular virus progeny titre \((10^{9.9} \text{ TCID}_{50} \text{ per } 10^6 \text{ inoculated cells})\) and the percentage of virus-producing monocytes \((0.16\%\), it is estimated that \(10^{9.7} \text{ TCID}_{50} \text{ virus was formed per virus-producing cell, which demonstrates that even virus-producing monocytes are not fully productive. This is in contrast with HSV-1 (Nauwynck & Pensaert, 1994).}\

Our results with regard to the susceptibility of lymphocytes to EHV-1 infection are somewhat different from those obtained \textit{in vivo} by Scott et al. (1983). They suggested that EHV-1 was mainly T-lymphotropic in unstimulated PBMC. We found that most of the unstimulated lymphocytes were refractory to infection. Very low percentages of infected lymphocytes were

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Table 2. EHV-1 infection in a subpopulation of T- and B-lymphocytes stimulated with different mitogens

<table>
<thead>
<tr>
<th>Mitogen*</th>
<th>h.p.i.</th>
<th>EHV-1 antigen-positive cells (%)</th>
<th>Intracellular virus titres ((\log_{10} \text{TCID}_{50}/10^6 \text{cells}))</th>
<th>Extracellular virus titres ((\log_{10} \text{TCID}_{50}/10^6 \text{cells}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>(0 \pm 0.8)</td>
<td>(\leq 1.5 \pm 0.0)</td>
<td>(\leq 1.5 \pm 0.0)</td>
</tr>
<tr>
<td>ConA</td>
<td>1</td>
<td>(1.0 \pm 0.3)</td>
<td>(\leq 1.5 \pm 0.0)</td>
<td>(\leq 1.5 \pm 0.0)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>(2.0 \pm 0.3)</td>
<td>(1.0 \pm 0.3)</td>
<td>(1.6 \pm 0.1)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>(3.0 \pm 1.2)</td>
<td>(2.0 \pm 0.3)</td>
<td>(2.5 \pm 0.3)</td>
</tr>
<tr>
<td>ConA</td>
<td>1</td>
<td>(0 \pm 0.8)</td>
<td>(\leq 1.5 \pm 0.0)</td>
<td>(\leq 1.5 \pm 0.0)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>(1.0 \pm 0.3)</td>
<td>(\leq 1.5 \pm 0.0)</td>
<td>(\leq 1.5 \pm 0.0)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>(2.0 \pm 0.3)</td>
<td>(1.0 \pm 0.3)</td>
<td>(1.6 \pm 0.1)</td>
</tr>
<tr>
<td>ConA</td>
<td>1</td>
<td>(0 \pm 0.8)</td>
<td>(\leq 1.5 \pm 0.0)</td>
<td>(\leq 1.5 \pm 0.0)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>(1.0 \pm 0.3)</td>
<td>(\leq 1.5 \pm 0.0)</td>
<td>(\leq 1.5 \pm 0.0)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>(2.0 \pm 0.3)</td>
<td>(1.0 \pm 0.3)</td>
<td>(1.6 \pm 0.1)</td>
</tr>
</tbody>
</table>

* Stimulation for 48 h. Abbreviations: ConA, concanavalin A; PHA, phytohaemagglutinin; PWM, pokeweed mitogen; and IONO/PDB, ionomycin and phorbol dibutyrate.
detected and less than 0.05% produced infectious virus on co-culture.

EHV-1 showed an increased replication in mitogen-stimulated lymphocytes, which is consistent with the results of Scott et al. (1983). Mitogens mimic the initial signals required to initiate cell proliferation or to induce a state of competence (Terada et al., 1991). The ability of T-lymphocytes to support virus replication following mitogen stimulation has been recognized for other herpesviruses as well (Nyaga & McKercher, 1980; Teute et al., 1983; Wang et al., 1988). HSV has long been known to replicate more efficiently in actively dividing than in growth-arrested cells. Recently, Schang et al. (1998) found that olomoucine and roscovitine inhibit HSV replication. Both substances exert an influence on the cell cycle by inhibiting certain cyclin-dependent kinases. The authors suggested that one or more of these kinases, which are active during the cell cycle from late G$_1$ onward, are required for HSV replication. For EHV-1, similar specific cell cycle events may play a role in virus replication.

Activation of T-lymphocytes may be an important pathogenic feature during an EHV-1 infection. McCulloch et al. (1993) demonstrated an increase in the percentage of blastic cells of up to 40% in the blood circulation of horses from 4–8 days and on day 10 after experimental inoculation with EHV-1. This coincides with the viraemic phase of an EHV-1 infection. Blastic transformation of lymphocytes, induced in vitro by mitogens or induced in vivo during an EHV-1 infection, most likely provides a signal for the virus to start its replication. The factor(s) inducing the proliferation of lymphocytes in vivo during an EHV-1 infection will be further examined.

Scott et al. (1983) experimentally infected ponies and collected blood samples at different time intervals after infection. Blastic transformation of lymphocytes may have taken place during infection before culturing the cells in vitro, which may explain why EHV-1 replication was found in T-lymphocytes even without mitogen stimulation. In our experiments, PBMC were obtained from healthy horses and were infected in vitro. It is plausible that almost no blastic transformation occurred before mitogens were added in vitro. The very low percentage of infected cells that we detected in the unstimulated lymphocytes may represent a small fraction of lymphoblasts, present in the blood of healthy horses.

We demonstrated a clear plasma membrane expression of EHV-1 antigens on mitogen-stimulated leukocytes. Such an expression makes infected cells recognizable for antibodies. After binding of the antibodies to their respective antigens, anchored in the plasma membrane, cell lysis occurs by the activation of complement or phagocytes. However, EHV-1-induced viremia occurs in the presence of virus-neutralizing antibodies (Doll & Bryans, 1963; Mumford et al., 1987). How EHV-1-infected leukocytes escape elimination by the host’s immune system is unknown and will be examined in the future.

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References


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