Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis

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Porcine alveolar macrophages (AMΦ) are the dominant cell type that supports the replication of porcine reproductive and respiratory syndrome virus (PRRSV) in vivo and in vitro. In order to determine the characteristics of the virus–receptor interaction, the attachment of PRRSV to cells was examined by using biotinylated virus in a series of flow cytometric assays. PRRSV bound specifically to AMΦ in a dose-dependent manner. Binding of PRRSV to AMΦ increased gradually and reached a maximum within 60 min at 4 °C. By confocal microscopy, it was shown that different degrees of PRRSV binding exist and that entry is by endocytosis. Virus uptake in vesicles is a clathrin-dependent process, as it was blocked by the addition of cytochalasin D and co-localization of PRRSV and clathrin was found. Furthermore, by the use of two weak bases, NH4Cl and chloroquine, it was demonstrated that PRRSV uses a low pH-dependent entry pathway. In the presence of these reagents, input virions accumulated in large vacuoles, indicating that uncoating was prevented. These results indicate that PRRSV entry into AMΦ involves attachment to a specific virus receptor(s) followed by a process of endocytosis, by which virions are taken into the cell within vesicles by a clathrin-dependent pathway. A subsequent drop in pH is required for proper virus replication.

Introduction

Virus infection of cells begins with the recognition and attachment of viral particles to specific receptors on the cell surface membrane and is followed by the delivery of viral genomic material into the appropriate subcellular compartment. In the case of enveloped animal viruses, viruses enter cells either by direct fusion of the viral envelope with the plasma membrane or by a process of endocytosis, where virions are taken into the cell within vesicles and subsequently undergo a pH-dependent fusion event (as reviewed by Marsh & Helenius, 1989; Marsh & Pelchen-Matthews, 1994). Viruses may be taken up by clathrin-coated vesicles, as for Semliki Forest virus (SFV) (Marsh & Pelchen-Matthews, 1994), by caveolae, as for simian virus 40 (SV40) (Anderson et al., 1996), or by non-coated vesicles, as for lymphocytic choriomeningitis virus (Borrow & Oldstone, 1994).

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family Arteriviridae, is an enveloped RNA virus (Meulenberg et al., 1993; Conzelmann et al., 1993). It demonstrates a high tropism for cells of the monocyte/macrophage lineage both in vitro and in vivo. Of the porcine cells tested, only alveolar macrophages (AMΦ) and some cultivated peripheral blood monocytes (BMo) support productive replication of PRRSV (Benfield et al., 1992; Kim et al., 1993; Voicu et al., 1994; Duan et al., 1997a). The state of differentiation and activation of macrophages dramatically affects their susceptibility to PRRSV. When BMo and AMΦ are aged in vitro, their susceptibility to PRRSV increases significantly, whereas infection can be completely blocked by treatment of AMΦ with phorbol 12-myristate 13-acetate (PMA) without changing the binding of virus to the cells (Duan et al., 1997b). Little is known about the regulation of macrophage tropism and susceptibility to PRRSV.

PRRSV, like other enveloped viruses, infects cells after attachment to a cellular receptor. A putative virus receptor has
been identified on AMΦ that determines part of the specific virus macrophage tropism (Duan et al., 1998a, b). The next steps in the entry process of PRRSV have already been studied in a non-porcine susceptible cell line, MARC-145. It was found that endocytosis and low pH are necessary for proper virus replication (Kreutz & Ackermann, 1996). However, these findings are difficult to extrapolate to AMΦ. Indeed, in contrast to cells of the continuous cell line MARC-145, AMΦ forms a heterogeneous population of cells with many special biological characteristics. Furthermore, monoclonal antibodies (MAbs) against the putative receptor of PRRSV on AMΦ did not react with MARC-145 cells, suggesting that PRRSV enters MARC-145 and AMΦ cells after binding to different receptors.

In order to elucidate the initial interaction between PRRSV and macrophages further, the rate of PRRSV attachment and the specific mode of entry into AMΦ were examined in the present study.

Methods

**Cells.** Porcine AMΦ were obtained from 4- to 6-week-old conventional Belgian Landrace pigs from a PRRSV-negative herd according to the method previously described by Wensvoort et al. (1991). The cells consisted of >98% macrophages, as demonstrated with the marker 74.22.15.

MARC-145 cells were used to produce PRRSV in large quantities. MARC-145 cells were maintained in Eagle’s minimum essential medium (MEM) supplemented with 10% foetal calf serum (FCS) and antibiotics.

**Virus.** Two PRRSV isolates were used; the Lelystad strain of PRRSV was kindly provided by G. Wensvoort (Institute for Animal Science and Health, Lelystad, The Netherlands). A Belgian isolate of PRRSV, designated as 94V360, was adapted to grow in MARC-145 cells. A thirteenth passage of Lelystad strain of PRRSV grown in AMΦ cells (10^6 TCID50/ml) and a fifth passage of 94V360 grown in MARC-145 cells (10^8 TCID50/ml) were used in this study. No difference was found in the percentage of infected AMΦ between the two virus strains when inoculation was performed at the same m.o.i.

**Virus purification and biotinylation.** One batch of culture fluids of PRRSV-infected (94V360) MARC-145 cells was clarified by centrifugation at 75000 g for 20 min at 4 °C. Virus was collected by centrifugation at 4000 g for 20 min at 4 °C. Virus was collected by centrifugation at 4000 g for 20 min at 4 °C. Virus was collected by centrifugation at 4000 g for 20 min at 4 °C.

Virus was collected by centrifugation at 75000 g for 3 h in a Beckman T35 rotor. The pellet was resuspended in 1/100 the original volume of TNE buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7-4) and centrifuged on a 0.5–1.5 M discontinuous sucrose gradient in an SW41 rotor at 110000 g for 16 h. After centrifugation, the virus band was harvested and its purity was determined by SDS-PAGE. The titre of the resulting virus preparation was approximately 5 × 10^8 TCID50/ml, as determined by a CPE assay in MARC-145 cells. The protein concentration was 2.5 mg/ml, as determined by the Bradford assay with BSA as a standard (Guttenberger, 1994). The number of virus particles, as determined by negative-staining electron microscopy, was 1–2 × 10^13 per mg virus protein.

Purified PRRSV in solution was labelled with biotin by using a protein biotinylation kit (Amersham). The virus pellets were resuspended in biotinylation buffer (40 mM Na2CO3, pH 8.0) at a protein concentration of 1 mg/ml. After brief sonication, 40 μl biotin reagent was added per mg PRRSV protein. The mixture was shaken for 1 h at 4 °C and the reaction was terminated by addition of Tris–HCl (pH 8.5) to a final concentration of 50 mM. Biotinylated virus was collected after purification on a Sephadex G-25 column and diluted in PBS at a concentration of 0.2 mg/ml. Biotinylated virions were stored at −30 °C. Biotinylation did not decrease the virus infectivity significantly (from 5 × 10^7 to 2.5 × 10^7 TCID50/ml).

**Analysis of PRRSV binding to AMΦ by flow cytometry.** To characterize the attachment of PRRSV to AMΦ, direct virus-binding studies were carried out with biotinylated PRRSV. AMΦ were washed three times with cold PBS containing 0.2% BSA (PBSA) (Sigma) and incubated in a 96-well V-bottomed plate on ice for 10 min. Different concentrations of biotinylated PRRSV in PBSA were mixed with 2 × 10^5 cells per well and incubated on ice for 60 min. To determine the time-course of virus binding, 2 × 10^5 cells were incubated with 5 μg biotinylated PRRSV for 10, 20, 30, 40, 50, 60, 90, 120 and 150 min. The cells were washed three times with cold PBSA and incubated with 50 μl 1:50-diluted streptavidin–fluorescein isothiocyanate (FITC) conjugate (Amersham) for 60 min on ice. The cells were washed once and resuspended in 1% formaldehyde in PBS at room temperature for 1 min. The cells were washed once in PBS plus 2% FCS plus 1 μg propidium iodide (PI) per ml and once in PBS plus 2% FCS only. Finally, the relative fluorescence intensity of each sample was scanned on a FACSCalibur flow cytometer (Becton Dickinson), in which dead cells labelled with PI were excluded. Specificity of binding was demonstrated in a competition experiment, in which 2 × 10^5 AMΦ were pre-incubated with different amounts of unlabelled virus in a final volume of 100 μl before performing the PRRSV-binding assay as described above.

Saturability of binding was analysed by incubation of a constant number (2 × 10^5) of cells with increasing amounts of biotinylated virus (0.5–50 μg) for 60 min on ice, followed by measurement of the amount of both bound and unbound virus. Quantification of AMΦ-bound virus was performed by flow cytometry as described above. To measure the amount of unbound virus and to demonstrate the specificity of binding of biotinylated PRRSV to AMΦ, swine anti-PRRSV IgG-coated insoluble protein A (anti-PRRSV protein A) was used. This was prepared by mixing 16 mg swine anti-PRRSV IgG with 10 mg insoluble protein A (Sigma) and incubating the mixture at 37 °C for 60 min with constant shaking. Afterwards, the protein A was collected by centrifuging at 10000 g for 1 min and washed three times with PBSA. The capacity of the prepared anti-PRRSV protein A to adsorb PRRSV was about 1 mg biotinylated virus per mg protein A. Supernatants which contained unbound PRRSV were mixed with 50 μl 1 mg/ml anti-PRRSV protein A and incubated at 37 °C for 60 min. The anti-PRRSV protein A was washed three times with PBSA and 50 μl 1:50-diluted streptavidin–FITC conjugate was added. After a further 60 min incubation and three washings, the anti-PRRSV protein A was suspended in 500 μl PBS and the fluorescence intensity was measured by flow cytometry. Fluorescence intensity was compared with a virus particle standard curve that had been made by adsorbing a series of known amounts of virus particles with anti-PRRSV protein A, followed by incubation with streptavidin–FITC conjugate and analysis by flow cytometry as described above. Specificity of adsorption was demonstrated by using normal swine IgG-coated insoluble protein A prepared by the same procedure as described above.

**Investigation of the effect of acidotropic agents and cytochalasin D on PRRSV infection.** Stock solutions of the acidotropic agents ammonium chloride (1 M) (UCB) and chloroquine (100 mM) (Sigma) were prepared in RPMI medium and filter-sterilized before use, while a stock of cytochalasin D (10 mM) (Sigma) was prepared in ethanol. Afterwards, working dilutions of each product were prepared in RPMI medium containing 10% FCS. AMΦ (1 × 10^6) were washed twice with PBS. Different concentrations of the
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Visualization of the PRRSV entry process by confocal microscopy. Approximately 10 μg biotinylated PRRSV was added to 2 × 10^5 AMΦ that were untreated or treated with 10 mM ammonium chloride, 50 μM chloroquine or 50 μM cytochalasin D. After 60 min incubation on ice, the cells were washed once with cold PBSA and were incubated with 1:20-diluted goat serum to block non-specific staining. They were then incubated with a mixture of the anti-PRRSV NP MAbs WBE1 and WBE4-6 (Drew et al., 1995) and then incubated with goat anti-mouse–FITC conjugate (Amersham). The smears were washed three times with PBS between each incubation. To confirm the specificity of staining, two mouse ascites fluids containing isotype-matched unrelated MAbs against suid herpesvirus type 1 (Nauwynck & Pensaert, 1995) were used as negative controls. Positive cells were counted with a Leica DM RBE fluorescence microscope.

Fig. 1. Semi-logarithmic graphs (Klotz plot) of the binding of PRRSV to AMΦ. (a) PRRSV binding is presented as the mean fluorescence intensity of bound virus vs the number of viral particles added per cell. (b) Specific PRRSV binding was calculated by subtracting the number of unbound viruses from the total viral particles added per cell. Each point is the arithmetic mean ± 3 SD from three independent experiments.
competition between biotinylated PRRSV and untagged PRRSV for binding to AM. AMΦ (2 × 10⁵) were pre-incubated with different amounts of unlabelled virus before adding 5 µg biotinylated PRRSV. Each bar represents the mean ± SD from three independent experiments.

Fig. 3. Time-course of binding of biotinylated PRRSV to AMΦ. Each point shown is the arithmetic mean ± SD from three independent experiments.

The effect of acidotropic agents on PRRSV infection

30 mM NH₄Cl or 100 µM chloroquine, 95% of the cells were viable after 24 h continuous treatment of AMΦ. Both agents inhibited PRRSV replication in a concentration-dependent manner and as little as 5 mM NH₄Cl or 10 µM chloroquine was sufficient to block virus replication completely (Table 1).

PRRSV replication was completely blocked when AMΦ were treated with 10 mM NH₄Cl and 50 µM chloroquine at 1 h before and at 0, 1, 2, 3 and 4 h after inoculation. The number of PRRSV-positive cells was identical in cells treated 6 h or later after inoculation compared with untreated cells (Table 2). Finally, it was demonstrated that NH₄Cl and chloroquine treatment of AMΦ did not affect binding of biotinylated PRRSV to AMΦ, as determined by flow cytometry analysis, after 1 and 24 h continuous treatment of AMΦ with 10 mM NH₄Cl or 50 µM chloroquine. Therefore, it was concluded that the effect of NH₄Cl and chloroquine on PRRSV replication was achieved by blocking post-adsorption steps in the entry process of the virus.

Table 1. Effect of NH₄Cl and chloroquine on PRRSV replication

<table>
<thead>
<tr>
<th>Concentration</th>
<th>% NP-positive cells</th>
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<tbody>
<tr>
<td>NH₄Cl</td>
<td></td>
</tr>
<tr>
<td>0·1 mM</td>
<td>23·8 ± 4·7</td>
</tr>
<tr>
<td>0·2 mM</td>
<td>22·1 ± 5·4</td>
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<td>12·4 ± 4·0</td>
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<td>1 mM</td>
<td>6·6 ± 1·7</td>
</tr>
<tr>
<td>2 mM</td>
<td>3·9 ± 0·6</td>
</tr>
<tr>
<td>5 mM</td>
<td>0</td>
</tr>
<tr>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>Chloroquine</td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td>28·5 ± 3·3</td>
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<tr>
<td>2 µM</td>
<td>12·3 ± 1·4</td>
</tr>
<tr>
<td>5 µM</td>
<td>7·9 ± 1·6</td>
</tr>
<tr>
<td>10 µM</td>
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</tr>
<tr>
<td>20 µM</td>
<td>0</td>
</tr>
<tr>
<td>50 µM</td>
<td>0</td>
</tr>
<tr>
<td>100 µM</td>
<td>0</td>
</tr>
<tr>
<td>No treatment</td>
<td>30·2 ± 3·2</td>
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</table>

The effect of acidotropic agents on PRRSV infection

To investigate whether a pH drop was an essential step in the infectious entry of PRRSV into AMΦ, the effect of NH₄Cl and chloroquine on PRRSV infection was examined by quantifying PRRSV NP-positive cells after PRRSV inoculation of AMΦ treated with these agents. At concentrations below 30 mM NH₄Cl or 100 µM chloroquine, 95% of the cells were viable after 24 h continuous treatment of AMΦ. Both agents inhibited PRRSV replication in a concentration-dependent manner and as little as 5 mM NH₄Cl or 10 µM chloroquine was sufficient to block virus replication completely (Table 1).

PRRSV replication was completely blocked when AMΦ were treated with 10 mM NH₄Cl and 50 µM chloroquine at 1 h before and at 0, 1, 2, 3 and 4 h after inoculation. The number of PRRSV-positive cells was identical in cells treated 6 h or later after inoculation compared with untreated cells (Table 2). Finally, it was demonstrated that NH₄Cl and chloroquine treatment of AMΦ did not affect binding of biotinylated PRRSV to AMΦ, as determined by flow cytometry analysis, after 1 and 24 h continuous treatment of AMΦ with 10 mM NH₄Cl or 50 µM chloroquine. Therefore, it was concluded that the effect of NH₄Cl and chloroquine on PRRSV replication was achieved by blocking post-adsorption steps in the entry process of the virus.

The effect of cytochalasin D on PRRSV replication

AMΦ were not infected by PRRSV in the presence of 20–100 µM cytochalasin D. In the presence of low concentrations of cytochalasin D (1–10 µM), only 1–5 NP-positive cells were detected in an AMΦ smear containing approximately 1 × 10⁵ cells at 12 h after PRRSV inoculation. Concentrations of cytochalasin D (1–10 µM) were pre-incubated with different amounts of unlabelled virus before adding 5 µg biotinylated PRRSV. Each bar represents the mean ± SD from three independent experiments.

Fig. 2. Competition between biotinylated PRRSV and untagged PRRSV for binding to AM. AMΦ (2 × 10⁵) were pre-incubated with different amounts of unlabelled virus before adding 5 µg biotinylated PRRSV. Each bar represents the mean ± SD from three independent experiments.
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Table 2. Effect of NH₄Cl, chloroquine and cytochalasin D added at various times pre- or post-inoculation on PRRSV replication

Acidotropic agents were added at 10 mM (NH₄Cl) or 50 µM (chloroquine and cytochalasin D). Percentage inhibition of virus replication was calculated as 100 x (% cells expressing NP in the absence of agent — % cells expressing NP in presence of agent)/(% cells expressing NP in absence of agent). Data are means ± SD from three independent experiments.

<table>
<thead>
<tr>
<th>Time of addition (h post-inoculation)</th>
<th>% Inhibition of virus replication</th>
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<tbody>
<tr>
<td></td>
<td>NH₄Cl</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>—1</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>+1</td>
<td>100</td>
</tr>
<tr>
<td>+2</td>
<td>100</td>
</tr>
<tr>
<td>+3</td>
<td>100</td>
</tr>
<tr>
<td>+4</td>
<td>100</td>
</tr>
<tr>
<td>+5</td>
<td>58.4±4.5</td>
</tr>
<tr>
<td>+6</td>
<td>3.1±3.5</td>
</tr>
<tr>
<td>+9</td>
<td>0.0±5.8</td>
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</table>

Fig. 4. Binding of labelled PRRSV and entry into AMΦ. Binding of PRRSV to the plasma membrane of AMΦ after 1 h incubation at 4 °C (a) and entry into the cell after 1 h (b) and 3 h (c) incubation at 37 °C are shown.

The entry process visualized by confocal microscopy

Taking advantage of the fact that biotin-tagged virus can be stained with streptavidin–FITC, the process of PRRSV entry into AMΦ was visualized by confocal microscopy. After AMΦ were incubated with biotinylated PRRSV at 4 °C for 60 min, under which conditions maximal virus binding without penetration was reached, AMΦ were stained with streptavidin–FITC and observed under the microscope. Fluorescent spots representing bound virus were observed on the membrane of almost all AMΦ (Fig. 4a), but with different degrees of fluorescence intensity on different individual cells. After a further incubation at 37 °C for a few minutes, fluorescent spots were widely distributed inside the cells. Intense fluorescence that was restricted to cytoplasmic vacuoles was observed after 1–2 h of incubation at 37 °C (Fig. 4b). The number of fluorescing spots in AMΦ decreased at 3–5 h post-inoculation (Fig. 4c). After 6–12 h of incubation, fluorescence was no longer detected in almost all viable AMΦ. In NH₄Cl- and chloroquine-treated AMΦ, the kinetics of attachment and endocytosis were not altered. However, at least 80% of the cells still showed intense fluorescence in the cytoplasm after 6–12 h of incubation (Fig. 5).

Co-localization of PRRSV and clathrin

In order to characterize further which components of microfilaments are involved in uptake of PRRSV, the association between PRRSV and clathrin or caveolae was examined by double immunofluorescence staining. Clear co-
localization was observed between PRRSV and clathrin after a short period of incubation at 37 °C (5 min) (Fig. 6, arrowheads), whereas no co-localization was found between PRRSV and caveolin (data not shown), suggesting that PRRSV uptake is a clathrin-dependent process. At a later stage of virus entry (30 min at 37 °C), clathrin was uncoated from endocytic vesicles. This can be seen in Fig. 6 (arrows) as endocytic vesicles containing virus in the cytoplasm with no co-localized clathrin.

**Discussion**

This study shows that entry of PRRSV into AMΦ occurred by specific binding to the outer cell membrane followed by uptake into the cell via clathrin-dependent endocytosis. Furthermore, low pH during the early entry process was essential for proper virus replication.

The initial interaction between PRRSV and AMΦ appears to be complex. Binding of PRRSV to AMΦ is dose dependent and is competed for by homologous virus. Binding activity increased gradually during a 60 min incubation at 4 °C. However, it was impossible to saturate all binding sites. One explanation for this may be that the number of receptors on AMΦ exceeds $10^4$ per cell, the maximal concentration of biotin-labelled virus that can be reached. This number of receptors is not exceptionally high when other viruses are considered; $2.5 \times 10^3$, $0.4 \times 10^4$ and $6.0 \times 10^4$ receptors per susceptible cell were found for murine polyomavirus, adenovirus type 5 and murine leukaemia virus, respectively (Herrmann et al., 1997; Hong et al., 1997; Johnson & Rosner, 1986).

After incubation of AMΦ with biotinylated PRRSV and streptavidin–FITC labelling, different numbers of regularly distributed fluorescent spots were seen under the microscope on each individual AMΦ, indicating that the number of areas on the membrane that contain virus receptors is quite variable. The fact that AMΦ are a heterogeneous population of cells may be the reason for the variation in membrane expression of
the virus receptor. Therefore, variable expression of virus receptors on the surface of each AMΦ may be one of the important factors that determines the rate of virus entry into cells and the susceptibility of cells to PRRSV infection. This may also be the reason for the observation that AMΦ with different densities and in different states of differentiation and activation have variable susceptibility to PRRSV (Choi et al., 1994; Duan et al., 1997b).

The presence of a functional cell-surface receptor for PRRSV has been recognized as an important factor determining the highly restricted macrophage tropism of this virus (Duan et al., 1997b, 1998a). However, it was observed that MAbs that recognize a 210 kDa protein and block PRRSV infection cannot prevent the binding of PRRSV to AMΦ completely (Duan et al., 1998a). This suggests that PRRSV–AMΦ binding is mediated by the interaction of one ligand with different receptors or, alternatively, by the interaction of multiple viral ligands with their respective receptors.

Following binding to cell-surface receptors, PRRS virions undergo receptor-mediated endocytosis through clathrin-coated pits and vesicles and are delivered intact into endosomes. These endocytic events were clearly demonstrated by confocal microscopy. When AMΦ were infected with labelled virus and visualized under the microscope, labelled virus particles were found inside the cells after incubation at 37 °C. Intensely fluorescing spots were observed after 1–2 h of incubation, suggesting that virions accumulate in distinct vacuoles in the cytoplasm. Degradation of viral particles took place at 6–12 h post-incubation, since no fluorescence of labelled virus could be observed and newly synthesized viral proteins were detected after this time. This entry process is microfilament dependent, since cytochalasin D blocked virus replication. Cytochalasin D is a microfilament-disrupting compound that inhibits clathrin-mediated endocytosis, phagocytosis and macropinocytosis in AMΦ. Clathrin-mediated endocytosis of PRRSV was proven directly by co-localization of PRRSV and clathrin at an early stage of entry. At a later stage of entry, clathrin was uncoated from the endocytotic vesicles. Many cell-surface receptors are known to interact with clathrin in coated pits and to mediate endocytosis (Marsh & Pelchen-Matthews, 1994). It is thought that, after PRRSV binding, the virus receptors are concentrated in clathrin-coated pits to form coated vesicles, which may lead to the formation of virus-containing endosomes.

It was found that low pH is essential for proper PRRSV replication. Although there is no direct evidence to explain why low pH is critical for PRRSV replication, it is generally believed that acidification of virus-carrying endosomes may induce conformational changes in the envelope glycoproteins or capsid structures that trigger membrane fusion or penetration reactions (Marsh & Pelchen-Matthews, 1994), as for SFV (Garoff et al., 1994) and influenza virus (Skehel et al., 1995). Whether similar mechanisms are involved in the uncoating of PRRSV capsid structures remains to be studied.

The pH-dependent endocytotic pathway of PRRSV entry into MARC-145 cells that has been described previously (Kreutz & Ackermann, 1996) closely resembles that described in AMΦ in the present study. NH₄Cl, chloroquine and

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**Fig. 6.** Co-localization of PRRSV with clathrin. AMΦ were incubated with FITC-labelled PRRSV for different times (0, 5 and 30 min) at 37 °C before fixation, permeabilization and staining of clathrin with Texas red. (a) FITC-labelled PRRSV was excited at 488 nm and (b) Texas red-labelled anti-clathrin was excited at 568 nm (arrowheads, co-localization; arrows, no co-localization).
cytochalasin all blocked the entry process in both cell types and the concentrations of these compounds required were similar. The time during which NH4Cl and chloroquine were effective was quite different, however. In MARC-145 cells, virus replication was inhibited when acidotrophic agents were added during the first 30 min post-inoculation, whereas in AMφ this was extended until 4 h post-inoculation. This difference may be attributable to the m.o.i. and the cell type. Indications of this were found with influenza virus, another virus that infects cells using endocytotic entry and a pH drop. With an increase of the m.o.i. of influenza virus in CHO cells, it became more difficult to block infection by inhibiting the acidification of the endosomes (Martin & Helenius, 1991). The chance that one virus penetrates quickly increases with a larger m.o.i. and this was suggested as a possible explanation for this observation. A similar effect of the m.o.i. might explain the shorter effective time-window reported for PRRSV by Kreutz & Ackermann (1996). The m.o.i. used by these authors was one-hundred times higher than that used in the present study. The cell type may also be important. As for PRRSV, it has been shown for influenza virus that virus penetration into cells of continuous cell lines is faster than in primary macrophages (Martin & Helenius, 1991; Meyer & Horisberger, 1984; Koff & Knight, 1979).

Although attachment and endocytosis of PRRSV was observed in almost all inoculated AMφ, viral antigens were only produced in 30% of these cells. This discrepancy may be attributable to a blockage in the virus replication cycle at the level of fusion, transcription or translation. Further studies will be performed to clarify this specific point.

References


Received 12 May 1998; Accepted 28 September 1998