In vitro reactivation of latent equid herpesvirus-1 from CD5 /CD8 leukocytes indirectly by IL-2 or chorionic gonadotrophin

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IL-2 and equine chorionic gonadotrophin (eCG) initiated reactivation of equid herpesvirus-1 (EHV-1) from venous lymphocytes at a frequency of 1/10⁵. Indirect immunofluorescence showed that >80% of virus-positive leukocytes were CD5/CD8 with the remaining 20% being CD5/CD8/CD4. Co-cultivation demonstrated that the reactivated virus was infectious. In addition, virus was reactivated in vitro from leukocytes of >70% of horses by the mitogens phytohaemagglutinin (PHA) and pokeweed mitogen (PWM). Transfer of supernatants showed that IL-2 and eCG acted indirectly by causing the release of other mediators from adherent cells; these mediators then reactivated EHV-1 from T cells. Blocking experiments with anti-IL-2 showed that PWM and PHA acted via IL-2 but that eCG did not. This is the first clear definition of the lymphoid cells that harbour latent EHV-1 in vivo and correlates with current RT–PCR and in situ hybridization of latency-associated transcripts in lymphocytes. This method of reactivation in vitro can be used to detect horses carrying latent EHV-1 in vivo and also has the potential to dissect the sequence of events involved in reactivation in vitro.

Introduction

Equid herpesvirus-1 (EHV-1) is a member of the alpha-herpesvirus subfamily (Allen & Bryans, 1986; Roizman et al., 1992; Telford et al., 1992) with the classical features of latency following acute respiratory disease (rhinopneumonitis), abortion, or more rarely, ataxia. The clinical signs of the acute infection last between 2 and 7 days, with EHV-1 replicating in epithelial cells of the respiratory tract and local lymph nodes. Following this acute respiratory infection there is a leukocyte-associated viraemia (Scott et al., 1983) and leukopenia (Bungardner et al., 1982; Allen & Bryans, 1986). During the viraemia EHV-1 has been isolated for up to 3 weeks post-infection from peripheral blood mononuclear cells, predominantly from T lymphocytes and to a lesser extent monocytes (Bungardner et al., 1982; Kydd et al., 1994). This cell-associated viraemia in an acute infection has been shown to be a prerequisite for abortion and paresis by initiating replication of EHV-1 in endothelial cells in the pregnant uterus and CNS (Patel et al., 1982; Edington et al., 1986, 1991).

Leukocytes also have been identified as a site of latency for EHV-1 (Edington et al., 1985, 1994; Welch et al., 1992). However, neither the type of the cell harbouring latent virus nor the cellular mechanisms by which EHV-1 is reactivated were described. In this paper we describe a method of reactivating EHV-1 in vitro from latently infected, circulating leukocytes using mitogens, hormones or cytokines. This work thus more specifically defines the sites of latency of EHV-1 in the horse and also indicates some of the pathways that might be involved in reactivation in the natural host.

Methods

Sources of tissues. Two sources were used: firstly, four Welsh mountain ponies which had been previously experimentally infected with an Ab4 isolate of EHV-1 (Patel & Edington, 1983); and secondly, abattoir horses of unknown virus and immune status. Samples of venous leukocytes were collected at post-mortem.

Cells. Rabbit kidney cells (RK13) and equine embryonic kidney cells (EEKs) were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% foetal calf serum (FCS), 50 mg/ml gentamicin, 0.2 mg/ml fungizone, 100 IU/ml penicillin and 100 mg/ml streptomycin. EEKs were used within 3 to 10 passages, since sensitivity to EHV-1 decreases in cells of higher passages. Plates (24-well) were seeded at 1 x 10⁶ cells per well.

Hormones, cytokines and mitogens. The following reagents were used at the final concentrations indicated: phytohaemagglutinin,

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Peripheral blood mononuclear cells were isolated by Nycoprep separation. A total of $4 \times 10^7$ of these cells in 200 µl anti-equine CD3 plus 100 µl PBS–1% BSA–5 mM EDTA was incubated at 4°C for 15 min. The lymphocytes were washed and resuspended in 400 µl PBS–BSA–EDTA buffer and then incubated for 15 min at 4°C with 20 µl MACS magnetic anti-mouse microbeads per 10^7 lymphocytes. The cells were washed again, pelleted and resuspended in 500 µl PBS–BSA–EDTA buffer. The CD3-positive cells which had attached to the magnetic column were eluted by washing the column with PBS–BSA–EDTA buffer. The purity of the CD3+ cells was analysed by flow cytometry (Coulter Epics) after staining with an anti-mouse FITC-conjugated antibody. The positive fraction, the negative fraction and cells prior to separation were analysed with XL2 software using 10,000 events for each sample, and the percentage of labelled cells was determined.

**In vitro reactivation of EHV-1 from leukocytes.** Blood (20 ml) was collected from ten horses in 30 IU/ml heparin. For leukocyte isolation the blood was allowed to sediment at room temperature for 30–60 min. The leukocyte-rich plasma layer was then collected and centrifuged for 10 min at 1000 rpm and the cell pellet washed twice in PBS before being resuspended, at a concentration of 2 × 10^6 cells/ml, in RPMI 1640 medium containing 10% FCS, t-glutamine and antibiotics as above. For mononuclear cell isolation, whole blood was layered onto a Nycoprep (Nycomed) gradient and the cells of the interface band collected after centrifugation for 30 min at 700 g. These cells were washed as for the leukocytes and resuspended at the same concentration in an identical medium.

Both the whole leukocytes and the mononuclear cells were added to a 25 ml tissue culture flask (NUNC) with optimal concentrations of each mitogen, hormone or cytokine (see above) and incubated at 37°C in 5% CO₂ in air for up to 5 days. At 2 h intervals for up to 12 h, and at 24 h, and thereafter daily for up to 5 days, cells were taken and cytokins made. Virus isolation was made by co-cultivation on monolayers of EEKs and RK13 as described previously (Patel et al., 1982). The cell surface markers of cells harbouring virus were detected using two-colour IIF or by secondary staining with alkaline phosphatase-labelled antibody on cytokins of 2 × 10^6 cells from each time-point. RT–PCR for mRNA of EHV-1 gB subsequently was made on whole leukocyte cultures taken 24 h after exposure to PHA or IL-2.

**Blocking of IL-2 and eCG.** When it had been established that mitogens, IL-2 and eCG were the principal inducers of virus replication, blocking of this action was attempted using venous leukocytes collected from four abattoir horses. The leukocytes were incubated for 24 h with either 20 IU/ml IL-2, 100 µg/ml PWM, 10 µg/ml PHA or 100 µg/ml eCG. Duplicate samples of each reagent were exposed to fivefold titrated amounts of antibody to either human recombinant IL-2 or eCG. The reactivation of EHV-1 was detected by IIF and by co-cultivation at 24 h (see above), and by RT–PCR (see below).

**Reactivation of EHV-1 in adherent and non-adherent cell populations.** Reactivation was carried out on whole and subpopulations, using total leukocytes as described above, or cells removed by overnight adherence to acid-washed coverslips, or the non-adherent population. Each population was stimulated with optimal levels (see above) of PWM, IL-2, eCG and medium alone. Virus was detected by IIF and co-cultivation as above. The adherent population, by morphology, contained 63% monocytes/dendritic cells, 22 ± 5% lymphocytes and < 5% polymorphonuclear cells (Siedek, 1998).
Reactivation of EHV-1 in T cells using supernatants from different leukocyte populations. Whole peripheral blood mononuclear cells, neutrophils, CD3+ lymphocytes, adherent and non-adherent cell populations were purified (see above) from the blood of four abattoir horses and stimulated for 24 h with either IL-2 or eCG. Supernatants from each of these cultures were then added for 24 h to a population of purified T cells (see above) to investigate which cells were supplying the mediators that initiated reactivation of EHV-1. Whole peripheral blood mononuclear cells were isolated by Nycoprep separation; CD3+ lymphocytes were isolated using magnetic cell separation (see above); and neutrophils were collected from the Nycoprep pellet. Adherent and non-adherent cells were isolated as described above.

The T cells were also stimulated with whole leukocyte supernatant as a positive control and with IL-2 or eCG directly as a negative control. Virus was detected in the target T cells by IIF on cytopsins and co-cultivation as above.

RT–PCR. Total RNA was extracted from washed leukocytes using the method of Chomczynski & Sacchi (1987) 24 h after the leukocytes had been exposed to IL2 or PHA (see above). For RT–PCR 1 µg total RNA and 100 pmol random hexamers pd(N)6 in RT buffer with 200 nmol dTT and 50 mM of each deoxynucleoside triphosphate was heated to 65 °C for 5 min and then chilled on ice for 5 min. After the addition of 5 units of RNase inhibitor and 100 units of Moloney murine leukemia virus RT, the reaction mixture was incubated for 1 h at 37 °C and chilled on ice for 10 min. Primer sequences of the conserved gB gene of EHV-1 were used as described by Welch et al., (1992): 1L, GGA AAG GAT ACA GCC ATA CGT C; 3R, CGT ACA CAA TAT CAC CGG TGG A.

Amplifications used the Perkin-Elmer DNA Thermal cycler on 5 µl cDNA, 59-6 µl 10 x NH4 buffer, 2 4 µl of 50 mM MgCl2, 25 pmol of each primer, 50 mM of each deoxynucleoside triphosphate and 5 U BIOTAQ polymerase. Each reaction was overlaid with 80 µl of autoclaved mineral oil. Thirty cycles of denaturation at 94 °C for 1 min were followed by annealing at 51 °C for 2 min, polymerization at 72 °C for 3 min, with an additional final incubation of 7 min to complete all extensions. Completed reactions were held at 4 °C until they could be resolved by electrophoresis in a 3% NuSieve agarose gel and visualized under UV light after ethidium bromide staining. Positive controls were in vitro EHV-1-infected RK13 cells; negative controls were either RNA from unstimulated leukocytes or stimulated leukocyte RNA to which RT was not added.

Results
Identification and frequency of cells harbouring latent virus

IIF identified EHV-1-positive venous leukocytes in 8/10 equines after the leukocytes were exposed to the reagents PHA, PWM, IL-2 and eCG. The first three reagents also each initiated proliferative responses in venous leukocytes at the concentrations used to cause virus reactivation, but eCG did not. IFN-γ, TGF-β, oestrogen, thyroxin, testosterone and progesterone did not reactivate latent EHV-1 (Table 1). Co-cultivation of leukocytes with monolayers confirmed that the reactivated virus was infectious. To detect infectious virus at least 10⁶ leukocytes were needed, whereas IIF the frequency of EHV-1-positive leukocytes was 10–50 per 10⁶ (Table 1).

EHV-1-positive cells could be seen by IIF as soon as 6 h after exposure to mitogen (Fig. 1). At 24 h there was a peak of fluorescence, but after 48 h all preparations were negative by IIF. Virus could be recovered by co-cultivation between 2 and 4 days after exposure to the mediators. All these virus-positive cells were shown to be CD5+ and by parallel analysis 80 ± 6% of the virus-positive cells were also CD8+ (Fig. 2). Virus was also detected in CD5+/CD8− T cells.

Detection by IIF of EHV-1 at different time-points in leukocytes from four horses after in vitro exposure to mitogens, hormones or cytokines was detected by immunofluorescence (IIF) and co-cultivation.

Table 1. Frequency of detection of EHV-1-positive cells

<table>
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<tr>
<th>Reagent*</th>
<th>IIF (+ve/10⁶ cells)†</th>
<th>Co-cultivation‡</th>
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<tr>
<td>PHA (10 µg/ml)</td>
<td>10 ± 3</td>
<td>+</td>
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<tr>
<td>PWM (100 µg/ml)</td>
<td>50 ± 5</td>
<td>+</td>
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<tr>
<td>eCG (100 µg/ml)</td>
<td>10 ± 5</td>
<td>+</td>
</tr>
<tr>
<td>IL-2 (20 IU/ml)</td>
<td>30 ± 3</td>
<td>+</td>
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* Progesterone, oestrogen, thyroxin, testosterone, TGF-β1 and IFN-γ all failed to reactivate detectable virus by IIF or co-cultivation.
† Mean number of positive cells per 10⁶ leukocytes from ten horses (±SD). Frequencies were similar whether a MAb to gB or a rabbit polyclonal antiserum to whole virus was used.
‡ Leukocytes (10⁶) added to monolayers of EEEK or RK13 cells and examined for cytopathic effect over 7 days, with one subsequent subculture.

Fig. 1. Detection by IIF of EHV-1 at different time-points in leukocytes from four horses after in vitro exposure to PHA (■), PWM (△), IL-2 (●) or eCG (×). For each sample MAb anti-EHV-1 gB, followed by rabbit anti-mouse-FITC was used on 1-2 × 10⁶ cells. Bars show SD from the mean of four horses. All reagents reactivated EHV-1 with the virus first detected at 6 h and peaking at 20–23 h, but disappearing in all but PWM by 48 h.
Fig. 2. Co-expression of CD5 (a) or CD8 (c) with EHV-1 (b, d). Virus was detected by anti-EHV-1 rabbit polyclonal and FITC-conjugated ovine anti-rabbit IgG antibodies after exposure of whole blood leukocytes to IL-2 for 24 h. CD5 and CD8 were subsequently detected on the same preparations by anti-equine CD MAbs and alkaline phosphatase conjugated to ovine anti-murine IgG. Magnification, ×1600.

CD8⁻/CD4⁻ cells, but these cells were less frequent (20 ± 6% of the CD5⁺ cells). Significantly higher numbers of virus-positive cells were seen with the mitogens than with eCG (P > 0.05).

Blocking of reactivation

Reactivation of EHV-1 by either IL-2, or the mitogens PHA or PWM (data not shown), could be blocked using antibody to IL-2 (Fig. 3a), but was not blocked by anti-eCG. On the other hand eCG-stimulated reactivation was blocked by anti-eCG (Fig. 3b), but not by anti-IL-2 antibodies.

Mediators of reactivation from different leukocyte populations

EHV-1 consistently could be reactivated from whole leukocyte cultures after 24 h stimulation by IL-2 or eCG (Table 2). However, neither reagent reactivated virus from either purified T lymphocytes, or adherent cells alone, or leukocyte cultures with the adherent population removed. However, if the supernatant from stimulated adherent cells (63 % monocytes/dendritic cells), or stimulated whole blood leukocytes, was added to purified CD3⁺ T cells (93 % pure), then reactivation was detected by IIF and by co-cultivation (Table 2). Supernatants from other separated populations of cells
Fig. 3. Blocking of IL-2 (black bars), PWM (grey bars) or eCG (white bars) reactivation of EHV-1 with antibody to IL-2 (a) or eCG (b). The IL-2 or eCG was initially titrated to find the minimum reactivating dose. Bars represent the standard deviation of the means from leukocytes taken from four horses. (a) The antiserum to IL-2 at concentration ≥ 0.05 IU blocks both PWM and IL-2, but has no effect on eCG. (b) The antiserum to eCG at concentration ≥ 0.01 IU blocks eCG, but has no effect on either IL-2 or PWM.

(neutrophils, non-adherent cells, CD3⁺ cells or T lymphocyte cultures) failed to reactivate virus.

**RT–PCR**

RT–PCR on RNA extracted from leukocytes 24 h after exposure to IL-2 or PHA yielded a 370 bp fragment which co-
migrated with the gB fragment observed on preparations from in vitro EHV-1-infected RK13 cells (Fig. 4). All negative controls were negative.

**Discussion**

 Reactivated EHV-1 could be detected by IIF predominantly in CD5⁻/CD8⁺ (80 ± 6%) and less frequently in CD5⁺/CD8⁻/CD4⁻ (20 ± 6%) cells in venous blood. RT–PCR for mRNA of gB on RNA from stimulated leukocytes and the detection of infectious virus by co-cultivation with RK13 cells confirmed that an infectious cycle of replication was occurring in the leukocyte population. CD5 is a marker for T lympho-

<table>
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<tr>
<th>Stimulant</th>
<th>No. of EHV-1-positive cells per 10⁶ CD3⁺ cells*</th>
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<tr>
<td>Medium</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>IL2</td>
<td>0</td>
</tr>
<tr>
<td>eCG</td>
<td>0</td>
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* Mean number of positive cells from four horses (± SD).
cytes (Blanchard-Chanell et al., 1994) and is also found on a small population of B lymphocytes in humans. CD8 is a marker for cytotoxic T cells and also, in the horse, for natural killer cells (Kydd & Antzak, 1991). As all the reactivated EHV-1-positive cells were shown to also be CD5+, this indicates that 80% of latent EHV-1 is harboured in cytotoxic T cells, but 20% is in a subpopulation that can only be characterized as CD5+. This is the first clear definition of the cells which are specifically harbouring latent EHV-1 in the horse. While both lymphocytes and monocytes were identified as infectious in acute infections by Scott et al. (1983) and Kydd et al. (1994), our evidence indicates that T cells are a predominant site for latency, which is consistent with the detection of latency-associated transcripts (LATs) in leukocytes (Chester et al., 1997). The rapid appearance (at 6–12 h) of the reactivated virus is consistent with this being a primary event, and not a secondary cycle from monocytes infected during the acute phase. The absence of EHV-1 in the adherent cell population also supports monocytes not being a source of latent virus.

The tenfold difference in the frequency of reactivated virus detected by IIF (using either a polyclonal antiserum to whole virus or a MAb to gB) and co-cultivation may be due to the different sensitivities of the assays. But it is also possible that not all cells identified as expressing gB by IIF produce infectious virus. Further work is needed to define this. The peaks of virus detection by IIF (24 and 48 h) terminated abruptly, yet infectious virus could be detected up to 4 days after infection. These results were interpreted as indicating that cells in which virus was reactivated were rapidly lysed.

While leukocytes are primary targets of the alphaherpesvirus of Marek’s disease (Li et al., 1994), and have been recognized as sites of latency with pseudorabies virus of pigs (Saboo & Rajcani, 1976), it is only recently that leukocytes have been recognized as sites of latency in bovine herpesvirus type 1 in cattle (Mweene et al., 1996) and in the rabbit model of herpes simplex virus type 1 (Seto et al., 1997). The reverse is true for EHV-1 in the horse, where leukocytes were first identified as the site of latency (Edington et al., 1985, 1994; Welch et al., 1992), while detection in the trigeminal ganglia has shown a low frequency of neurones expressing LATs (Baxi et al., 1995) and with either no recovery or only low frequencies of infectious virus from latently infected neurones (Burrows & Goodridge, 1984; Edington et al., 1994; Slater et al., 1994). The present work is consistent with the range of variation in the predominant sites of latency that is seen between alphaherpesviruses in different hosts.

Hormones were investigated as possible mediators of reactivation in the horse in order to explain the specificity of EHV-1 infection of the endothelium of the endometrium in pregnancy and of other endocrine glands (Smith et al., 1992; Whitwell & Blunden, 1992). CG, which is one of the major hormones released during early pregnancy, is able to reactivate latent EHV-1. While we postulate that the microenvironment of the endometrium will have higher levels of eCG than other tissues, the precise mechanisms by which eCG acts remains to be identified. However, eCG was not mitogenic and its ability to reactivate was independent of IL-2 mediation. The persistence of latent EHV-1 in T lymphocytes and its reactivation by eCG is consistent with EHV-1 infecting the endothelial lining of the endometrium in pregnant mares (Edington et al., 1986; Smith et al., 1992), particularly when abortion has occurred weeks or months after detectable viraemia (Gleeson & Collins, 1980).

The evidence from culturing separate leukocyte populations indicates that both IL-2 and eCG reactivated EHV-1 indirectly. Thus, EHV-1 could only be reactivated by IL-2 or eCG from whole blood leukocytes and not from purified CD3+ T cells or any of the other separated populations. However, when the supernatant from IL-2- or eCG-stimulated adherent cells was added to an enriched population (93%) of CD3+ T cells, reactivated EHV-1 was detected (Table 2). This suggests that both IL-2 and eCG bound to receptors on monocytes and caused the release of other mediators which in turn have acted on the CD3+ lymphocytes to initiate reactivation of the virus. Candidate mediators are numerous and will be the subject of further investigation, but IL-1 would be a likely candidate since both T and B cells have IL-1 receptors on their surface and IL-1 stimulates activation of many different cell types, including T cells (Dinarello, 1984; Dower et al., 1985). Unfortunately, functional equine IL-1 was not available to investigate this hypothesis, and species specificity prevents the use of heterologous preparations. Apart from eCG, all the mediators of reactivation were mitogenic, which may have influenced the higher frequency of virus-positive cells with these latter agents; but it was beyond the remit of the present investigation to investigate this in any detail.

Prevention of reactivation with PWM and PHA by antibody to IL-2 showed that these mitogens were acting by stimulating the production of IL-2 in a mixed cell population. Anti-IL-2, however, had no effect on the action of eCG nor vice versa. This indicated that eCG and IL-2 had independent access to monocytes.

This present work supports and further defines earlier studies using genomic PCR and co-cultivation of explanted leukocytes to detect latent virus in leukocytes from venous blood and from lymph nodes of the respiratory tract (Welch et al., 1992), and more recent work which has identified LATs in these cells using in situ hybridization and RT–PCR (Chester et al., 1997).

The present work has shown that latent EHV-1 is predominantly harboured in CD5+/CD8+ T lymphocytes in circulating leukocytes. It seems most likely that IL-2 and eCG indirectly stimulate host factors and therefore initiate immediate early transcription leading to the reactivation of the virus via host factors (Jones et al., 1985; Everett, 1987; Goding & O’Hare, 1989; Kemp et al., 1990; Lin et al., 1991; Purewal et al., 1992). The model of reactivation described will be useful for future studies in defining the interaction of infected
leukocytes and endothelial cells in EHV-1 infections of the horse and will also be a useful model for further defining mechanisms involved in alphaherpesvirus latency.

We acknowledge the abattoir staff for their help and co-operation in collecting leukocyte samples; and Mark Holmes, Cambridge University, for the use of CVS5 and CVS4 antibodies. The assistance of the staff of the Biological Services unit at Royal Veterinary College is also greatly appreciated. Financial support for this work was from the Horserace Betting Levy Board.

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Received 15 April 1998; Accepted 29 July 1998