Antibody-induced internalization of viral glycoproteins and gE–gI Fc receptor activity protect pseudorabies virus-infected monocytes from efficient complement-mediated lysis

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Pseudorabies virus (PRV)-infected blood monocytes are able to transport virus throughout the body of vaccination-immune pigs. PRV-infected monocytes express viral glycoproteins in their plasma membrane that can be recognized by virus-specific antibodies. Recently, it has been shown that addition of PRV-specific polyclonal immunoglobulins to PRV-infected monocytes at 37 °C induces internalization of the majority of plasma membrane-expressed viral glycoproteins. This study investigated whether this process may interfere with efficient antibody-dependent complement-mediated lysis (ADCML) of infected monocytes. Therefore, an ADCML assay was set up in vitro. A significant decrease in the percentage of cells lysed by ADCML was observed when antibody-induced internalization of PRV glycoproteins occurred (P < 0.005). Furthermore, it is shown (i) that the PRV gE–gI complex, which, like certain other alphaherpesvirus orthologues, possesses IgG-binding capacity, aids in avoiding efficient ADCML of PRV-infected monocytes and (ii) that the efficiency of PRV gE–gI-mediated evasion of ADCML can be decreased by the presence of gE–gI-specific antibodies.

INTRODUCTION

Pseudorabies virus (PRV), a member of the subfamily Alphaherpesvirinae, causes Aujeszky’s disease in its natural host, the pig. Clinical signs depend on the age of the pig and are characterized by nervous signs in newborn pigs, respiratory disorders in fattening pigs and reproductive failure in sows. Abortion may be an important consequence of PRV infection in pregnant sows (Pensaert & Kluge, 1989). In the presence of a vaccination-induced immunity, PRV may still replicate in the respiratory tract and draining lymph nodes, resulting in a restricted viraemia (Wittmann et al., 1980). The limited replication in immune animals generally does not cause problems. However, abortion may occur as a result of cell-mediated transplacental spread and intrafoetus replication. It has been shown that blood monocytes are essential to transport the virus to the pregnant uterus in vaccination-immune pigs (Nauwynck & Pensaert, 1992, 1995a) but little is known on exactly how these infected monocytes survive in the blood of a vaccination-immune animal.

PRV-infected monocytes (and PRV-infected cells in general) express viral envelope proteins on their plasma membrane (Favoreel et al., 1999). Antibodies bind to these viral glycoproteins, which should induce antibody-dependent lysis of infected cells (Sissons & Oldstone, 1980). A major component of the antibody-dependent immune system is the complement cascade (reviewed by Sim & Dodds, 1997). Complement components bind to the Fc region of antibodies, which leads to a cascade of events and results finally in lysis of antibody-covered infected cells (antibody-dependent complement-mediated cell lysis or ADCML) (reviewed by Müller-Eberhard, 1984).

Recently, we described a process of how PRV-infected monocytes may avoid efficient lysis by ADCML. Addition of PRV-specific antibodies to PRV-infected monocytes in vitro results in aggregation of the majority of plasma membrane-bound viral glycoproteins, followed by internalization of these glycoprotein–antibody complexes (Favoreel et al., 1999). The use of wild-type PRV and isogenic deletion mutants showed that the viral glycoproteins gB and gD play a crucial role during this antibody-induced internalization process (Favoreel et al., 1999; Van de Walle et al., 2001). Recently, it was shown that a tyrosine-based motif in the cytoplasmic tail of gB is crucial for the correct functioning of gB during the internalization process (Favoreel et al., 2002). The internalization process is fast and efficient and leaves the infected monocyte with only few viral protein–antibody complexes on its surface. The purpose of the current study was to create an ADCML assay in vitro to investigate
whether this antibody-induced clearance of the majority of plasma membrane-anchored viral glycoproteins results in inefficient ADCML of PRV-infected monocytes.

Furthermore, we also tested the importance of another potential strategy of PRV to resist ADCML: glycoprotein complex gE–gI Fc receptor activity. For herpes simplex virus (HSV), it has been shown already that its gE–gI Fc receptor binding is important for evasion of antibody-dependent components of the immune system in vitro and in vivo (Johnson et al., 1988; Frank & Friedman, 1989; Nagashunmugam et al., 1998). PRV gE–gI has been shown also to display Fc receptor activity (toward porcine IgG) (Favoreel et al., 1997) but its role in antibody-dependent immune evasion had not been studied yet. Therefore, besides studying the effect of antibody-induced internalization of viral cell surface glycoproteins on ADCML efficiency, we also investigated whether PRV gE–gI binding capacity interferes with efficient ADCML of PRV-infected monocytes.

**METHODS**

**Viruses.** PRV strains 89V87, NIA3 and Kaplan (Ka) and its gE–gI deletion mutant (Ka_gE-gI null) were used. All strains were described earlier (Kaplan & Vatter, 1959; McFerran & Dow, 1975; Mettenleiter et al., 1987; Nauwynck & Pensaert, 1992).

**Antibodies.** In most experiments, unlabelled or FITC-labelled protein A-purified IgG antibodies were used, derived from a PRV deletion mutant (Ka gE–gI null). All strains were described (Nauwynck & Pensaert, 1995b). When mentioned, protein A-purified IgG antibodies were used, derived from convalescent sera obtained (Nauwynck & Pensaert, 1995b). When mentioned, protein A-purified IgG antibodies were used, derived from convalescent sera obtained from pigs originating from a PRV-negative farm; sera were collected at 21 days after inoculation with 10⁶ TCID₅₀ PRV (NIA3, Ka or from pigs originating from a PRV-negative farm; sera were collected to display Fc receptor activity (toward porcine IgG) (Favoreel et al., 1997) but its role in antibody-dependent immune evasion had not been studied yet. Therefore, besides studying the effect of antibody-induced internalization of viral cell surface glycoproteins on ADCML efficiency, we also investigated whether PRV gE–gI binding capacity interferes with efficient ADCML of PRV-infected monocytes.

**Porcine complement.** Serum from a pig originating from a PRV-negative farm (C-SN titre of <2) was used as the source for porcine complement.

**Isolation of blood monocytes.** PRV-negative pigs were used as blood donors. Monocytes were separated on Ficoll–Paque (Amersham Pharmacia), seeded on 4-well multidishes (Nunc) and washed at 24 h post-seeding to remove non-adhering lymphocytes, all as described before (Favoreel et al., 1999). Purity of monocytes was always ≥70%, as assessed by flow cytometric analysis using the monocyte marker 74.22.15 (Pescovitz et al., 1984).

**Infection of blood monocytes.** Monocytes were infected with different PRV strains at an m.o.i. of 10 in 0.5 ml monocyte medium without heparin (MMWH) (Favoreel et al., 1999). Cells were incubated further at 37 °C with 5% CO₂. For all strains used and for all experiments, between 80 and 90% of monocytes were infected and less than 5% of the non-monocytes were infected.

**ADCML assay.** In general, at 13 h post-inoculation (p.i.) with PRV, cells were centrifuged (10 min at 500 g), washed and resuspended in MMWH. Cells were incubated with IgG derived from porcine PRV-specific polyclonal hyperimmune serum (0.9 mg ml⁻¹, except where indicated otherwise) for 1 h at 37 °C with 5% CO₂. Cells were washed twice in RPMI-1640 (Gibco-BRL) and incubated with porcine complement (a 5% dilution in MMWH, except where indicated otherwise) for 1 h at 37 °C with 5% CO₂. Except where mentioned otherwise, 50 µg genistein ml⁻¹ (Sigma) was added 45 min before the addition of antibody and also during antibody and complement incubation. Genistein inhibits tyrosine kinase activity and therefore antibody-induced internalization of viral glycoproteins (Favoreel et al., 1999). After complement incubation, cells were washed again in RPMI-1640 and resuspended finally in 0.5 ml RPMI-1640 supplemented with 10 µg propidium iodide (PI) (Molecular Probes), which specifically stains dead cells. The intensity of fluorescence of the cells was analysed by flow cytometry after 5 min incubation with PI.

To determine the percentage of infected cells lysed by ADCML, the following formula was used: [(per cent dead cells after the ADCML assay—per cent dead cells before the ADCML assay)/total per cent infected cells] × 100.

**Enrichment of infected cells with antibody-induced internalization of viral cell surface proteins by magnetic cell sorting.** PRV-infected monocytes (13 h p.i.) were incubated with 0.9 µg FITC-labelled PRV-specific antibodies ml⁻¹ (without genistein) for 1 h at 37 °C with 5% CO₂. Cells were washed with PBS and the supernatant was removed completely. Cells were then resuspended in 90 µl buffer, consisting of PBS at pH 7.2 supplemented with 0.5% BSA and 2 mM EDTA. After washing, 10 µl MACS (magnetic antibodies cell separation) anti-FITC Microbeads (Miltenyi Biotec) were added and cells were incubated for 30 min at 4°C. Cells were washed, resuspended in 500 µl buffer and sorted with MiniMACS (Miltenyi Biotec), according to the manufacturer’s instructions. The flow-through fraction, enriched in cells with complete internalization, was collected, washed in RPMI-1640 and used in the ADCML assay.

**Definition of viral glycoprotein distribution.** The distribution of viral glycoproteins was scored as described before (Favoreel et al., 1999; Van de Walle et al., 2001): (i) ‘no internalization’, when the fluorescence label exhibited a homogeneous or aggregated cell surface cover without internalized vesicles; (ii) ‘partial internalization’, when the labelled viral glycoproteins formed randomly distributed aggregates on the cellular surface and when some were localized in internalized vesicles; (iii) ‘complete internalization’, when all visible viral glycoproteins were located in vesicles inside the cell, without any remaining on the plasma membrane. Quantitative results were obtained by examining the distribution of fluorescence on at least 200 cells using fluorescence microscopy (Leica DM IL, Leica). All assays were run independently at least three times.

**Indirect immunofluorescence staining of viral glycoproteins on the cell surface.** PRV-infected monocytes (13 h p.i.) were centrifuged for 10 min at 500 g, washed and fixed in 1% paraformaldehyde for 10 min. Cells were washed and incubated with 0.9 µg porcine PRV-specific antibodies ml⁻¹ (originating from different hyperimmune sera, as described in the text) for 1 h at 37 °C, followed by a 1:300 dilution of FITC-labelled rabbit α-swine antibodies (Nordic Immunological Laboratories) for 1 h at 37 °C. Finally, the cells were washed thoroughly and analysed by flow cytometry.

**PRV gE–gI IgG-binding assay.** PRV gE–gI IgG binding was assessed on PRV-infected swine kidney (SK) cells (13 h p.i.) using 0.4 µg biotinylated PRV-negative porcine IgG ml⁻¹ (in the presence of 50 µg genistein ml⁻¹), essentially as described before (Favoreel et al., 1997). To investigate the effect of different PRV-specific hyperimmune sera on the efficiency of gE–gI IgG binding, SK cells (13 h p.i.) were incubated first with biotinylated PRV-negative IgG antibodies, as described above, followed by incubation for 1 h at
37°C with 0.9 mg IgG antibodies ml⁻¹ (in the presence of 50 μg genistein ml⁻¹) derived from hyperimmune sera of pigs inoculated with either PRV Ka_wild-type or PRV Ka_gI–gE null, followed by streptavidin–FITC (in the presence of 50 μg genistein ml⁻¹) for 1 h at 37°C and flow cytometric analysis.

**Flow cytometric analysis.** The intensity of fluorescence of the cells was analysed with a FACScalibur (Becton-Dickinson). Approximately 5000 cells were analysed for each sample and forward-scattered light versus side-scattered light dot plots were used to identify monocyte populations. Statistical analysis was performed with SPSS (SPSS).

**RESULTS**

**Effect of antibody and complement concentration on ADCML of PRV-infected monocytes**

To optimize the ADCML assay, PRV-infected (Ka, 89V87 or NIA3) porcine blood monocytes (13 h p.i.) were incubated with different concentrations (0–1.8 mg ml⁻¹) of PRV-specific antibodies or different concentrations (0–10%) of porcine complement. Genistein (50 μg ml⁻¹) was used to avoid internalization and had no effect on cell viability, as assessed by the use of PI and flow cytometric analysis. ADCML was shown to be specific, since (i) incubating mock-infected cells with 0.9 mg PRV-specific IgG ml⁻¹ and subsequently with 5% porcine complement resulted in background levels of lysis (3.8 ± 2.5% dead cells) and (ii) incubating PRV Ka-infected cells with 0.9 mg IgG ml⁻¹ derived from a PRV-negative pig and subsequently with 5% porcine complement also resulted in background levels of lysis (0.9 ± 1.6% dead cells).

Fig. 1(a, b) shows that ADCML of PRV-infected monocytes increases with increasing concentrations of antibodies and complement. A steady-state level of cell lysis was observed when using at least 0.9 mg antibodies ml⁻¹ and 5% of porcine complement. These concentrations were therefore used in all further experiments. A low percentage (2.1–9.7%) of dead cells was observed when adding 5% of porcine complement without the addition of antibodies. To investigate further if this percentage could be attributed to the activation of the alternative complement pathway, experiments with the chelators EGTA and EDTA were performed. The Ca²⁺- and Mg²⁺-chelator EDTA blocks the dead PRV-infected monocytes was analysed by flow cytometry. Data are means ± SD of triplicate assays. (c) Effect of antibodies derived from different hyperimmune sera on ADCML efficiency of PRV-infected monocytes. Monocytes infected with 89V87, NIA3 or Kaplan were incubated at 13 h p.i. for 1 h at 37°C with antibodies derived from hyperimmune sera against 89V87 (black bars), NIA3 (white bars) or Ka (grey bars). Afterwards, cells were incubated with 5% of porcine complement for 1 h at 37°C. Finally, PI was added for 5 min and the percentage of dead PRV-infected monocytes was analysed by flow cytometry. Data are means ± SD of triplicate assays.
activation of both the classical and the alternative pathway and was used to examine if the observed cell death during ADCML is indeed complement-mediated lysis (Joseph et al., 1975; Friedman et al., 2000). The Ca\(^{2+}\)-chelator EGTA only blocks the activation of the classical pathway and was used to determine if the low percentage of cell lysis observed when no PRV-specific antibodies were added in the ADCML assay is due to activation of the alternative complement pathway (Joseph et al., 1975; Friedman et al., 2000). PRV Ka-infected cells were incubated with 10 mM EDTA or EGTA (in combination with 1 mM Mg\(^{2+}\)) 15 min before and during complement incubation. Adding 10 mM EDTA (inhibitor of both classical and alternative pathways) during the ADCML assay reduced the percentage of dead cells from 49.0 ± 3.6% to 1.3 ± 1.5%, indicating that the observed lysis during ADCML is indeed mediated by complement. Adding 10 mM EGTA (inhibitor of the activation of the classical pathway), in combination with 1 mM Mg\(^{2+}\), resulted in a reduction in the percentage of lysed cells to 9.0 ± 1.0%. This percentage is comparable to the percentage of cell lysis we observe when only complement and no antibodies are added during the ADCML assay (7.3 ± 0.6%). Hence, we can conclude that the low percentage of dead cells when adding porcine complement without the addition of antibodies is most probably due to activation of the antibody-independent alternative complement pathway.

Furthermore, we tested whether the use of different virus strains and IgG purified from different hyperimmune sera resulted in different efficiencies of ADCML. The use of comparable concentrations of IgG antibodies derived from different porcine hyperimmune sera had no significant effect on the efficiency of ADCML nor did the use of three different PRV wild-type strains result in significantly different susceptibilities of the infected monocytes toward ADCML (Fig. 1c). There was also no increase in ADCML efficiency when incubating the cells with porcine complement for 2 or 3 h compared to 1 h (data not shown).

**Effect of antibody-induced internalization of viral cell surface glycoproteins on ADCML of PRV-infected monocytes**

Addition of PRV-specific polyclonal antibodies to PRV-infected monocytes induces internalization of the majority of plasma membrane-anchored viral glycoproteins (Favoreel et al., 1999). In order to investigate whether this process may interfere with ADCML of PRV-infected monocytes, PRV 89V87-infected monocytes (13 h p.i.) were incubated with 0.9 mg FITC-labelled antibodies ml\(^{-1}\) for 1 h at 37°C in the presence or absence of 50 μg genistein ml\(^{-1}\) and used subsequently in the ADCML assay. Genistein is a tyrosine kinase inhibitor which blocks the internalization process (Favoreel et al., 1999). The percentage of cells lysed by ADCML when internalization could occur (39.0 ± 2.0%) was lower than the percentage of lysis when no internalization could occur (46.0 ± 3.5%). Using a PRV strain with a point mutation in the tyrosine residue in the cytoplasmic tail of gB that is critical for efficient antibody-induced internalization (Favoreel et al., 2002) resulted in a similar increase in ADCML of PRV-infected monocytes (50.7 ± 1.6% cell lysis versus 41.4 ± 3.4% when using the wild-type virus). Although these data together indicate that antibody-induced internalization is beneficial for the virus to avoid ADCML of infected cells, the differences in ADCML are relatively small. This can be attributed possibly to the fact that (in the absence of genistein and using a wild-type virus) only 36% of cells had undergone complete internalization. To test this hypothesis, the population of infected cells with complete internalization was enriched by magnetic cell sorting (see Methods). This enrichment had no effect on the viability of the cells (as assessed by using PI and flow cytometric analysis) and resulted in an increase of cells with internalized viral glycoproteins in the flow-through fraction from 36.0 ± 6.2% to 74.3 ± 2.1% (Fig. 2a). Of the latter population of cells, only 21.0 ± 6.6% were lysed by ADCML (Fig. 2b), which is significantly less than the 46.0 ± 3.5% that were lysed by ADCML when no internalization could occur (P < 0.005, two-way ANOVA)(Fig. 2b). Taking the results of the experiments with and without genistein and with and without magnetic cell sorting together, we calculated that approximately 49% of cells with no partial endocytosis are lysed by complement, whereas only about 13% of cells with complete internalization are lysed by complement.

**Effect of gE–gI on ADCML of PRV-infected monocytes**

HSV gE–gI possesses Fc receptor activity, which has been shown to be important for immune evasion of the virus in vivo (Nagashunmugam et al., 1998). Fc receptor activity has been demonstrated for PRV gE–gI as well but its effect on immune evasion had not been investigated thus far (Favoreel et al., 1997). To examine the role of PRV gE–gI in ADCML evasion in vitro, Ka- and Ka\(_{gE–gI\ \text{null}}\)-infected monocytes were used in the ADCML assay. First, an indirect immunofluorescence assay (see Methods) was used to ensure that cells infected with the Ka gE–gI mutant (13 h p.i.) did not bind lower amounts of PRV-specific antibodies, compared to cells infected with the Ka wild-type strain (Fig. 3a, panels I and II). Second, cells infected with Ka and Ka\(_{gE–gI\ \text{null}}\) (13 h p.i.) were subjected to the ADCML assay using the same antibodies. Fig. 3(b, panel I) shows that cells infected with the Kaplan wild-type strain were only slightly, but significantly less, susceptible (P < 0.01) to ADCML compared to cells infected with the gE–gI null mutant.

A possible explanation for the rather small difference in ADCML susceptibility between Ka- and Ka\(_{gE–gI\ \text{null}}\)-infected monocytes could be that gE–gI-specific antibodies that are present in the hyperimmune serum used in the ADCML assay interfere with PRV gE–gI Fc receptor activity. Therefore, the same ADCML experiments were repeated
using antibodies derived from a hyperimmune serum of a pig infected with the Ka_gE–gI null mutant and which, therefore, does not contain gE–gI-specific antibodies. Fig. 3(b, panel II) shows that this resulted in a much more prominent difference in ADCML susceptibility between Ka- and KagE–gI null-infected monocytes (P < 0.001). Repeating the same experiments using another PRV strain (Becker), and its isogenic gE–gI null mutant (Whealy et al., 1993), gave similar results (data not shown). From the results of the ADCML assay using wild-type and gE–gI-negative hyperimmune sera and PRV_{wild-type} and PRV_{gE–gI null}-infected monocytes, it can be concluded (i) that PRV gE–gI IgG binding, like HSV gE–gI IgG binding, protects infected cells from efficient ADCML in vitro and (ii) that there are indications that gE–gI-specific antibodies can interfere with PRV gE–gI Fc receptor activity.

To investigate further whether gE–gI-specific antibodies can interfere with PRV gE–gI Fc receptor activity, we performed a displacement assay on PRV Ka-infected SK cells. SK cells, in contrast to monocytes, do not express natural Fc receptors on their cell surface and are therefore better suited to study PRV gE–gI Fc receptor activity. The displacement assay consisted of preincubation of PRV-infected SK cells with PRV-negative IgG, followed by incubation of the cells with PRV-specific IgG derived from

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<td>93.3 ± 1.2 3.0 ± 0.0 3.6 ± 1.1</td>
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Fig. 2. Effect of antibody-induced internalization of viral cell surface glycoproteins on ADCML of PRV-infected monocytes. PRV-infected monocytes were incubated at 13 h p.i. with FITC-labelled PRV-specific antibodies for 1 h at 37°C in the presence or absence of genistein (internalization inhibitor). Afterwards, cells with internalization were enriched by magnetic cell sorting. (a) Percentage of cells with no, partial or complete internalization in the absence of genistein (before and after enrichment) or in the presence of genistein. Data are means ± SD of triplicate assays. (b) Representative flow cytometric contour plots showing the percentage of dead (PI-positive) infected (FITC-positive) monocytes after ADCML of monocytes without internalization of antibodies (+ genistein, I) or with internalization of antibodies (− genistein, after enrichment by magnetic cell sorting, II). Percentages given are means ± SD of triplicate assays.
I. PRV-specific antibodies containing anti-gE-gI antibodies

(a)

II. PRV-specific antibodies containing no anti-gE-gI antibodies

(b)

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<th>% of dead PRV-infected monocytes</th>
<th>wild type</th>
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<td>33.0 ± 2.5</td>
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Fig. 3. (a) PRV Ka_wild-type_ or Ka_gE-gI_null-infected monocytes bind equivalent amounts of antibodies. Fluorescence intensity of monocytes infected with PRV Ka_wild-type_ (grey histogram) or with Ka_gE-gI_null (black line) and stained at 13 h p.i. using 0.9 mg porcine PRV-specific antibodies ml⁻¹ containing gE-gI-specific antibodies (I) or containing no gE-gI-specific antibodies (II) for 1 h at 37°C, followed by incubation with FITC-labelled rabbit α-swine antibodies for 1 h at 37°C. Mock-infected monocytes were used as a negative control (dotted plots). Intensity of fluorescence was analysed by flow cytometry. (b) Effect of deletion of gE-gI on ADCML of PRV-infected monocytes when using PRV-specific antibodies containing gE-gI-specific antibodies (I) or containing no gE-gI-specific antibodies (II). Monocytes infected at 13 h p.i. with PRV Ka_wild-type_ (grey bars) or Ka_gE-gI_null (white bars) were used in the ADCML assay. Afterwards, PI was added for 5 min and percentage of dead PRV-infected monocytes was analysed by flow cytometry. Data are means ± SD of triplicate assays.

hyperimmune sera of pigs inoculated with wild-type or gE-gI null PRV. If there are antibodies present among the PRV-specific antibodies that are directed against epitopes on gE that are important for Fc receptor activity, it is to be expected that they will be able to displace the PRV-negative IgG, which were previously bound with their Fc side; the affinity of IgG antibodies to their antigen is typically higher than that of the Fc sides of antibodies to Fc receptors (Fanger et al., 1998; Friguet et al., 1998). Fig. 4(a) shows that wild-type PRV-infected SK cells exhibit IgG-binding capacity, whereas gE-gI null-infected SK cells do not, as has been shown before (Favoreel et al., 1997). This was determined using a FACS assay, as described in Methods. Fig. 4(b) shows the results of the displacement assay. Here, PRV_wild-type-infected cells were incubated with 0.4 mg biotinylated PRV-negative IgG ml⁻¹ and subsequently with 0.9 mg IgG ml⁻¹ derived from either wild-type or gE-gI-negative hyperimmune sera, followed by incubation with streptavidin–FITC and flow cytometric analysis of the fluorescence intensity. Antibodies, derived from pigs inoculated with wild-type PRV, were able to efficiently displace the biotinylated PRV-negative IgG, whereas antibodies derived from gE-gI null-infected pigs were not, supporting our hypothesis that gE-gI-specific antibodies can at least partially interfere with PRV gE-gI Fc receptor activity.

**DISCUSSION**

PRV-infected blood monocytes are known to be able to transport PRV in well-vaccinated pigs (Wittmann et al., 1980; Nauwynck & Pensaert, 1992). These infected monocytes express viral glycoproteins on their plasma membrane and should therefore be susceptible to ADCML.

Recently, a mechanism has been postulated of how PRV-infected monocytes may potentially avoid ADCML: the antibody-induced internalization of the majority of viral
plasma membrane proteins (Favoreel et al., 1999). The aim of the current study was therefore to evaluate the importance of this mechanism in the ADCML evasion of PRV-infected monocytes in vitro. Furthermore, a second potential ADCML evasion strategy has been described for several alphaherpesviruses, including PRV: gE–gI Fc receptor activity. For HSV, the prototypical alphaherpesvirus, this gE–gI IgG binding has been shown to interfere with efficient antibody-dependent lysis in vitro and in vivo (Johnson et al., 1988; Frank & Friedman, 1989; Nagashumugam et al., 1998). Here, we also investigated the effect of PRV gE–gI Fc receptor activity on the efficiency of ADCML of PRV-infected monocytes in vitro. A third potential mechanism of complement evasion, gC complement binding, has also been described for several alphaherpesviruses, including HSV and PRV (Friedman et al., 1984; Huemer et al., 1993). For HSV, it has been shown already that gC complement binding is important for evasion of the antibody-independent, alternative complement activation pathway rather than for the antibody-dependent classical pathway (ADCML) (Friedman et al., 1984, 1996; Lubinski et al., 1999; Friedman et al., 2000). Therefore, this mechanism was not considered in the current study.

An in vitro ADCML assay was set up using PRV-infected porcine blood monocytes, porcine PRV-specific antibodies and porcine complement to simulate the in vivo situation. During the set up of this ADCML assay, we observed that a low percentage (up to 10 ± 2 %) of PRV-infected monocytes was lysed when porcine complement was added to PRV-infected monocytes without prior addition of antibodies. This was not seen when using mock-infected cells and the low percentage of lysed cells was confirmed to be caused by activation of the alternative complement pathway, which does not require the presence of antibodies (reviewed by Pangburn & Müller-Eberhard, 1984). Our results therefore suggest that, although complement-mediated lysis of PRV-infected monocytes via the alternative pathway indeed does occur, it seems to be of rather minor importance. Efficient antibody-independent complement-mediated lysis of PRV-infected cells may perhaps be disturbed by binding of viral glycoprotein gC to complement factor C3b (Huemer et al., 1993). Activation of the alternative complement pathway by PRV-infected cells has been described before (Kimman et al., 1992). The percentage of infected cells lysed by the alternative complement activation in this study was approximately 20 %, which is higher than the percentage observed in our study. The differences in cell type (SK cells) and complement source (rabbit complement) can probably account for these differences.

Antibody-induced internalization of plasma membrane-bound viral glycoproteins in PRV-infected monocytes leaves the infected monocyte with only few viral protein–antibody complexes on its surface (Favoreel et al., 1999). Since ADCML of infected cells depends principally on the density of antibody–antigen complexes on the cell surface (reviewed by Sissons & Oldstone, 1980), our hypothesis was that this internalization process may interfere with efficient ADCML. To investigate this, PRV-infected monocytes were incubated with PRV-specific antibodies in the absence or presence of genistein (a tyrosine kinase inhibitor that blocks the internalization) (Favoreel et al., 1999) prior to incubation with complement. From these experiments, it could be concluded that cells with internalized viral glycoproteins are
significantly less susceptible toward ADCML than cells that
did not undergo internalization (P<0.005); 49 % of the
cells without internalization of viral cell surface proteins
were lysed by ADCML, compared to only 13 % of the
cells that had undergone internalization of viral cell surface
glycoproteins. Since allowing antibody-induced internalization
of antibody–antigen complexes to protect PRV-infected monocytes from efficient ADCML in vitro, and since the internalization process in PRV-infected monocytes is fast and efficient, starting within minutes after antibody addition (Favoreel et al., 1999), it is tempting to speculate that this process may be significant for the survival of infected monocytes in vaccinated animals. Further research will clarify whether the short time-span between antibody binding and internalization is short enough to allow a significant percentage of infected monocytes to successfully avoid lysis by antibody-dependent components of the immune system.

For HSV, varicella-zoster virus and PRV, the gE–gI complex
has been demonstrated to display Fc receptor activity
(Johnson et al., 1988; Litwin et al., 1992; Favoreel et al.,
1997). Expression of the gE–gI complex results in a species-
specific high affinity binding of IgG and it has been
suggested that this Fc receptor activity may cause ‘antibody
bipolar bridging’, resulting in disarmed bound antibodies
that are unable to activate the classical complement pathway
(Frank & Friedman, 1989). For HSV, gE–gI IgG binding has
been shown to be important for immune evasion of the
virus in vivo (Nagashunugam et al., 1998). In the current
report, we show that, as expected, porcine blood monocytes
infected with gE–gI-deleted PRV mutants are more
susceptible to ADCML, compared to wild-type-infected
cells. This difference in percentage of lysed infected cells was
significant (P<0.01) but surprisingly low. However, the
difference in ADCML between wild-type and gE–gI null-
infected monocytes became much more prominent when
the ADCML assay was performed using antibodies derived
from a hyperimmune serum of a pig infected with the
Kα gE–gI null mutant. Additionally, we showed that antibodies
derived from hyperimmune serum of a pig infected with
wild-type PRV, but not from a pig infected with the
PRV gE–gI null mutant, can at least partially interfere with efficient PRV gE–gI IgG binding. Taking all these findings
together indicates (i) that the PRV gE–gI Fc receptor activity
aids in protecting PRV-infected monocytes from efficient ADCML in vitro and (ii) that gE–gI-specific antibodies
can at least partially interfere with efficient IgG binding by
PRV gE–gI.

During all ADCML assays we performed, even when
blocking internalization and using a gE–gI null virus to
avoid Fc binding of the antibodies, ADCML of infected cells
never reached 100 %. This may suggest that, besides the two
mechanisms studied in this report, PRV may have other
tools at its disposal to avoid efficient ADCML of infected
cells. One such mechanism could be PRV gC complement
binding (Huemer et al., 1993). The binding of gC to the C3
factor of the complement cascade was first described for
HSV (Friedman et al., 1984) and has been shown to be
important for immune evasion of HSV in vivo (Lubinski
et al., 1999). Although HSV gC has been shown to interfere
only with the antibody-independent and not the antibody-
dependent (ADCML) pathway of complement activation
(Fries et al., 1986; Friedman et al., 1996), a possible role for
this glycoprotein in protecting PRV-infected cells from
ADCML cannot at present be ruled out and may be worth
investigating in further detail. Several other mechanisms of
ADCML evasion of infected cells have been described for
other viruses and include the expression of viral proteins
that mimic the function of cellular inhibitors of comple-
ment activation as well as infection-mediated upregulation
of such cellular inhibitors of complement activation (Mold
et al., 1988; Albrecht & Fleckenstein, 1992; Isaacs et al.,
1992; Russo et al., 1996; Spiller et al., 1996; Safiuddin
et al., 1997; Kapadia et al., 1999). Further research will be
necessary to elucidate whether PRV makes use of such or other
mechanisms to avoid ADCML of infected cells.

Furthermore, even when using a non-mutated virus and
allowing antibody-induced internalization to proceed, a
substantial number of PRV-infected cells (39.0±2.0 %)
were lysed by ADCML. This most likely can be attributed to
the fact that none of the complement evasion mechanisms is
absolute. Antibody-induced internalization, which removes
antibody–antigen complexes from the cell surface, only
occurs completely in 36 % of PRV-infected monocytes,
leaving the other 64 % unprotected. Also, the gE–gI Fc
receptor activity may be partly inhibited to perform its
function by the presence of gE-specific antibodies in serum
(see Fig. 4). As discussed above, gC complement binding has
been shown to be predominantly important for evading
antibody-independent complement activation. These three
arguments together possibly explain why a significant
percentage of PRV-infected monocytes is sensitive towards
ADCML. However, immune evasion strategies of PRV still
allow a significant fraction of PRV-infected monocytes to
avoid ADCML in vitro. This indicates that, although not
perfect, PRV complement evasion strategies may still
provide PRV-infected cells with an important tool to
extend their lifespan and time to transmit virus throughout
the body.

In conclusion, this report shows that antibody-induced
internalization of PRV plasma membrane proteins, together
with PRV gE–gI Fc receptor activity, are of significant
importance in protecting PRV-infected monocytes from
efficient ADCML in vitro.

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