Short Communication

Absence of viral antigens on the surface of equine herpesvirus-1-infected peripheral blood mononuclear cells: a strategy to avoid complement-mediated lysis

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Equine herpesvirus-1 (EHV-1), a member of the genus Alpha herpesvirus, is an important pathogen of horses. After exposure, EHV-1 replicates in the respiratory tract. Replication is followed by a leukocyte-associated viraemia which enables EHV-1 to reach internal organs where its replication can result in abortion, neonatal death or nervous system disorders (Allen & Bryans, 1986). Viraemia may occur in the presence of virus-neutralizing antibodies in infection-immune (Doll & Bryans, 1963; Gleeson & Coggins, 1980; Mumford et al., 1987) and vaccination-immune (Bürki et al., 1990; Heldens et al., 2001) horses. Apparently, recognition of circulating EHV-1-infected peripheral blood mononuclear cells (PBMCs) by the antibody-mediated immune system is inefficient.

Following infection of cells with enveloped viruses, viral glycoproteins are incorporated into cellular membranes. Binding of virus-specific antibodies to glycoproteins present in the plasma membrane makes infected cells recognizable for the classical complement pathway, phagocytes and natural killer cells, leading to lysis of the cell (Harper, 1994). Several herpesviruses have developed strategies to avoid antibody-dependent cell lysis. For pseudorabies virus (PRV)-infected monocytes, addition of PRV-specific antibodies results in clearance of viral glycoproteins from the plasma membrane by antibody-induced internalization (Favoreel et al., 1999). Clearance of the plasma membrane renders infected monocytes significantly less susceptible towards antibody-dependent, complement-mediated lysis (G. R. Van de Walle, H. W. Favoreel, H. J. Nauwynck and M. B. Pensaert, unpublished results). In human cytomegalovirus (HCMV)-infected monocyte-derived macrophages, transport of viral glycoproteins to the plasma membrane is prevented due to the destruction of the microtubule network (Fish et al., 1996). The absence of HCMV glycoproteins on the cell surface may be another strategy of avoiding recognition by antibody-dependent immune responses. Finally, several herpesviruses are known to encode proteins that interfere with the activation of the complement cascade (reviewed by Favoreel et al., 2000).

The main purpose of the present study was to investigate how EHV-1-infected PBMCs are able to avoid recognition and destruction by antibody-dependent immune responses. PBMCs were isolated from infection-immune horses by density centrifugation on Ficoll–Hypaque. After isolation, PBMCs were incubated for 24 h in medium supplemented with 0·5 μM ionomycin (IONO) and 10 nM phorbol dibutyrate (PDB) (Sigma) to favour EHV-1 replication (van der Meulen et al., 2001). Rabbit kidney (RK13) and equine embryonic lung (EEL) cells were maintained in minimal essential medium supplemented with 5 % foetal bovine serum, 100 U penicillin ml\(^{-1}\), 0·1 mg streptomycin ml\(^{-1}\), 0·1 mg kanamycin ml\(^{-1}\) and 0·3 mg glutamine ml\(^{-1}\), until trypsinization and inoculation. All cells were inoculated...
with EHV-1 strain 97P70 at an m.o.i. of 10 (van der Meulen et al., 2000). After 1 h of incubation, the cells were washed and cultured at 37 °C. To ensure that the percentages of infected PBMCs obtained were not confounded by a possible reactivation in or re-infection of the horses during the sampling period, we always included stimulated, but non-inoculated, PBMCs. The expression of viral antigens was never detected in these control samples.

Cell surface and intracellular expression of EHV-1 antigens in PBMCs were examined at 0, 6, 9, 12 and 24 h post-inoculation (p.i.). PBMCs were fixed with 3 % paraformaldehyde and viral proteins on the cell surface were stained with polyclonal, FITC-labelled horse anti-EHV-1 IgGs. Intracellular viral proteins were stained upon permeabilization with 0-1 % saponin, using polyclonal, biotinylated anti-EHV-1 IgGs and a streptavidin–Alexa Fluor 350 substrate (Molecular Probes). Anti-EHV-1 IgGs were raised by the hyperimmunization of a horse. An infection-immune horse was inoculated intranasally with EHV-1 strain 97P70. After 2 weeks, a second intramuscular immunization was performed using the same virus strain mixed with adjuvants (Suvaxyn Aujeszky im o/w, Fort Dodge). After 2 weeks, serum was collected. IgGs were purified on a protein G column and labelled with FITC (Becton Dickinson) or biotinylated (Amersham Pharmacia), following the manufacturers’ instructions. Cells were analysed using an inverted fluorescence microscope (Leica DM IRBE). Cells were considered for further examination on possible surface expression only if intracellular expression of viral antigens was present. Total percentages of infected cells were 2±0.1 % at 6 h p.i., 4±3±1 % at 9 h p.i., 6±6±1 % at 12 h p.i. and 14±2±4±3 % at 24 h p.i. Of these infected PBMCs, 4±0±0±8 % showed cell surface expression at 6 h p.i., 14±4±3±7 % at 9 h p.i., 26±8±6±9 % at 12 h p.i. and 31±4±4±5 % at 24 h p.i. Thus, only a minority of EHV-1-infected PBMCs showed expression of viral antigens on their surface. Among these, two-thirds of the PBMCs expressed antigens on part of the cell surface only, designated ‘focal expression’, whereas the remaining cells expressed EHV-1 antigens on the entire surface, designated ‘general expression’. No differences were observed in the ratio of these expression patterns between the various time-points p.i. Confocal images of an EHV-1-infected PBMC without surface expression (Fig. 1a), with focal expression (Fig. 1b) and with general expression (Fig. 1c) are shown. These images were obtained using a Bio-Rad Radiance 2000 MP confocal laser scanning system attached to an inverted microscope (Eclipse TE300, Nikon).

To determine if the expression of viral antigens on the surface relied on the cell type, EEL and RK13 cells were examined at 12 h p.i. using the immunofluorescence staining technique described before. For PBMCs, an additional step was included prior to permeabilization, using monoclonal antibodies HT23A (anti-equine CD5), 1.9/3.2 (anti-equine B-lymphocytes) or DH59B (equine monocyte marker) (VMRD), followed by goat anti-mouse antibodies conjugated to Texas red, thus allowing identification of different PBMC subpopulations. EHV-1 antigens were present on the surface of 12±6±3±0 % of infected T-lymphocytes, 12±2±1±8 % of infected B-lymphocytes, 25±7±8±7 % of infected monocytes, 11±0±5±6 % of infected EEL cells and 13±0±1±0 % of infected RK13 cells. Thus, cell surface expression was absent on the majority of infected cells independently of the cell type.

To examine the effect of antibody and complement on the viability of EHV-1-infected PBMCs with and without surface expression, PBMCs were infected for 24 h and incubated subsequently for 1 h at 37 °C with various concentrations of the FITC-labelled, anti-EHV-1 antibodies described previously. Afterwards, different concentrations of unheated EHV-1-negative horse serum were added for 1 h at 37 °C as a source of complement. Ethidium monoazide bromide (EMA) (Molecular Probes) was then added for 30 min at 4 °C to stain the nucleus of lysed cells. No photo-crosslinking of EMA was performed, since it

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**Fig. 1.** Expression patterns of viral antigens on the cell surface of EHV-1-infected PBMCs. PBMCs were stimulated for 24 h with IONO/PDB. Cells were then inoculated with EHV-1. At 0, 6, 9, 12 and 24 h p.i., cells were fixed and immunofluorescently stained using polyclonal, FITC-labelled horse anti-EHV-1 IgGs to detect cell surface expression of viral antigens. Subsequently, intracellular viral proteins were stained upon permeabilization, using polyclonal, biotinylated anti-EHV-1 IgGs and streptavidin–Alexa Fluor 350. Confocal images of an EHV-1-infected PBMC without surface expression (a), with expression on part of the cell surface (focal expression) (b) and with expression on the entire cell surface (general expression) (c) are shown. Bar, 5 μm.
reduced the intensity of fluorescence of the FITC-labelled antibodies and did not affect the observed percentages of lysed cells when compared to non-photo-crosslinked samples (data not shown). Finally, intracellular viral proteins were stained upon fixation and permeabilization as described earlier. In order to visualize surface expression in PBMCs incubated in the absence of FITC-labelled antibodies during the first step of the assay, an additional labelling step was included in between fixation and permeabilization. For this, the polyclonal, FITC-labelled anti-EHV-1 IgG was used. PBMCs were analysed using an inverted fluorescence microscope (Leica DM IRBE). A minimal number of 100 cells was scored per data-point. Statistical analysis was based on analysis of variance (rejection level 0.05). Table 1 presents the percentages of lysed PBMCs when using varying antibody and complement concentrations. In non-infected PBMCs, between 1-2 and 2-2 % of the cells were lysed and the addition of antibodies and complement had no effect on viability. For infected PBMCs without cell surface expression, percentages of lysed cells were similar to those in non-infected PBMCs. For infected PBMCs showing focal surface expression, the percentage of lysed cells was only significantly higher compared to non-infected PBMCs when antibody concentrations of 1-6 mg ml⁻¹ and serum concentrations of 20 % were used (8-3 %). For infected PBMCs showing general surface expression, between 19-4 and 31-2 % of the cells were lysed in the presence of antibodies and complement, which was significantly higher compared to non-infected cells. When complement was added in the absence of antibodies, 10-5 % of PBMCs showing general surface expression were lysed, whereas the addition of antibodies in the absence of complement resulted in 5-4 % lysed cells. In contrast to infected PBMCs without and with focal surface expression, PBMCs showing general surface expression are apparently destroyed by three different immune mechanisms. Binding of antibodies to the surface of these infected PBMCs in the absence of complement triggered destruction, most likely via antibody-dependent, cell-mediated cytotoxicity. Lysis by complement in the absence of antibodies most likely occurred via the antibody-independent, complement-mediated pathway, whereas the addition of antibodies increased the percentage of lysed cells by activation of the antibody-dependent, complement-mediated pathway.

To determine whether lysis was mediated by complement, the assay was repeated in the presence of EDTA. EDTA was a Ca²⁺ and Mg²⁺-chelator which blocks activation of the complement system in different species, including the horse (Joseph et al., 1975; Leid et al., 1985; Friedman et al., 2000). EDTA (10 mM) was added 30 min before and during complement incubation. Antibody and serum concentrations used were 0-8 mg ml⁻¹ and 20 %, respectively. The addition of EDTA significantly reduced the percentage of lysed PBMCs showing general surface expression from 27-6 ± 3-6 to 8-8 ± 2-8 %, thereby demonstrating the involvement of complement in the process of lysis. The percentages of lysed cells in infected PBMCs without and with focal surface expression showed a slight increase to 3-9 ± 1-3 and 5-6 ± 2-3 %, respectively. This increase was due to cytotoxic effects of EDTA, since it was also observed when EDTA was used alone, in the absence of antibodies and complement.

The present study describes a potential strategy of immune evasion for EHV-1-infected PBMCs. We demonstrate that

**Table 1. Complement-mediated cell lysis in EHV-1-inoculated PBMCs**

The percentages of lysed cells, expressed as the mean ± SD of at least three experiments, are given. The effects of antibodies and complement on cell lysis are shown for infected or uninfected PBMCs.

<table>
<thead>
<tr>
<th>Antibodies (mg ml⁻¹)</th>
<th>Complement (% horse serum)</th>
<th>Non-infected PBMCs</th>
<th>Infected PBMCs (%)</th>
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<tr>
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<td>Without surface expression</td>
<td>With focal surface expression</td>
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<tr>
<td>Antibodies</td>
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<td>0</td>
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<td>1-2 ± 0.4</td>
<td>1-2 ± 0.5</td>
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<tr>
<td>0</td>
<td>20</td>
<td>2-2 ± 0.8</td>
<td>1-5 ± 0.8</td>
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<td>0-4</td>
<td>20</td>
<td>1-7 ± 0.8</td>
<td>3-6 ± 0.7</td>
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<td>0-8</td>
<td>20</td>
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<tr>
<td>1-6</td>
<td>20</td>
<td>1-9 ± 0.4</td>
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<td>Complement</td>
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<td>0</td>
<td>0</td>
<td>1-2 ± 0.4</td>
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<td>0-8</td>
<td>5</td>
<td>1-2 ± 0.6</td>
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<td>0-8</td>
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<td>1-7 ± 0.3</td>
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the majority of EHV-1-infected PBMCs lacks cell surface expression of viral antigens. Absence of surface expression protects infected PBMCs from lysis by equine complement.

Infected PBMCs showing focal cell surface expression could escape from complement-mediated lysis as well. Circumvention of antibody-dependent, complement-mediated lysis may be explained by the fact that efficiency of lysis depends on the amount of antibody bound per infected cell (Joseph et al., 1975; Perrin et al., 1976; Sissons et al., 1979). In our study, the extent of antigen expression on the surface of infected PBMCs showing focal expression varied from only a small area of expression to an almost complete surface expression. It is possible that only cells with more extensive antigen expression may bind enough IgGs to induce cell lysis. How infected PBMCs without surface expression and with focal surface expression escape antibody-independent, complement-mediated lysis seems less obvious. Analogous to the antibody-dependent pathway, the extent of antigen expression on the surface of infected cells may be important in turning the scale for either prevention or activation of the antibody-independent, complement-mediated pathway.

The maximal percentage of lysed PBMCs showing general surface expression was 31.2 %. This suggests that the efficiency of equine complement to lyse EHV-1-infected PBMCs is rather low. A low efficiency of equine complement, both of the antibody-independent and the antibody-dependent pathway, compared to other mammalian species has already been described by Ish et al. (1993). Low efficiency of complement in our study may also result from virus interference with the complement cascade. For herpes simplex virus (HSV), varicella-zoster virus (VZV) and PRV, it has been demonstrated that the viral glycoprotein complex gE–gI displays Fc-receptor activity, which interferes with efficient antibody-dependent complement activation (Johnson et al., 1988; Frank & Friedman, 1989; Litwin et al., 1992; Favoreel et al., 1997; Nagashunmugam et al., 1998; G. R. Van de Walle, H. W. Favoreel, H. J. Nauwynck and M. B. Pensaert, unpublished results). Homologues of gE and gl are also expressed by EHV-1 (Audonnet et al., 1990; Elton et al., 1991; Telford et al., 1992) but whether they exert Fc-receptor activity is not known yet. Glycoprotein gC of HSV, VZV, EHV-1 and -4, PRV and bovine herpesvirus-1 is capable of binding with complement factor C3, a pivotal component of complement activation, which may result in inhibition of the antibody-independent pathway (Friedman et al., 1984; Harris et al., 1990; Huemer et al., 1992, 1993, 1995).

In conclusion, at least two-thirds of EHV-1-infected PBMCs lack cell surface expression of viral antigens. Absence of surface expression was observed independently of the subtype of PBMCs and protects infected cells from antibody-dependent and antibody-independent lysis by equine complement.

ACKNOWLEDGEMENTS

We thank Carine Boone, Chantal Vanmaecker and Chris Bracke for their excellent technical assistance. We also thank Professor Dr. P. Van Oostveldt from the Department of Molecular Biotechnology at Ghent University for his help with the confocal microscopy and Armand De Smet from the Veterinary and Agrochemical Research Centre for labelling the horse anti-EHV-1 antibodies with FITC.

REFERENCES


