Mitogen stimulation favours replication of equine herpesvirus-1 in equine blood mononuclear cells by inducing cell proliferation and formation of close intercellular contacts

Karen M. van der Meulen, Hans J. Nauwynck and Maurice B. Pensaert

In the present study, equine herpesvirus-1 (EHV-1)-infected cells were identified in ionomycin/phorbol dibutyrate (IONO/PDB)-stimulated peripheral blood mononuclear cells (PBMC) and the mechanism by which stimulation increases the percentage of infected cells was examined. In the population of viral antigen-positive PBMC, 38.4±4.5% were CD5+ T-lymphocytes (18.1±3.2% CD4+), 18.1±5.4% were B-lymphocytes, 8.5±3.9% were monocytes and 35% remained unidentified. The role of the cell cycle in the increased susceptibility to EHV-1 upon stimulation was examined by stimulating PBMC for 0, 12, 24 or 36 h prior to inoculation. A high correlation was found between the increase of cells in the S- (r = 0.974) and G2/M-phase (r = 0.927) at the moment of inoculation and the increase of infected cells at 12 h post-inoculation (p.i.). This suggests that a specific stage of the S-phase or S- and G2/M-phase facilitates virus replication. At 24 h p.i. lower correlations were found, suggesting that other effects are involved. From 12 h after addition of IONO/PDB, formation of clusters of PBMC became manifest. We examined whether close intercellular contacts in these clusters facilitated cell-to-cell transmission of EHV-1. Between 8 and 17 h p.i., the percentage of clusters containing adjacent infected cells increased from 1.6 to 13.4% and the maximal number of adjacent infected cells increased from two to four. Confocal microscopy visualized close intercellular contacts between adjacent infected cells. It can be concluded that mitogen stimulation favours EHV-1 infection of PBMC (i) by initiating specific cell cycle events and (ii) by inducing formation of clusters, thereby facilitating transmission of virus between cells.

Introduction

Equine herpesvirus-1 (EHV-1), a member of the Alpha-herpesvirinae, is an important pathogen of horses, causing abortion, neonatal foal death, nervous system disorders and less frequently respiratory disorders. An extensive cell-associated viraemia during EHV-1 infection enables the virus to reach internal organs, even in the presence of virus-neutralizing antibodies (Doll & Bryans, 1963; Mumford et al., 1987). During viraemia, the virus is carried by peripheral blood mononuclear cells (PBMC), mainly by lymphocytes and to a lesser extent by monocytes, as described by Scott et al. (1983).

Somewhat different from these results obtained in vivo by Scott et al. (1983), we found during in vitro studies that the majority of the EHV-1-infected equine PBMC are monocytes, whereas most of the lymphocytes are refractory to infection (van der Meulen et al., 2000). However, mitogen stimulation of PBMC prior to EHV-1 infection increases the percentage of infected T-lymphocytes (van der Meulen et al., 2000). The identity of the EHV-1-infected subtypes of mitogen-stimulated T-lymphocytes has not been determined so far.

The finding that mitogen stimulation increases the number of infected T-lymphocytes led to the hypothesis that EHV-1 replication in T-lymphocytes might benefit from a specific phase of the cell cycle, induced by mitogen stimulation. Cell cycle-dependent replication has already been demonstrated for several herpesviruses. For example, herpes simplex virus (HSV) needs one or more cyclin-dependent kinases, active from late G1 onward and required for cellular progression into the S-phase, for the accumulation of HSV transcripts, viral DNA replication and production of infectious virus (Schang et al.,...
1998, 1999, 2000). Cyclin-dependent kinases are also required for the replication of human cytomegalovirus (HCMV) (Bresnahan et al., 1997). An effect of the cell cycle on the replication of EHV has already been described by Lawrence (1971) in a human carcinoma cell line. He showed that synthesis of EHV DNA takes place only when the cells are in or just entering the S-phase of the cell cycle. McCulloch et al. (1993) demonstrated an increase in the percentage of blastic cells of up to 40% in the blood circulation of experimentally inoculated horses during a time period coinciding with the viraemic phase of an EHV-1 infection. Cell cycle-dependent replication of EHV-1 may be an important pathogenic feature during an infection in vivo.

The main purpose of this study was to identify the EHV-1-infected subtype(s) of mitogen-stimulated PBMC in vitro and to obtain better insights in the mechanism(s) by which mitogen stimulation affects the course of an EHV-1 infection.

Methods

PBMC. Blood samples were obtained from infection-immune horses by jugular venipuncture and collected on heparin (Leo, Zaventem, Belgium). PBMC were then isolated by density centrifugation on Ficoll-Paque (Pharmacia). Cells were washed three times with PBS and resuspended in medium [RPMI 1640, 10% foetal calf serum, 0.05 mM 2-mercaptopoethanol, 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, 1% glutamine]

Virus and in vitro infection. The Belgian EHV-1 strain 97P70 was used in all experiments (van der Meulen et al., 2000). Virus used for inoculation was at the fifth passage on equine embryonic lung (EEL) cells and one subsequent passage on rabbit kidney (RK13) cells.

PBMC were infected at an m.o.i. of 10. After 1 h of incubation at 37 °C, the cells were washed thoroughly with RPMI and cultured in medium supplemented with 10 U/ml heparin with or without addition of mitogens.

Mitogen stimulation. In a previous study, we demonstrated that the highest number of EHV-1 antigen-positive PBMC was obtained after stimulation with a combination of the phorbol ester phorbol 12,13-dibutyrate (PDB), and the calcium ionophore ionomycin (IONO) (van der Meulen et al., 2000). Therefore, IONO and PDB were used throughout the experiments in the current study. PBMC were stimulated with 0.5 µM IONO and 10 nM PDB (Sigma) in medium as described earlier. Mitogen stimulation was performed before or during EHV-1 infection, depending on the experiment.

Detection of viral antigen-positive PBMC. The percentage of EHV-1 antigen-positive cells was determined by indirect immuno-fluorescence staining on acetone-fixed cell smears. Cells were incubated for 1 h at 37 °C first with mouse polyclonal antibodies against EHV-1 and after three washing steps in PBS with goat anti-mouse antibodies labelled with fluorescein isothiocyanate (FITC). Samples were analysed with a Leica DM BRE fluorescence microscope. At least 200 cells were counted for each sample.

Cell cycle analysis. Cells were fixed in ice-cold ethanol (70%) for 30 min, washed with PBS and stained for 30 min at room temperature with a solution containing 10 µg/ml propidium iodide, 0.1% Triton X-100, 2 mM MgCl2, 0.1 M NaCl, 10 mM PIPES buffer and 20 units/ml RNase A in distilled water. The cells were analysed by flow cytometry using a Becton Dickinson FACS Calibur and BD Cellquest software. The following parameters were stored for further analysis: FSC, SSC, FL2-W and FL2-A. Gates were placed, based on FSC and SSC profiles, to include viable cells only. At least 5000 cells were analysed for each sample.

Identification of the EHV-1-infected, mitogen-stimulated PBMC. A double immunofluorescence staining was performed in two steps. First, the cells were fixed in paraformaldehyde (3%), washed with PBS and incubated for 1 h with one of the following monoclonal antibodies: HT23A (anti-equine CD5), HB61A (anti-equine CD4), 73/6.9.1 (anti-equine CD8) or DH59B (equine monocyte marker) (VMRD, Pullman, WA, USA). Then, an FITC-conjugated goat anti-mouse antibody was added for 1 h. To stain the B-lymphocytes an FITC-labelled goat anti-horse IgM antibody (Kirkegaard & Perry Laboratories) was used. After the first step, the cells were washed in PBS and permeabilized with saponin (0.1%). Then, the cells were incubated for 1 h with protein G-purified and biotinylated horse antibodies raised against EHV-1 and finally with Texas Red- or phycoerythrin-labelled streptavidin (Molecular Probes). An irrelevant monoclonal antibody 41D3 against a putative receptor for a porcine arterivirus (Duan et al., 1998) as well as a non-infected sample were included as controls.

Staining was performed either in suspension or within the chambered coverglass (Nunc) in which some PBMC samples had been stimulated with mitogens and subsequently infected; the latter procedure prevented loss of existing contacts between cells. For cells in suspension, all manipulations were performed at 4 °C. Samples were analysed by flow cytometry. Four parameters were stored for further analysis: FSC, SSC, FL1 and FL2. Gates were placed, based on FSC and SSC profiles, to include viable cells only. At least 10000 cells were analysed for each sample. Within the chambered coverglass, all manipulations during the staining were performed at room temperature. Cells were analysed within their recipient with a Leica DM IL inverted fluorescence microscope. At least 100 cells or cell groups were counted for each sample. Additionally, cells were analysed with a Bio-Rad Radiance 2000 confocal laser scanning system linked to a Nikon Diaphot 300 microscope.

Results

Identification of the EHV-1-infected, IONO/PDB-stimulated PBMC by flow cytometry

PBMC were simultaneously stimulated and infected. Cells were collected at 48 h post-inoculation (p.i.) and a double immunofluorescence staining was performed. The percentages of the different cell types within the total population of PBMC and within the population of EHV-1 antigen-positive PBMC are shown in Table 1.

The total population of PBMC consisted mainly of CD5+ T-lymphocytes (including CD4+ and CD8+ T-lymphocytes), with B-lymphocytes the next largest fraction. Monocytes formed the smallest fraction of the PBMC. With the markers used in this experiment, 36-9% of the PBMC could not be identified.

Most of the EHV-1 antigen-positive cells were CD5+ T-lymphocytes, with B-lymphocytes the next largest fraction. The smallest fraction of the EHV-1 antigen-positive cells were monocytes. Thirty-five percent of the infected cells could not be identified. The composition of EHV-1-infected PBMC reflected well the composition of the total population of PBMC.
Table 1. Identification of the EHV-1-infected cells in IONO/PDB-stimulated PBMC

PBMC were simultaneously stimulated and infected; cells were collected at 48 h.p.i. All data are expressed as the mean value of at least three experiments ± SD.

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Percentage of cells identified as:</th>
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<tr>
<td></td>
<td>CD5+ T-lymphocytes</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Total population of PBMC</td>
<td>46±2</td>
</tr>
<tr>
<td>EHV-1-infected PBMC</td>
<td>38±4</td>
</tr>
</tbody>
</table>

* 100 – (% CD5+ T-lymphocytes + % B-lymphocytes + % monocytes).

Table 2. Replication of EHV-1 in PBMC stimulated with IONO/PDB during different time periods prior to inoculation

<table>
<thead>
<tr>
<th>Horse no.</th>
<th>Stimulation with IONO/PDB</th>
<th>Prior to EHV-1 inoculation (h)</th>
<th>After EHV-1 inoculation</th>
<th>Cell cycle distribution at EHV-1 inoculation (%)</th>
<th>EHV-1 antigen-positive cells at … h p.i. (%)</th>
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</thead>
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<tr>
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<td>No (0)</td>
<td>No</td>
<td>G0/G1</td>
<td>S</td>
</tr>
<tr>
<td>1†</td>
<td></td>
<td>No (0)</td>
<td>No</td>
<td>98±2</td>
<td>1±6</td>
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<tr>
<td></td>
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<td>Yes (12)</td>
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<td>Yes</td>
<td>95±7</td>
<td>3±0</td>
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<td></td>
<td>Yes (36)</td>
<td>Yes</td>
<td>92±7</td>
<td>5±8</td>
</tr>
</tbody>
</table>

* Mean value of three experiments ± SD.
† Value of a single experiment.

Replication of EHV-1 in PBMC stimulated with IONO/PDB for different time periods prior to inoculation

PBMC were stimulated for 0, 12, 24 or 36 h and subsequently infected. Stimulation was continued after inoculation. Non-stimulated PBMC were included as a control. Cells were collected at 0, 12 and 24 h.p.i. Table 2 shows the distribution of cells in the different stages of the cell cycle (G0/G1, S, G2/M) at the time-point of inoculation with EHV-1 and the percentage of EHV-1 antigen-positive cells at different time-points after inoculation for the PBMC of three different infection-immune horses.

Based on the results of the five experiments, the mean percentage of PBMC in the G0/G1-phase of the cell cycle decreased from 98±2 ±1-0% before stimulation to 93±3 ±1-3% after 36 h of stimulation. The mean percentage of PBMC in the S- and G2/M-phase increased from 1±5 ±1-2% and 0±4 ±0-1% respectively before stimulation to 5±7 ±0-8% and 1±1 ±0-5% respectively after 36 h of stimulation.

Owing to the large biological variation observed between the individual experiments, significant differences were not
Formation of clusters of equine PBMC, induced by stimulation with IONO/PDB, facilitates cell-to-cell transmission of EHV-1. PBMC were stimulated for 24 h with IONO/PDB and infected afterwards. At 8, 17 and 24 h p.i. immunofluorescence staining using biotinylated horse anti-EHV-1 polyclonal antibodies and streptavidin–Texas red was performed to detect viral antigen-positive cells. Clusters of PBMC are shown in (a) as detected by light microscopy. Bar, 10 µm. Panels (b), (c) and (d) are 3-dimensional images of a cluster containing one, two and three adjacent infected cells respectively, constructed by superimposing the images obtained at 20 different sections throughout the cell by confocal microscopy. Bar, 5 µm.

found between the absolute mean percentages of EHV-1 antigen-positive cells in the 0, 12, 24 or 36 h-prestimulated samples and the non-stimulated control at 12 h p.i. However, when relative values were calculated based on the data from the individual experiments and with the non-stimulated control as a reference value of 1, a significant difference was observed between the 36 h-prestimulated PBMC and the control (relative values were 0·9 ± 0·2, 1·9 ± 1·0, 2·3 ± 0·7 and 3·1 ± 2·3 for the 0, 12, 24 and 36 h-prestimulated samples respectively). At 24 h p.i., a significant difference was observed between the absolute percentages of viral antigen-positive cells in the 12, 24 and 36 h-prestimulated samples and the non-stimulated control. For the relative values, a significant difference was found between the 24 and 36 h-prestimulated samples on the one hand and the non-stimulated control on the other hand (relative values were 1·6 ± 0·5, 5·7 ± 2·9, 9·1 ± 5·3 and 9·7 ± 7·8 for the 0, 12, 24 and 36 h-prestimulated samples respectively). Statistical analysis was based on the least significant difference (LSD; rejection level 0·050).

A correlation coefficient (r; Pearson’s correlation) between the increase in the percentage of cells in the S- or G2/M-phase at the moment of inoculation with EHV-1 and the increase in the percentage of EHV-1 antigen-positive cells was calculated for 0, 12, 24 and 36 h-prestimulated PBMC. Therefore, the relative values were used for both the percentage of viral antigen-positive cells and the percentage of cells in the S- and G2/M-phase. The highest correlation was observed between the relative value of cells in the S-phase at the moment of inoculation and the relative value of EHV-1 antigen-positive cells at 12 h p.i. (r = 0·974). A lower correlation was found between the relative value of cells in the G2/M-phase and the EHV-1 antigen-positive cells at 12 h p.i. (r = 0·27), between the relative value of cells in the S-phase and the EHV-1 antigen-positive cells at 24 h p.i. (r = 0·909) and, finally, between the relative value of cells in the G2/M-phase and the EHV-1 antigen-positive cells at 24 h p.i. (r = 0·865). However, all calculated correlations were significant (t-test; rejection level 0·050).

### Formation of clusters of PBMC by stimulation with IONO/PDB

When PBMC were stimulated with IONO/PDB, intercellular contacts were initiated which resulted in the formation of aggregates or clusters of PBMC, consisting of at least two to more than 50 cells (Fig. 1a). Clusters became manifest...
EHV-1 replication within clusters of IONO/PDB-stimulated PBMC

PBMC were stimulated for 24 h prior to EHV-1 inoculation. All data are expressed as the mean value of five experiments ± SD.

<table>
<thead>
<tr>
<th>Time post-inoculation (h)</th>
<th>Percentage of clusters of PBMC with adjacent infected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>83.3 ± 2.7</td>
</tr>
<tr>
<td>17</td>
<td>62.6 ± 9.5</td>
</tr>
<tr>
<td>24</td>
<td>60.6 ± 5.2</td>
</tr>
</tbody>
</table>

Table 3. EHV-1 replication within clusters of IONO/PDB-stimulated PBMC

between 12 and 24 h after addition of IONO/PDB and remained clearly visible till at least 60 h after IONO/PDB addition (end of the experiment).

**EHV-1 replication in clusters of IONO/PDB-stimulated PBMC**

PBMC were stimulated for 24 h and infected afterwards. At 8, 17 and 24 h p.i., a double immunofluorescence staining was carried out. The results are shown in Table 3. At 8 h p.i., most of the infected clusters contained one viral antigen-positive cell (Table 3; Fig. 1 b). Between 8 and 17 h p.i., a 2- to 2-fold increase in the percentage of clusters with infected cells was seen and the number of adjacent infected cells per cluster increased from a maximum of two to a maximum of four (Table 3; Fig. 1 c, d). The percentages obtained at 24 h p.i. were similar to those obtained at 17 h p.i. By using confocal laser scanning microscopy, the presence of close intercellular contacts between adjacent infected cells was visualized in more detail, as shown in Fig. 2 (a, b).

Between 8 and 17 h p.i., an increase was seen in the percentage of clusters containing one infected cell (Table 3). Identification with DH59B, an equine monocyte marker, revealed that this increase was caused by an increase in the percentage of clusters containing one infected cell, not belonging to the monocytes (from 10.5 ± 4.1% at 8 h p.i. to 19.3 ± 4.0% at 17 h p.i.). The percentage of clusters containing one infected monocyte, on the other hand, remained at a constant level (4.6 ± 1.3% at 8 h p.i. and 4.9 ± 1.0% at 17 h p.i.). The percentages obtained at 24 h p.i. were similar to those obtained at 17 h p.i.

A similar pattern was seen in the population of cells that remained individual in the recipient, without any visual contact to other cells. In the population of cells not belonging to the monocytes an increase was seen in the percentage of viral antigen-positive cells between 8 (2.2 ± 1.1%) and 24 h p.i. (6.9 ± 6.0%), whereas a constant number of monocytes was infected during the entire course of the EHV-1 infection (10.0 ± 4.0, 11.0 ± 2.1 and 11.1 ± 3.4% at 8, 17 and 24 h p.i. respectively).

In clusters with two or more infected cells in contact, the infected cell groups consisted of either monocytes and non-monocytes or non-monocytes only (maximum 6.8 ± 4.0 and 7.6 ± 3.6% of all the clusters respectively). Clusters consisting of two or more infected monocytes formed 1.3 ± 1.0% of all the clusters.

**Discussion**

The present study shows that stimulation of equine PBMC with IONO/PDB affects an EHV-1 infection in two ways. First, stimulation initiates proliferation events in lymphocytes,
which enable EHV-1 to replicate in a limited number of cells. Second, stimulation induces the formation of aggregates or clusters of PBMC. The close interactions between the PBMC within these clusters facilitate the transmission of EHV-1 from an infected cell to an uninfected adjacent cell.

EHV-1 infection was studied in a population of PBMC stimulated with IONO/PDB for different time periods prior to virus inoculation. PBMC were isolated from infection-immune, seropositive horses; therefore they may be assumed to be latently infected. It has been reported that latent EHV-1 can be reactivated in vitro from leukocytes by the mitogens phyto-haemagglutinin and pokeweed mitogen, as detected by cocultivation and immunofluorescence (Smith et al., 1998). Since IONO/PDB also acts as a mitogen, it is reasonable to suggest that these products induced reactivation of EHV-1 in the leukocytes of the three infection-immune horses, thereby confounding the results of the present study. However, to exclude an influence of reactivation of latently infected leukocytes on the percentage of viral antigen-positive cells, we always determined the percentage of EHV-1 antigen-positive cells in uninoculated PBMC after 0, 12, 24 and 36 h of stimulation (0 h p.i.). No viral antigen-positive cells were detected (Table 2). Therefore, we may conclude that the stimulation with IONO/PDB did not cause a reactivation of EHV-1 and did not affect the percentage of EHV-1 antigen-positive cells.

Based on the relative values of viral antigen-positive cells at 12 h p.i., a significant difference was observed between the 36 h-prestimulated PBMC and the non-stimulated control. This suggests that stimulation with IONO/PDB induces one or more specific events that enable EHV-1 to replicate in a limited number of cells. Viral transmission from cell to cell, as discussed below, cannot account for the increase, since groups consisting of two or more adjacent viral antigen-positive cells occurred mainly from 14 h p.i. onwards (data not shown). Moreover, when EHV-1 replication was studied at 8 and 17 h p.i. in prestimulated PBMC, a 2-fold increase in the percentage of clusters containing one infected lymphocyte (from 10±5% at 8 h p.i. to 19±3% at 17 h p.i.) as well as in the percentage of individually located, infected lymphocytes (from 2±2% at 8 h p.i. to 4±4% at 17 h p.i.) was observed. Since there is no direct interaction with other infected cells, these observations support the idea that the increase in the percentage of infected cells is not caused by cell-to-cell transmission of the virus, but by another effect induced by the mitogens, for example one or more cell cycle-dependent events.

To determine whether the effect induced by IONO/PDB was related to the S-phase or the G2/M-phase of the cell cycle, the correlation coefficient (r) was calculated between the relative value of cells in the S- or G2/M-phase of the cell cycle at the moment of inoculation and the relative value of EHV-1 antigen-positive cells. A very high correlation (r = 0.974) was observed between the relative value of cells in the S-phase and the relative value of EHV-1 antigen-positive cells at 12 h p.i.

This means that an increase in the percentage of cells in the S-phase after a certain time interval of prestimulation is accompanied by a similar increase in the percentage of EHV-1 antigen-positive cells at 12 h p.i. Therefore, we assume that virus replication is facilitated during a specific stage of the S-phase of the cell cycle. This is consistent with a report by Lawrence (1971), who found evidence for a relationship between EHV DNA synthesis and the S-phase of the cell cycle in KB cells, a human carcinoma cell line. Since most cells entering the S-phase will continue into the G2/M-phase, a significant, but lower correlation was found between the relative proportion of cells in the G2/M-phase and the relative proportion of EHV-1 antigen-positive cells at 24 h p.i. At 24 h p.i., there was still a significant correlation between the different phases of the cell cycle and the relative percentage of EHV-1 antigen-positive cells. However, the correlation coefficients were clearly lower than at 12 h p.i., suggesting that other effects are also involved at this time-point after inoculation.

We hypothesized that the close intercellular interactions formed within the clusters of IONO/PDB-stimulated equine PBMC favoured the transmission of EHV-1 from an infected to an uninfected adjacent cell. Our results show that EHV-1 was indeed transferred from infected cells to adjacent cells. At 8 h p.i., only 1-6% of the clusters contained a group of adjacent infected cells, whereas this percentage increased significantly to 13±4% at 17 h p.i. Moreover, the number of adjacent infected cells per cluster increased from a maximum of two at 8 h p.i. to a maximum of four at 17 and 24 h p.i. With confocal laser scanning microscopy the existence of close intercellular contacts between adjacent infected cells was confirmed. The exact mechanism by which EHV-1 is transmitted from one cell to another within the clusters of PBMC remains to be determined. Since addition of virus-neutralizing antibodies to the medium during infection did not alter the percentage of infected clusters or the number of adjacent infected cells per cluster (data not shown), we suggest that a direct cell-associated spread of the virus is involved. Since cluster formation seems to be required for transmission and since cluster formation depends on discrete interactions between cell adhesion molecules (reviewed by Hogg & Landis, 1993), it seems likely that EHV-1 transmission relies on the close contacts initiated by cell adhesion molecules, as described for human immunodeficiency virus (Fais et al., 1995; Tsunetsugu-Yokota et al., 1997).

The results of the present study concerning the subfractions of lymphocytes that were scored as infected differed from those obtained by Scott et al. (1983). However, it is important to mention that the study of Scott et al. was performed in vivo. They experimentally infected ponies to detect and quantify the in vivo presence of the virus in leukocytes. Cultivation and identification were performed on cells obtained from the infected ponies. In the present study, the interaction between leukocytes and EHV-1 was studied in vitro. Leukocytes were isolated from healthy horses and afterwards stimulated and
infected in vitro. Furthermore, Scott et al. used infectious centre assays and plaque assays to detect the infected cells and therefore their results represent the ‘productively infected’ leukocytes. In our experiments, immunofluorescence staining was used and therefore the results represent the ‘viral antigen-positive’ leukocytes. In our previous report, we demonstrated that only 13·7% of the viral antigen-positive monocytes and 5·3% of the viral antigen-positive lymphocytes were productively infected. This means that the number of viral antigen-positive cells will be quite different from the number of productively infected cells. The above mentioned differences may be the cause of the disparity between the study of Scott et al. and the present study.

Remarkably, one-third of the PBMC could not be identified by the markers used in our experiments. This finding cannot be attributed to a specific subset of cells (30·1±5·7% unidentified). Modulation of the expression of cell surface molecules in response to phorbol esters has been described in horses by Zhang et al. (1994). Since they found that PMA-mediated down-regulation of EHV-1 from cell to cell.

We can conclude from this study that mitogen stimulation positively influences an EHV-1 infection in two ways: (i) by initiating specific cell cycle events and (ii) by inducing the formation of clusters of PBMC, thereby facilitating transmission of EHV-1 from cell to cell.

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References


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