Pseudorabies virus (PRV)-specific antibodies suppress intracellular viral protein levels in PRV-infected monocytes

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Blood monocytes infected with pseudorabies virus (PRV), a swine alphaherpesvirus, are not eliminated efficiently by antibody-dependent immunity and may occasionally transport PRV to the pregnant uterus of vaccinated animals. This study examines in vitro the long-term fate of PRV-infected monocytes cultivated in the presence of porcine PRV-specific antibodies. All monocytes were infected and expressed viral late proteins, and 30 % of PRV-infected monocytes cultivated with PRV-specific antibodies survived up to 194 h post-infection (p.i.), the end of the experiment (compared to 0 % for cells cultivated with PRV-negative antibodies). Of these surviving cells, ±75 % no longer expressed microscopically detectable viral late proteins from 144 h p.i. onwards. Remarkably, monocytes infected with a PRV gB-null virus did not survive in the presence of PRV-specific antibodies. These data suggest that PRV-specific antibodies suppress viral protein levels in infected monocytes, perhaps helping the virus to persist and reach internal organs in vaccinated animals.

The alphaherpesvirus pseudorabies virus (PRV) causes Aujeszky’s disease in its natural host, the pig. The disease is characterized by nervous signs in newborn pigs, respiratory disorders in fattening pigs and reproductive failure in sows (Wittmann et al., 1980). Abortion can be an important consequence of PRV infection in susceptible pregnant sows (Pensaert & Kluge, 1989). Even in the presence of vaccination-induced immunity, PRV inoculation may result in infection of the respiratory tract, involving mononuclear cells in draining lymph nodes. These cells can enter the bloodstream, resulting in a restricted viraemia (Wittmann et al., 1980), which, in general, does not cause problems. However, in rare instances, abortion in immune animals can occur as a result of transplacental spread and intrafoetal replication. Infected blood monocytes are essential to transport the virus in vaccination-immune pigs to enable the virus to reach the pregnant uterus (Nauwynck & Pensaert, 1992, 1995a). Different components of immunity, including antibody-dependent immune effectors, should be able to lyse PRV-infected monocytes in the blood stream of vaccinated animals. Indeed, PRV-infected monocytes express viral proteins in their plasma membrane (Favoreel et al., 1999b) and binding of antibodies to these antigens should induce antibody-dependent cell lysis (Sissons & Oldstone, 1980). One of the mechanisms that may aid PRV in masking infected blood monocytes from antibody-dependent cell lysis consists of the internalization of antibody–antigen complexes (Favoreel et al., 1999b). During this process, binding of PRV-specific antibodies to viral cell surface proteins in PRV-infected monocytes results in rapid internalization of antibody–antigen complexes (immune-masked monocytes). This internalization process is fast and efficient, is mediated by viral proteins gB and gD and has been shown to reduce the sensitivity of PRV-infected monocytes towards antibody-dependent, complement-mediated cell lysis in vitro (Van de Walle et al., 2001, 2003a; Favoreel et al., 2002). Such immune-masked monocytes have already been shown in vitro to be capable of transmitting virus to vascular endothelial cells via direct cell-to-cell spread in the presence of neutralizing antibodies as a possible first step for PRV to reach internal organs (Van de Walle et al., 2003b).

To date, antibody-induced internalization of viral cell surface proteins has only been studied in vitro over relatively short periods post antibody addition (≤ 2 h) and post inoculation (≤ 17 h). However, the circulation of PRV-infected monocytes in vivo occurs in the continuous presence of PRV-specific antibodies. Therefore, the aim of the current study was to investigate the effect of the continuous presence of porcine PRV-specific antibodies on PRV-infected monocytes over an extended period of time, examining both the viability of the infected cells as well as expression level of viral proteins in infected cells.
Pigs from a PRV-negative farm were used as blood donors. Blood was collected from the vena jugularis on heparin (15 U ml⁻¹) (Leo) and monocytes were isolated, seeded onto 4-well multidish plates (Nunc) and inoculated with PRV at an m.o.i. of 10, all as described before (Van de Walle et al., 2003b). At 1 h post-inoculation (p.i.), cells were washed three times with RPMI 160 (Gibco-BRL) and incubated in medium supplemented with porcine PRV-specific or PRV-negative antibodies (0·4 mg IgG ml⁻¹). PRV-specific protein A-purified IgG antibodies were derived, as described earlier, from a PRV (89V87)-inoculated pig originating from a PRV-negative farm (Nauwynck & Pensaert, 1995b), had an antibody titre of 512, as determined with a serum neutralization (SN) test (Andries et al., 1978), and were able to neutralize all input virus at the concentration used in our experiments. PRV-negative protein A-purified IgG antibodies were derived from a PRV-negative pig and had an antibody titre of <2 with the SN test.

First, we determined the percentage of monocytes initiating expression of viral proteins upon PRV infection. Therefore, cells were acetone-fixed at different time-points p.i., stained with FITC-labelled porcine PRV-specific polyclonal antibodies (Van de Walle et al., 2003b), analysed and scored by fluorescence microscopy (Leica DM RBE microscope, Leica). Polyclonal antibodies were derived from hyperimmune serum of a PRV-vaccinated and -infected pig (Van de Walle et al., 2003b), had high reactivity against PRV late proteins gB, gD and gE (data not shown). Fig. 1 shows that PRV inoculation at a high m.o.i. resulted in the microscopically detectable expression of viral antigens in 100 % of monocytes at 96 h p.i. both if monocytes were cultivated in the presence of either PRV-specific or PRV-negative antibodies. Hence, all monocytes initiated the expression of PRV proteins irrespective of the addition of PRV-specific antibodies to the medium at 1 h p.i.

Then, we evaluated how long swine blood monocytes survive a PRV infection if they are cultivated in the continuous presence of PRV-specific or PRV-negative antibodies. Therefore, at different time-points p.i. with PRV at an m.o.i. of 10 (0, 24, 48, 72, 96, 120 and 196 h p.i.), monocytes were incubated for 30 min on ice with ethidium mono-azide bromide (EMA) (Molecular Probes) (100 μg ml⁻¹), which specifically stains the nuclei of dead cells (red), followed by acetone fixation and staining of all nuclei with Hoechst 33342 (Molecular Probes) for 10 min at room temperature (blue). Fig. 2 shows that the viability of PRV-infected monocytes decreased dramatically until 72 h p.i., in the presence of both PRV-specific and PRV-negative antibodies. However, from 120 h p.i. onwards, all PRV-infected monocytes incubated with PRV-negative IgG antibodies were lysed, whereas approximately 30 % of PRV-infected monocytes incubated with PRV-specific polyclonal IgG antibodies remained viable (Fig. 2, box). These results indicate that in the continuous presence of PRV-specific antibodies, a subpopulation of PRV-infected
monocytes remains viable for unusually long periods of time. Interestingly, the processes of antibody-induced internalization of antibody–antigen complexes that we described earlier and the antibody-induced survival of PRV-infected monocytes that we describe here may perhaps be related, since the viral protein gB seems to play crucial roles during both processes. Indeed, gB is necessary for efficient internalization of antibody–antigen complexes (Van de Walle et al., 2001) and, as shown in Fig. 2, monocytes infected with a gB-deleted PRV strain (grown on gB-complementing cells) cannot survive a PRV infection in the presence of PRV-specific antibodies (Fig. 2, dotted line); the gB-null virus and gB-complementing cells were described earlier (Rauh & Mettenleiter, 1991). This indicates that antibody binding to gB may be a key event in initiating internalization of antibody–antigen complexes as well as in allowing a subpopulation of monocytes to survive a PRV infection for extended periods of time.

Next, we evaluated microscopically the expression of viral antigens in the subpopulation of PRV-infected monocytes that survived for unusually long periods of time in the presence of PRV-specific antibodies (Fig. 2, box). At different times p.i., monocytes were incubated with EMA to stain the nuclei of dead cells (red), as described above. Cells were then acetone-fixed and stained to detect viral antigens (anti-PRV-FITC, green) and all nuclei (Hoechst 33342, blue), both as described above. Fig. 3(A) shows that the expression of viral antigens in the majority of these surviving PRV-infected cells decreased gradually. All of the surviving monocytes were microscopically

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**Fig. 3.** (A). Expression of viral proteins in long-term surviving PRV-infected monocytes. Monocytes ($n=10^6$), at 1 h p.i. with PRV, were incubated with 0.4 mg PRV-specific polyclonal IgG antibodies ml$^{-1}$. From 72 h p.i. onwards, expression of viral proteins in surviving PRV-infected monocytes was determined at different time-points, as described in the text. The total number ($\times 10^4$) of surviving monocytes is shown (○). The number of surviving monocytes with microscopically detectable viral antigen expression (○) decreased gradually, whereas the number of surviving monocytes without microscopically detectable viral antigen expression (△) increased over time. Data represent the means ± SD of triplicate assays. (B) Confocal sections through the middle of triple-stained monocytes at 72 (left and middle columns) or 144 (right column) h p.i. with PRV, in the continuous presence of PRV-specific antibodies. Cells were stained with Hoechst 33342 (blue, stains all nuclei), EMA (red, stains nuclei of dead cells) and FITC-labelled PRV-specific antibodies (green, viral antigens), as described in the text. Bar, 5 μm.
positive for viral antigens at 72 h p.i., whereas by 194 h p.i. less than 20% of surviving cells showed visually detectable viral antigen expression. Fig. 3(B) shows confocal images of monocytes, at different times p.i. with PRV, and cultivated in the continuous presence of PRV-specific polyclonal IgG antibodies. Fig. 3(B, left column) shows a PRV-infected monocyte, at 72 h p.i., that shows expression of viral antigens and is still alive (positive on Hoechst 33342 and anti-PRV-FITC but negative on EMA). Fig. 3(B, middle column) shows a PRV-infected monocyte, at 72 h p.i., that shows viral antigen expression and is dead (positive on all fluorescence channels). Fig. 3(B, right column) shows another PRV-infected monocyte, at 144 h p.i., that shows no microscopically detectable viral antigen expression and is alive (only positive on Hoechst 33342). The results from these experiments indicate that the presence of PRV-specific antibodies suppresses the expression of PRV proteins in the majority of surviving PRV-infected monocytes.

The suppressed expression of viral antigens and extended cell survival induced by PRV-specific antibodies may perhaps aid PRV to persist in monocytes, circulate in the blood of vaccinated animals and reach internal organs. We showed recently that PRV-infected monocytes with internalized antibody–antigen complexes are able to transmit virus to vascular endothelial cells via direct cell-to-cell spread, in the presence of neutralizing antibodies, as a possible first step for the virus to reach internal organs (Van de Walle et al., 2003b). We determined whether the long-term surviving PRV-infected monocytes with suppressed expression of viral antigens we describe here are still able to transmit infectious virus to vascular endothelial via direct cell-to-cell spread by performing a co-cultivation assay similar to the one we described earlier (Van de Walle et al., 2003b). At 144 h p.i., surviving monocytes were collected and co-cultivated with porcine primary endothelial cells in the presence of PRV-neutralizing antibodies. After 30 h of co-cultivation, cells were methanol-fixed and stained for viral antigens, but no plaques of endothelial cells could be detected (data not shown). This indicates that PRV-infected monocytes with antibody-induced suppression of viral antigens are no longer able to transmit infectious virus via direct cell-to-cell spread. Relating this to the in vivo situation would mean that antibody-induced suppression of virus may perhaps help PRV to persist in monocytes, thereby allowing it to circulate in the blood of vaccinated pigs, but that external stimuli seem to be needed to allow the monocytes to resume expression and allow further transmission of the virus. We are currently examining which factors can stimulate such resumption of viral antigen expression and infectious virus production.

Current data suggest that the presence of PRV-specific antibodies may lead to a suppressed virus infection in a subpopulation of PRV-infected monocytes. Suppression of intracellular virus gene expression upon binding of specific antibodies to infected cells has been reported already for the closely related alphaherpesvirus herpes simplex virus type 1 (HSV-1) as well as for the alphaherpesvirus Sindbis virus and has been shown to extend the lifespan of Sindbis virus-infected cells (Chanas et al., 1982; Oakes & Lausch, 1984; Levine et al., 1991; Griffin et al., 1997, 2001). Although it is still largely unclear how virus-specific antibodies may suppress viral protein expression, for Sindbis virus it has been suggested that transmembrane signalling, triggered by the antibody-induced cross-linking of viral cell surface proteins, may be a crucial event (Ubol et al., 1995). In this context, it is intriguing that especially antibodies directed against HSV-1 proteins gB and gE (Oakes & Lausch, 1984) and, as suggested by our current results, against PRV protein gB, are able to suppress HSV-1 and PRV infection, since we and others showed recently that antibody-induced cross-linking of PRV gB and PRV and HSV gE may activate intracellular signal transduction pathways (Favoreel et al., 1999a, 2002; Rizvi & Raghavan, 2003). One hypothetical explanation of how such virus-mediated, antibody-induced signal transduction pathways can suppress virus infections may be found when considering the mechanisms of cytokine-mediated suppression of a virus infection. Binding of several cytokines (especially interferons) to their respective receptors on virus-infected cells has also been shown to activate intracellular signal transduction pathways, which, in turn, induce the expression of several cellular proteins that can interfere with different steps of the virus life cycle (reviewed by Guidotti & Chisari, 2000). Perhaps, signal transduction pathways activated by antibody-induced cross-linking of viral proteins on the cell surface may induce similar cellular responses. Alternatively, it is possible that the internalized virus-specific antibodies may have direct intracellular effects on virus replication. Further research will certainly be necessary to clarify this issue.

Combining the results from this study indicates that processes induced by the interaction between antibodies and viral cell surface proteins in PRV-infected monocytes may not only delay destruction of the cells by the immunity but may also enable the cells to survive a PRV infection for unusually long periods of time by suppressing the ongoing infection. In turn, this may aid PRV-infected monocytes to persist in vaccinated animals.

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