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Equine gammaherpesvirus 2 (EHV2) is latent in B lymphocytes

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Summary. Peripheral blood leukocytes were collected from 5 Thoroughbred horses and examined for the presence of EHV2 in sub-populations of mononuclear cells. Peripheral blood mononuclear cells were separated on Percoll gradients and then enriched for plastic adherent cells (predominantly monocytes), surface immunoglobulin positive (sIg^+) B lymphocytes and T lymphocytes, using panning techniques. The purity of each cell population was assessed by fluorescence activated cell scanning. In an infectious centre assay, each cell population was inoculated onto equine foetal kidney monolayer cell cultures which are fully permissive for the replication of EHV2. Only enrichment for sIg^+ B lymphocytes resulted in a marked increase in the number of infectious centres, indicating that EHV2 is present in B lymphocytes. Freeze-thawing of sIg^+ B lymphocytes, prior to inoculation onto EFK monolayer cell cultures, resulted in the complete abrogation of infectious centre formation, confirming that EHV2 is latent in B lymphocytes i.e., infectious free virus was not present in the cells. The number of EHV2 infected B lymphocytes varied considerably between horses from 4 to 780 per 10^6 cells. Evidence was also obtained that direct cell to cell contact between the epithelial cells and sIg^+ B lymphocytes was necessary for the production of infectious centres. The data indicate that EHV2, like other members of the *Gammaherpesvirinae*, is latent within B lymphocytes.

Introduction

Based on biological properties, herpesviruses are classified in three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* [21]. Viruses in the subfamily *Gammaherpesvirinae* are defined for their lymphotropism and are associated with aberrations of these cells in vivo including transient and chronic lymphoproliferative disorders and in vitro, with the immortalisation of B lymphocytes. Gammaherpesviruses generally have a narrow host range, slow replication cycles, and establish latency in B and/or T lymphocytes. Although the viral genome is replicated in lymphoblastoid cells, infection is frequently

non-productive. Some gammaherpesviruses are known to productively infect epithelial cells. Gammaherpesviruses are divided into two genera, the γ1 and γ2 herpesviruses based on genetic criteria that include GC content and genomic organisation. γ1-herpesviruses include Epstein Barr virus (EBV), the cause of infectious mononucleosis/glandular fever and several lymphoproliferative disorders of humans, and is tropic for B lymphocytes. γ2-herpesviruses include herpesvirus saimiri (HVS2) and are associated with lymphoproliferative disorders in lower primates. γ2-herpesviruses are typically associated with tropism for T cells, although murid herpesvirus strain 68 (MHV68) is tropic for B cells [25] and herpesvirus sylvilagus is tropic for both B and T cells [10].

Equine herpesvirus 2 (EHV2) is a recently classified member of the *Gammaherpesvirinae* [27] and the genome of 184 kbp has been completely sequenced [26]. EHV2 is a slowly cytopathic, highly cell associated virus and can be isolated from buffy coat cells or nasal secretions of up to 90% of adult horses [7, 20]. EHV2 has a relatively broad host-range in vitro in that it is known to replicate in rabbit, guinea pig, feline and ovine kidney cell cultures as well as equine cell cultures of kidney, thyroid, brain, lung, testis, spleen, lymph node, bone marrow, dermis and leukocytes (see [1]). The exact role of EHV2 in producing disease is not fully defined. However, it has been associated with immunosuppression, upper respiratory tract disease, conjunctivitis, general malaise and poor performance [1–3, 6, 17, 23, 24, 28]. It has also been suggested that EHV2 may play a role in transactivation and reactivation of latent alphaherpesviruses, EHV1 (equine abortion virus) and EHV4 (equine rhinopneumonitis virus) [30].

While EHV2 has been linked to a number of disease syndromes in the horse, little is known about the pathogenesis of the virus in causing disease. The reclassification of EHV2 as a gammaherpesvirus suggests new approaches to investigating the role of this virus in various diseases. In this study, we investigated the site and nature of latency of EHV2 and the mechanism of infection of permissive epithelial cells from latently infected B lymphocytes. We show that like EBV and some of the lower primate gammaherpesviruses, EHV2 is latent in B lymphocytes.

Materials and methods

Virus and cells

Equine foetal kidney (EFK) monolayer cell cultures are permissive for the growth of EHV2 and were used in infectious centre assays at passage 5. EFK cells were grown in minimal essential medium (MEM) containing Earle's salts, L-glutamine and non-essential amino acids and supplemented with 10% v/v foetal calf serum (FCS; Gibco BRL), 0.5% v/v lactalbumin hydrolysate (Sigma) and 0.006 M $NaHCO_3$. For infectious centre assays, EFK cells were maintained in MEM supplemented with 1% v/v FCS, 50 μg/ml ampicillin, 0.013 M $NaHCO_3$ and 0.015 M HEPES (Sigma) at pH 7.4 (maintenance medium; MM). A semi-solid overlay medium consisted of MM with 1% carboxy methyl cellulose.

EHV2 strain 94/01, isolated from peripheral blood mononuclear cells (PBMC) of a horse was propagated in EFK monolayer cell cultures for use as a positive control in Millicell (Millipore) experiments (see below) and was used at passage 2.

Horses

Peripheral blood and nasal swabs were collected from 19 Thoroughbred mares on a stud farm in Victoria. The horses were screened for EHV2 DNA in peripheral blood and nasal swabs by PCR. Five horses were chosen for this study, mares 3, 12, 14, 15, and 17.

Infectious centre assays

Peripheral blood was collected from adult horses in sterile tubes containing 15U/ml of preservative free heparin (Sigma). PBMCs were separated on 60% Percoll gradients (Pharmacia) by centrifugation at 400 **g** for 20 min. PBMCs harvested from the interface between the plasma and Percoll were washed three times in RPMI 1640 medium supplemented with 10% heat inactivated serum (56 °C, 30 min; FCS) and separated into populations enriched for plastic adherent cells, B lymphocytes and T lymphocytes by panning techniques. Plastic adherent cells were separated from the total PBMC population by placing approximately 10^7 PBMC/ml in a 60 mm tissue culture grade Petri dish for 1 h at 37 °C. Non-adherent cells were removed and enriched for B and T lymphocytes. For the positive selection of sIg^+ B lymphocytes, Fab2′ goat-(anti-horse IgG) (Chemicon) was used to coat 60 mm tissue culture grade Petri dishes (Nunc) (Mage et al. [11]; Mason et al. [12]). Petri dishes were coated overnight with a 10 μg/ml solution of antibody in PBS at 4 °C. Unoccupied sites were blocked with PBS containing 0.2% BSA for 1 h at 37 °C. Plates were washed 3 times with PBS before use in B lymphocyte pannings. Plastic non-adherent cells were placed on Fab2′ goat-(anti-horse IgG) coated petri dishes for 1 h at 4 °C. Non-adherent, surface immunoglobulin (sIg) negative cells were removed and used as the T-lymphocyte enriched fraction. Petri dishes containing plastic adherent cells or sIg^+ cells were washed with jets of PBS to remove bound cells. In experiments where lysed cells were used, lymphocytes were freeze-thawed 3 times at −70 °C and 37 °C.

The number of cells in each of the four fractions (PBMC, plastic adherent, sIg^+ B lymphocytes and T lymphocytes) was counted and between 10^6 and 10^7 cells from each cell population were inoculated onto EFK monolayer cell cultures and incubated at 37 °C. After 3 days incubation, and before the appearance of any visible cytopathic effect (CPE), the liquid medium was removed and replaced with MM containing 1% methyl cellulose overlay medium and incubated for a further 4–7 days. The identity of the plaques as due to EHV2 was confirmed by PCR analysis of a minimum of 3 plaques from each flask. Monolayers were then fixed with 10% formal saline and stained with 1% crystal violet for plaque enumeration.

Experiments investigating the mode of reactivation of EHV2 were performed by adding lymphocytes or infectious virus in 0.45 μm HA Millicell chambers (Millipore) which were then placed on EFK monolayer cell cultures in 6-well cluster dishes (Greiner). Millicells have a 0.45 μm membrane that allows the free exchange of medium and free virus across the membrane but prevents direct cell–cell contact.

To determine if the horse was shedding EHV2 at the time peripheral blood was collected for infectious centre assays, nasal swabs from each horse were obtained and placed in maintenance medium. The medium was filtered (0.45 μm), inoculated onto EFK monolayer cell cultures and incubated 7–14 days at 37 °C.

PCR analysis

Nested PCR was performed using primers specific for the EHV2 gB gene [18]. For the PCR analysis, approximately 10^6 peripheral blood leukocytes (PBL) from the buffy coat fraction were lysed in lysis buffer (50 μg/ml proteinase K, 0.45% Tween 20, 0.45% NP40 in

10 mM Tris-HCl, pH 8.3) for 18 h at 65 °C. Cellular debris was removed by centrifugation (10 000 **g**, 15 min) and DNA extracted with phenol/chloroform [22]. Plaques picked from infectious centre assays were diluted 1:1 with sterile water and boiled for 15 min before use in PCR. Nasal swabs collected from the horses were placed in 1 ml of sterile MM and used directly in PCR reactions.

FACscan analysis

For single colour FACscan analysis 10^7 cells/ml were stained with monoclonal antibody (F66.3F3) specific to equine $CD3^+$T lymphocytes [4] or with biotinylated Fab2′ goat-(anti-horse IgG) (Chemicon) diluted in PBSBA (phosphate buffered saline containing 0.2% BSA and 0.05% sodium azide) for 30 min at 4 °C. Excess antibody was removed by three washes in PBSBA. $CD3^+$ lymphocytes were stained with phycoerythrin labeled anti-mouse immunoglobulins and Ig^+ cells were stained with fluorescence labeled avidin. After 30 min incubation at 4 °C, cells were washed three times with PBSBA and then fixed in 1.5% paraformaldehyde and refrigerated. Cells were examined using a FACS analyser and Consort 30 computer (Becton Dickinson Immunocytometry Systems, Mountein View, CA) using CALIB settings.

Results

Isolation of EHV2 from lymphocytes

Sixteen of 19 Thoroughbred mares screened by nested PCR for the presence of EHV2 DNA in peripheral blood and nasal secretions were positive in either or both of these sites. Of these 16 EHV2 positive mares, 4 were chosen for further analysis to determine the site of latency of EHV2 and one mare was chosen from the EHV2 negative group.

EHV2 DNA was detected in both nasal secretions and PBL DNA of mare 15 suggesting that this mare was naturally infected with EHV2. To determine if the mare was actively infected with EHV2, nasal swab filtrate was placed on EFK monolayer cell culture but no virus could be cultivated from this site. PBMCs isolated from mare 15 were enriched for plastic adherent cells, sIg^+ B lymphocytes and T lymphocytes and examined in an infectious centre assay. The purity of each cell population was assessed by FACscan. The results (Table 1)

Table 1. Production of EHV2 infectious centres in enriched lymphocyte populations

	% Positive cells by FACscan[a]		Infectious centres/10^6 cells
	$CD3^+$	Ig^+	
Total PBMC	48	29	22
Plastic adherent cells	6	18	4
T lymphocyte enriched cells	57	12	12
B lymphocyte enriched cells	2	75	360

[a] 5000 events were collected for FACscan analysis

show that the total PBMC population contained 29% sIg^+ B lymphocytes and 48% $CD3^+$ T lymphocytes and produced 22 infectious centres per 10^6 cells. The plastic adherent cell population, which contained 18% sIg^+ B lymphocytes and 6% $CD3^+$ T lymphocytes, produced 4 EHV2 infectious centres per 10^6 cells. The T lymphocyte enriched fraction containing 57% $CD3^+$ T lymphocytes and 12% sIg^+ B lymphocytes produced 12 infectious centres. In contrast to these relatively low numbers of infectious centres the sIg^+ B lymphocyte fraction, which contained 75% B lymphocytes, produced 360 infectious centres per 10^6 cells. To examine the status of EHV2 in B lymphocytes, the cells were lysed before addition to the EFK monolayer. No infectious centres were produced when sIg^+ B lymphocytes were lysed before addition to EFK monolayer cell cultures (data not shown) confirming that EHV2 is latent in B cells.

The number of EHV2 infected B lymphocytes varies between horses

Peripheral blood was obtained from 4 other mares to examine whether the number of infected B lymphocytes varies in different horses. The mares were examined for the presence of EHV2 DNA in peripheral blood and nasal secretions by PCR. EHV2 DNA could be detected in either or both the nasal secretions or PBL DNA of three of these mares, 3, 12 and 14 (Table 2). No EHV2 DNA could be detected in mare 17 (Table 2). Purified lymphocytes were enriched for plastic adherent cells, sIg^+ B cells and $CD3^+$ T cells and added to EFK monolayer cell cultures in an infectious centre assay. In 3 of the 4 horses, there was an increase in the number of infectious centres by fractions enriched for sIg^+ B lymphocytes (data not shown) adding evidence that the site of latency of EHV2 was the B lymphocyte. The number of infectious centres formed varied considerably between animals and did not appear to correlate with the ability to detect EHV2 DNA by PCR performed on samples obtained up to three months prior to

Table 2. Comparison of the number of B lymphocytes infected with EHV2 in five mares

Mare no.	PCR status in PBL DNA/nasal swab[a]	Purity of B cells (%)[b]	No. of infectious centres/10^6 B cells[c]	Corrected No. of B cells carrying latent EHV2/10^6[d]
3	+/+	60	467	780
12	+/−	80	17	21
17	−/−	80	3	4
14	+/+	80	0	<0.6[e]
15	+/+	75	360	480

[a] Determined three months before lymphocyte analysis
[b] Determined by single colour FACscan analysis
[c] Infectious centres were enumerated 7–10 days after inoculation
[d] Corrected for % purity of B cells
[e] 2×10^6 lymphocytes placed on EFK monolayer

Table 3. B cells require direct contact with epithelial cells (EFKs) for infectious centre formation

B lymphocyte sample[a]		Infectious centres[b]
Unlysed cells (viable)	direct contact	+
	Millicell	−
Lysed cells (non-viable)	direct contact	−
	Millicell	−
EHV2 infectious virus	direct contact	+
	Millicell	+

[a] 1.6×10^6 B lymphocytes from Mare 15 were added either directly onto EFK monolayer cell cultures or into Millicell chamber

[b] EFK monolayer cell cultures were examined 7 days post inoculation for infectious centres

the infectious centre assays. EHV2 could not be isolated from any of the cell populations obtained from mare 14 despite the detection of EHV2 DNA in both PBL and nasal secretions. EHV2 was isolated from the sIg^+ B lymphocytes of 3 of the 4 mares tested including EHV2 DNA negative mare 17. The number of infectious centres varied between 4 (mare 17) and 780 (mare 3) per 10^6 B lymphocytes. Virus was not isolated from the nasal secretions taken from any of the horses tested.

B lymphocytes require direct contact with the EFK for the production of infectious centres

In infectious centre assays there is direct cell–cell contact between the lymphocytes and the EFK cells. To determine if this direct cell–cell contact is essential for the formation of infectious centres, B lymphocytes from mare 15, were placed in a Millicell chamber which was then placed on an EFK monolayer. Viable lymphocytes placed inside the Millicell chamber did not produce CPE on EFK monolayers (Table 3) indicating that direct cell–cell contact is necessary. As expected from the earlier lysis experiments no infectious centres were produced if cells were lysed before inoculation of the EFK monolayers either directly or by placing the lysate in a Millicell chamber. Cell-free, infectious EHV2 produced CPE when inoculated either directly on the EFK monolayer or when placed in the Millicell chamber confirming that free virus can move from the Millicell chamber and infect the EFK monolayer.

Discussion

The most important finding of this study was that enrichment of B lymphocytes from the total PBMC fraction of EHV2 infected horses resulted in a dramatic increase in EHV2 infectious centres. However, the increase in the number of

infectious centres is obviously related to the purity of the cell fractions which ranged from 60% to 80%. The ability to enrich particular cell populations is mainly dependent on the specificity of the antibodies used for separation. The fact that there are few monoclonal antibodies available to equine lymphocytes limited our ability to obtain lymphocyte subset fractions of higher purity. However, panning techniques are widely used to enrich lymphocyte subsets using polyclonal or monoclonal antibodies [11, 12, 25].

The sIg^+ lymphocyte enriched fraction from 4 of 5 mares, when added to permissive EFK monolayer cell cultures, produced increased numbers of infectious centres per 10^6 cells. Concomitantly, there was a decrease in the number of infectious centres produced by cell fractions depleted of sIg^+ B lymphocytes (Tables 1 and 2). The isolation of EHV2 from sIg^+ B lymphocytes did not appear to be associated with detectable reactivation of EHV2 in any of the donor horses as cell free virus was not isolated from the nasal secretions of the horses collected at the time the blood samples were obtained. Unambiguous evidence that EHV2 was latent in B lymphocytes was adduced from freeze-thaw experiments. When sIg^+ B lymphocytes were freeze-thawed, no cell free infectious virus was recovered in infectious centre assays. Detection of EHV2 DNA by PCR correlated in 3 out of 5 mares examined with the isolation of EHV2 from enriched sIg^+ B cell populations.

EHV2 was isolated from one mare (No. 17) in which EHV2 DNA could not be detected by PCR. The number of EHV2 infected cells at the time of assay was calculated to be 4 per 10^6 sIg^+ cells or approximately 4 infectious centres per 10^7 PBLs assuming an upper limit of 10% B cells in the PBL fraction. Such levels of infection are outside the detection limits of the PCR assay used (100 copies of genome) if each B lymphocyte carries only one copy of viral genome which explains the failure to detect EHV2 DNA in mare 17. A second apparent contradiction occurred in mare 14 where virus could not be isolated from any of the cell populations tested, even when 2×10^6 enriched B cells were inoculated onto EFK monolayers. The PCR performed three months earlier on this mare detected EHV2 DNA in both the buffy coat and a nasal swab. It is possible that at this time, EHV2 may have been reactivated and that the infection had reverted to latency at the time blood was collected for the infectious centre assay.

Of the 4 mares from which virus was isolated in vitro, the number of infected EHV2 B lymphocytes varied from 4 to 780 per 10^6 B lymphocytes. This wide variation in the number of infected lymphocytes may be influenced by a number of factors. It could be a consequence of the relative number of B lymphocytes infected at the time of primary infection. If it is assumed that latently infected B lymphocytes subsequently undergo clonal expansion and that a copy of the EHV2 genome passes to each daughter cell, this could in a stochastic manner, increase the number of infected cells. It is also possible that the number of infected B lymphocytes reflects the frequency of reactivation of EHV2 if reactivation leads to infection of more lymphocytes. Those horses with high numbers of infected B cells may have experienced a recent reactivation of virus boosting

numbers of latently infected B cells. Cytotoxic T lymphocytes (CTL) may also play an important role by removing infected B lymphocytes that reactivate EHV2 and express target antigens on their surface. It has been shown that the number of EBV infected B cells is tightly regulated by CTLs, that are specific for Epstein-Barr virus nuclear antigens (EBNAs) [8, 15, 16]. It is possible that immune surveillance of infected B cells is less efficient in those horses with high levels of latently infected B cells and hence the numbers of circulating infected cells in vivo is increased.

The formation of infectious centres in vitro when sIg^+ B lymphocytes were added to EFK cells indicates that cell–cell contact "reactivates" EHV2 in B lymphocytes and by some mechanism, either enveloped virus, viral nucleocapsid or viral genome is transferred to permissive EFK cells (epithelial cells). The mechanisms by which gammaherpesviruses are reactivated to form infectious centres on permissive cells is not known even though the replication cycle of EBV has been extensively studied [9, 13]. Studies of the life cycle of EBV have been limited because no permissive cell line is available that supports full cycle EBV replication. The finding in this study that B lymphocytes and permissive cells require direct cell–cell contact for infectious centre formation suggests that specific receptors, either virus or cell encoded, are involved in this process. It is likely that when infected lymphocytes contact a permissive epithelial cell, certain EHV2 genes are switched on that changes EHV2 from latency to a state that permits delivery of the virus, nucleocapsid or viral genome to the permissive epithelial cell. EHV2 may itself encode a protein, expressed during latency that facilitates this switch. Examination of the entire EHV2 sequence, which comprises 184 kbp containing 76 open reading frames (ORFs), has not revealed any obvious homologues to EBV latency associated genes or proteins although many (ORFs) have no homologues with other herpesvirus genes [26, 27]. It is evident that some EHV2 ORFs may represent in a generic sense "new" latency associated proteins.

EHV5, a second gammaherpesvirus of horses, can also be isolated from the leukocytes of horses and there is evidence that horses may be simultaneously infected with both EHV2 and EHV5 [18, 31]. The various clinical syndromes including poor performance with which EHV2 and EHV5 have been associated share many features in common with glandular fever/infectious mononucleosis of humans although there is no evidence that either of the equine viruses is associated with lymphoproliferative disorders or that they are capable of transforming lymphocytes in vitro (H. E. Drummer, unpubl. obs.). The identification of an IL10 gene homologue in EHV2 [19, 27], a homologue of which is also present in EBV [5, 14, 29], as well as genes encoding G-protein coupled receptors [26, 27] presents the prospect that EHV2 can modulate the immune response during infection. Taken together with the finding that EHV2 is latent in B lymphocytes, these genes may play a pivotal role in establishing latency and in reactivation of latent virus. In the light of these results, the viruses require further studies in order to better define their biology and establish their exact role in disease.

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