

# Porcine Arterivirus Infection of Alveolar Macrophages Is Mediated by Sialic Acid on the Virus

Peter L. Delputte and Hans J. Nauwynck\*

Laboratory of Virology, Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium

Received 30 October 2003/Accepted 19 March 2004

Recently, we showed that porcine sialoadhesin (pSn) mediates internalization of the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) in alveolar macrophages (Vanderheijden et al., *J. Virol.* 77:8207–8215, 2003). In rodents and humans, sialoadhesin, or Siglec-1, has been described as a macrophage-restricted molecule and to specifically bind sialic acid moieties. In the current study, we investigated whether pSn is a sialic acid binding protein and, whether so, whether this property is important for its function as a PRRSV receptor. Using untreated and neuraminidase-treated sheep erythrocytes, we showed that pSn binds sialic acid. Furthermore, pSn-specific monoclonal antibody 41D3, which blocks PRRSV infection, inhibited this interaction. PRRSV attachment to and infection of porcine alveolar macrophages (PAM) were both shown to be dependent on the presence of sialic acid on the virus: neuraminidase treatment of virus but not of PAM blocked infection and reduced attachment. Enzymatic removal of all N-linked glycans on the virus with N-glycosidase F reduced PRRSV infection, while exclusive removal of nonsialylated N-linked glycans of the high-mannose type with endoglycosidase H had no significant effect. Free sialyllactose and sialic acid containing (neo)glycoproteins reduced infection, while lactose and (neo)glycoproteins devoid of sialic acids had no significant effect. Studies with linkage-specific neuraminidases and lectins indicated that  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acids on the virion are important for PRRSV infection of PAM. From these results, we conclude that pSn is a sialic acid binding lectin and that interactions between sialic acid on the PRRSV virion and pSn are essential for PRRSV infection of PAM.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped positive-strand RNA virus that belongs to the family of the *Arteriviridae*, grouped together with the *Coronaviridae* and the *Roniviridae* in the order of the *Nidovirales*. It causes reproductive failure in sows and respiratory problems in pigs of all ages (5, 7, 31). Other members of the family are equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus. In vivo, PRRSV has a very selective tropism for subpopulations of differentiated macrophages (15). In vitro, only porcine alveolar macrophages (PAM) and African green Monkey kidney cells can be infected with the virus (4, 23, 46, 47). PRRSV has a positive-strand polyadenylated RNA genome that is 14.5 kb in length (40). The genome contains nine open reading frames (ORF) which encode the viral replicase (ORFs 1a and 1b), the membrane glycoproteins GP2a, GP3, GP4, and GP5 (encoded, respectively by ORFs 2a, 3, 4, and 5), the unglycosylated membrane protein P2b and the matrix (M) protein (encoded, respectively, by ORFs 2b and 6), and the nucleocapsid protein N (ORF 7) (26, 28, 30, 43). GP5 and M are the major membrane proteins, while GP2a, P2b, GP3, and GP4 are minor structural proteins.

On PAM, two PRRSV receptors have been identified. The glycosaminoglycan heparan sulfate is a PRRSV receptor that is involved in PRRSV attachment but probably not in virus up-

take (13). The viral M protein alone or in a complex with GP5 is presumed to be involved in attachment to the heparan sulfate receptor, since this protein binds to heparin, an analogue of heparan sulfate (13). Recently, we identified a second PRRSV receptor on PAM, porcine sialoadhesin (pSn), and showed that it is essential for PRRSV internalization in macrophages (42). A monoclonal antibody directed against pSn (41D3) completely blocks PRRSV infection of PAM, and expression of a recombinant pSn in PK-15 cells, which are resistant to viral entry, renders these cells capable of internalizing the virus (17, 42). Sialoadhesin, or Siglec-1, was originally defined as sheep erythrocyte receptor (8) and was subsequently identified as a sialic acid-dependent lectin-like receptor (Siglec) that specifically recognizes Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc (9, 10). In mice, humans, and swine, sialoadhesin is only expressed on discrete subsets of tissue macrophages that are found mostly in the spleen, lymph nodes, bone marrow, liver, colon, and lungs (9, 16, 17, 19, 42).

In this study, it was investigated whether pSn is a sialic acid binding protein and, if so, whether sialic acids that may be present on PRRSV virus are involved in attachment to pSn and infection of PAM.

## MATERIALS AND METHODS

**Viruses and cells.** A 13th and a 4th passage on PAM of the European prototype PRRSV strain Lelystad virus (LV; kindly provided by G. Wensvoort), and the Belgian PRRSV strain, 94V360 (15), respectively, and a fourth passage on Marc-145 cells of the American prototype PRRSV strain VR-2332 (7) were used. The pseudorabies virus Kaplan strain, kindly provided by T. C. Mettenleiter, was used as a control. For infection experiments, all viruses were used at a multiplicity of infection that resulted in an infection rate on PAM or Marc-145 cells of 50% at 10 h postinoculation.

\* Corresponding author. Mailing address: Laboratory of Virology, Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Salisburylaan 133, 9820 Merelbeke, Belgium. Phone: 32 9 264 73 66. Fax: 32 9 264 74 95. E-mail: hans.nauwynck@UGent.be.

For all experiments, virus was semipurified by ultracentrifugation at 100,000 × g for 3 h through a 30% sucrose cushion in an SW41 Ti rotor (Beckman Coulter Inc.). Virus pellets were resuspended in phosphate-buffered saline (PBS) in 1/100 of the original volume and stored at -70°C. For flow cytometric experiments, virus was labeled with biotin immediately after ultracentrifugation with a protein biotinylation kit (Amersham Biosciences Ltd.) as described earlier (17) and stored at -70°C.

PAM were isolated from 4- to 6-week-old conventional Belgian Landrace pigs from a PRRSV-negative herd as described by Wensvoort et al. (46). Unless mentioned otherwise, cells were cultivated in Earle's minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine (BDH Chemicals Ltd.), 1% nonessential amino acids (Gibco-BRL), 1 mM sodium pyruvate, and antibiotics in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. For all experiments, PAM were cultivated for 24 h before infection and attachment experiments. Marc-145 cells were maintained in minimal essential medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics in a humidified 5% CO<sub>2</sub> atmosphere at 37°C.

**Involvement of sialic acid and pSn in sheep erythrocyte binding to alveolar macrophages.** To assess the capacity of pSn to bind sialic acids, the ability of sheep erythrocytes to agglutinate to PAM was investigated. Whole blood from sheep was obtained by venipuncture and stored at 4°C after addition of an equal volume of Alsever's solution. Sheep erythrocytes were washed three times with PBS immediately before use.

To investigate sheep erythrocyte binding to PAM, cells were washed twice with RPMI and incubated for 30 min at 37°C with a 0.1% solution of sheep erythrocytes and washed again to remove unbound sheep erythrocytes, and the number of PAM binding four or more sheep erythrocytes was counted with a light microscope.

To evaluate the importance of sialic acid, PAM or sheep erythrocytes were treated for 90 min at 37°C with *V. cholerae* neuraminidase (Roche), an enzyme that removes sialic acids, and washed twice before sheep erythrocyte addition to the PAM.

PAM were incubated for 30 min with either monoclonal antibody 41D3, directed against pSn (17, 42), or the isotype-matched (immunoglobulin G1) control monoclonal antibody 13D12, directed against pseudorabies virus glycoprotein gD (33) or with monoclonal antibody 74-22-15, reactive with SWC3 on porcine monocytes, macrophages, and neutrophils (35). All monoclonal antibodies were purified with a protein G-Sepharose column (Amersham Biosciences) and dialyzed against PBS.

**Neuraminidase treatments.** To remove sialic acid from the virus, semipurified virus was incubated with different concentrations of soluble neuraminidase from *V. cholerae* (Roche; specific for  $\alpha$ 2-3,6,8-linked sialic acids) or from *Arthrobacter urefaciens* (Calbiochem; specific for  $\alpha$ 2-3,6,8,9-linked sialic acids) in RPMI for 90 min at 37°C. For some experiments, a recombinant *Salmonella* neuraminidase (New England Biolabs), which has a 260-fold preference for  $\alpha$ 2-3-linked sialic acids over  $\alpha$ 2-6-linked sialic acids, was used. PAM or Marc-145 cells were incubated with the virus-neuraminidase mixtures for 1 h at 37°C, washed extensively with RPMI supplemented with 5% fetal bovine serum to remove unbound virus and neuraminidase, and further incubated for 9 h in medium. The buffer in which the neuraminidase was provided was used as a negative control (0 units of neuraminidase). To remove sialic acids from the cells before infection, PAM or Marc-145 cells were incubated with the soluble *V. cholerae* neuraminidase for 1 h at 37°C and washed extensively to remove the enzyme prior to inoculation. *V. cholerae* neuraminidase attached to agarose beads (Sigma) was used under the same experimental procedures to treat the virus except that the beaded neuraminidase was washed three times with RPMI before use and removed by low-speed centrifugation prior to addition of the virus to the PAM. As a negative control, heat-inactivated (10 min of incubation in boiling water) beaded neuraminidase was used.

**Enzymatic removal of N-glycans.** Semipurified virus was treated with N-glycosidase F (New England Biolabs) or with endoglycosidase H (New England Biolabs) in RPMI for 90 min at 37°C to remove all N-glycans (high-mannose and complex, sialic acid-containing glycans) or N-glycans of the high-mannose type, respectively. To remove N-glycans from PAM, cells were treated with the enzymes for 1 h at 37°C in RPMI, washed to remove enzyme, and then infected with PRRSV. After 1 h, cells were washed to remove unbound virus and enzyme, then further incubated at 37°C, and fixed at 10 h postinoculation.

**Effect of monovalent sialoside and sialoconjugates on PRRSV infection.** PAM were incubated for 1 h prior to infection with fetuin or asialofetuin, a mixture of  $\alpha$ 2,3- and  $\alpha$ 2,6-sialyllactose or lactose (Sigma), or with the neoglycoprotein N-acetyllactosamine-bovine serum albumin (BSA) or 3'-N-acetylneuraminyl-N-acetyllactosamine-BSA (Calbiochem). PRRSV was added, and the cells were

incubated at 37°C. After 1 h, cells were washed to remove unbound virus and sialoconjugates, further incubated at 37°C, and fixed 10 h postinoculation.

**Lectin competition experiments.** The following lectins were used in the experiments: *Trichomonas mobilensis* lectin (Calbiochem), which recognizes both  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acid; wheat germ agglutinin lectin (EY Laboratories), which has the capacity to interact with a broad range of sialoconjugates; *Sambucus nigra* lectin (EY Laboratories), which binds to  $\alpha$ 2-6-linked sialic acids and to a lesser degree to  $\alpha$ 2-3-linked sialic acids; *Maackia amurensis* lectin (EY Laboratories), which binds exclusively to  $\alpha$ 2-3-linked sialic acids; and the *Galanthus nivalis* lectin (EY Laboratories), which does not bind to sialic acids but interacts with mannose residues. Lectin buffer was exchanged for RPMI medium (Gibco) with Biomax centrifugal devices with a 5-kDa molecular mass cutoff (Millipore). PRRSV was incubated with different concentrations of lectin in RPMI for 1 h at 37°C, and PAM were incubated with the virus-lectin mixtures for 1 h, washed to remove unbound virus and lectin, and further incubated for 9 h. To investigate whether sialic acids on PAM are involved in PRRSV infection, PAM were incubated with lectin for 1 h, washed to remove free lectin, and subsequently incubated with virus for 1 h at 37°C. Afterwards, cells were washed to remove unbound virus, further incubated at 37°C, and fixed 10 h postinoculation.

**Immunoperoxidase staining of infected PAM.** Macrophages were fixed and stained as described previously (13). Briefly, cells were fixed in an acetone-methanol mixture (50:50), and infected cells were detected by immunoperoxidase staining with the anti-nucleocapsid protein monoclonal antibody P3/27 (48), followed by incubation with horseradish peroxidase-labeled goat anti-mouse secondary antibody and development with a substrate solution containing 3-amino-9-ethylcarbazole. Viral antigen-positive cells and total cells were counted with an Olympus light microscope (Olympus Optical Co., Hamburg, Germany), and the percentage of infected cells was calculated. Three microscopic fields and a minimum of 200 cells per field were counted for each experimental condition.

**Flow cytometric analysis.** PAM were suspended by flushing, washed with cold RPMI, and finally resuspended in cold RPMI. Cell suspensions remained on ice throughout the experiment. Biotinylated PRRSV was incubated with soluble neuraminidase from *V. cholerae* (Roche) in RPMI for 90 min at 37°C, chilled to 4°C, added to PAM, and incubated for 1 h at 4°C. PAM were washed twice with PBS-3% fetal bovine serum (PBS-F) to remove unbound virus, and cells were incubated for 1 h at 4°C with fluorescein isothiocyanate-labeled streptavidin (1:50 dilution in PBS-F) to detect biotinylated virus. Finally, cells were washed twice with PBS-F and analyzed with a Becton-Dickinson FACScalibur flow cytometer, equipped with a 15-mW air-cooled argon ion laser and interfaced with a Macintosh Quadra 650 computer. Data were analyzed with Becton Dickinson Cellquest software. Ten thousand cells were analyzed for each sample, and three parameters were used for further analysis: forward light scatter, sideward light scatter, and green fluorescence. The median fluorescence intensity was used to measure the number of virus particles bound to the PAM.

## RESULTS

**Sheep erythrocytes bind to pSn on alveolar macrophages in a sialic acid-dependent way.** To investigate whether sialic acids can bind to pSn, we incubated PAM for 30 min at 37°C with sheep erythrocytes. Cells were then washed to remove unbound sheep erythrocytes, and the number of PAM with four or more erythrocytes attached was counted.

As shown in Fig. 1A and 1B, approximately 80% of cultured PAM bound four or more sheep erythrocytes. Preincubation of the macrophages with monoclonal antibody 41D3, directed against pSn, reduced the number of PAM with attached sheep erythrocytes to a minimum of 1.4% at 20  $\mu$ g of 41D3 per ml (Fig. 1A). Isotype-matched, irrelevant control monoclonal antibody 13D12 and monoclonal antibody 74-22-15, which binds to SWC3-positive cells (monocytes and macrophages), had no significant effect on sheep erythrocyte binding to PAM (Fig. 1A). To evaluate whether sheep erythrocyte attachment to sialoadhesin is dependent on the presence of sialic acids, sheep erythrocytes were treated for 90 min at 37°C with *V. cholerae* neuraminidase in RPMI, washed, and subsequently added to PAM. Neuraminidase treatment completely blocked binding

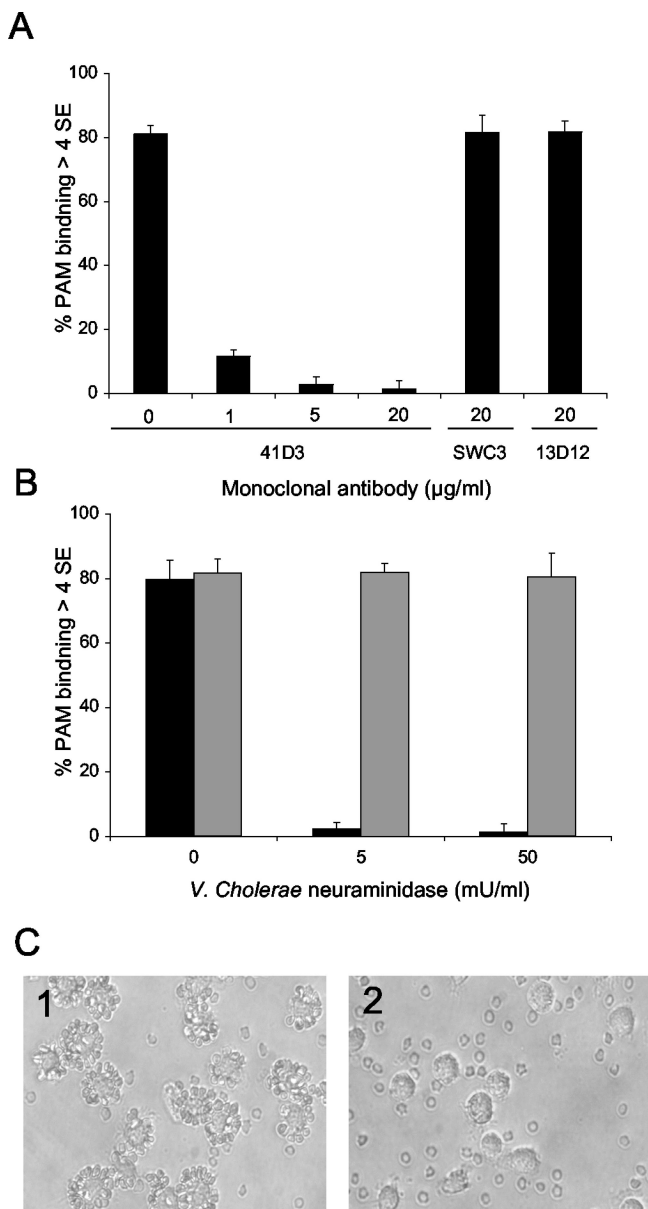


FIG. 1. Sheep erythrocyte (SE) binding to PAM. (A) Effect of different monoclonal antibodies on sheep erythrocyte attachment to PAM. PAM were incubated with sheep erythrocytes in the presence of pSn-specific monoclonal antibody 41D3, irrelevant isotype-matched monoclonal antibody 13D12, or monoclonal antibody 74-22-15, which reacts with SWC3 on PAM. (B) Effect of neuraminidase treatment of sheep erythrocytes (black bars) and PAM (gray bars) on sheep erythrocyte attachment to PAM. (C) A representative light microscopic image of the attachment of mock-treated (picture 1) and neuraminidase-treated (picture 2) sheep erythrocytes to PAM. Data represent means  $\pm$  standard deviation (error bars) of triplicate assays. % cells binding > 4 SE = percentage of cells binding more than four sheep erythrocytes.

of sheep erythrocytes. Treatment of PAM with neuraminidase before addition of sheep erythrocytes had no effect on binding (Fig. 1B). Figure 1C shows the typical rosette formation of sheep erythrocytes attached to PAM (picture 1). When sheep erythrocytes were treated with neuraminidase, attachment and

rosette formation were blocked (picture 2). The picture is also representative of the effect observed when PAM were incubated with monoclonal antibody 41D3. These results show that pSn can interact with sialic acids and that sialoadhesin-specific monoclonal antibody 41D3 blocks sialic acid-dependent interactions.

**Neuraminidase treatment of PRRSV reduces infection of alveolar macrophages but not of Marc-145 cells.** Since monoclonal antibody 41D3 blocks both PRRSV infection of macrophages and sialic acid-dependent binding of sheep erythrocytes to macrophages, we further investigated whether sialic acids on PRRSV are involved in infection of PAM by enzymatic removal of sialic acids from the surface of PRRSV.

European PRRSV strains LV and 94V360 and American PRRSV strain VR-2332 were incubated with different concentrations of soluble neuraminidase from *V. cholerae* before inoculation. Inoculated cells were fixed 10 h postinoculation to allow only one cycle of replication, and infected cells were stained by immunocytochemistry with anti-nucleocapsid monoclonal antibody P3/27. PRRSV infection was strongly reduced, even at low concentrations (1 mU/ml) of neuraminidase, and was almost completely blocked at the highest concentrations used (100 mU/ml) (Fig. 2A). Both the European and American strains were sensitive to the treatment, and no significant differences were observed between the virus strains tested. As shown in Fig. 2B, treatment of PRRSV LV with a recombinant neuraminidase from *Arthrobacter urefaciens*, certified to be free of other glycosidases, resulted in a similar dose-dependent reduction of infection. Treatment of virus with *Salmonella enterica* serovar Typhimurium neuraminidase, which preferentially cleaves  $\alpha$ 2-3-linked sialic acid, resulted in a lower reduction of infection ( $67 \pm 5\%$  reduction at 100 mU/ml; data not shown) compared to the other neuraminidases used. Neuraminidase treatment of PAM before infection did not reduce but slightly increased infection (data not shown).

To exclude the possibility that neuraminidase present in the virus suspension during inoculation has an effect on PAM that leads to a reduced PRRSV infection, PRRSV LV was incubated with a neuraminidase that was covalently linked to beaded agarose and removed from the virus suspension by low-speed centrifugation prior to inoculation of PAM. The effect of beaded neuraminidase on PRRSV isolate LV was similar to the effect observed with soluble neuraminidase, while control treatment with heat-inactivated beaded enzyme had no significant effect on infection (Fig. 2C).

To further ascertain that neuraminidase treatment did not modify any viral component essential for infection other than sialic acids, we tested the effect of neuraminidase treatment of PRRSV on infection of Marc-145 cells. Marc-145 cells are African green monkey cells sensitive to PRRSV infection, but the pSn-specific monoclonal antibody 41D3, which blocks PRRSV infection of PAM, does not react with and cannot block PRRSV infection of Marc-145 cells (17). Treatment of PRRSV LV and VR-2332 with *V. cholerae* neuraminidase prior to infection of Marc-145 cells did not result in a reduction of infection (data not shown). Since treated virions are still able to infect Marc-145 cells, we conclude that the components of the PRRSV virions that are essential for infection of a susceptible cell, other than sialic acids, are maintained during the

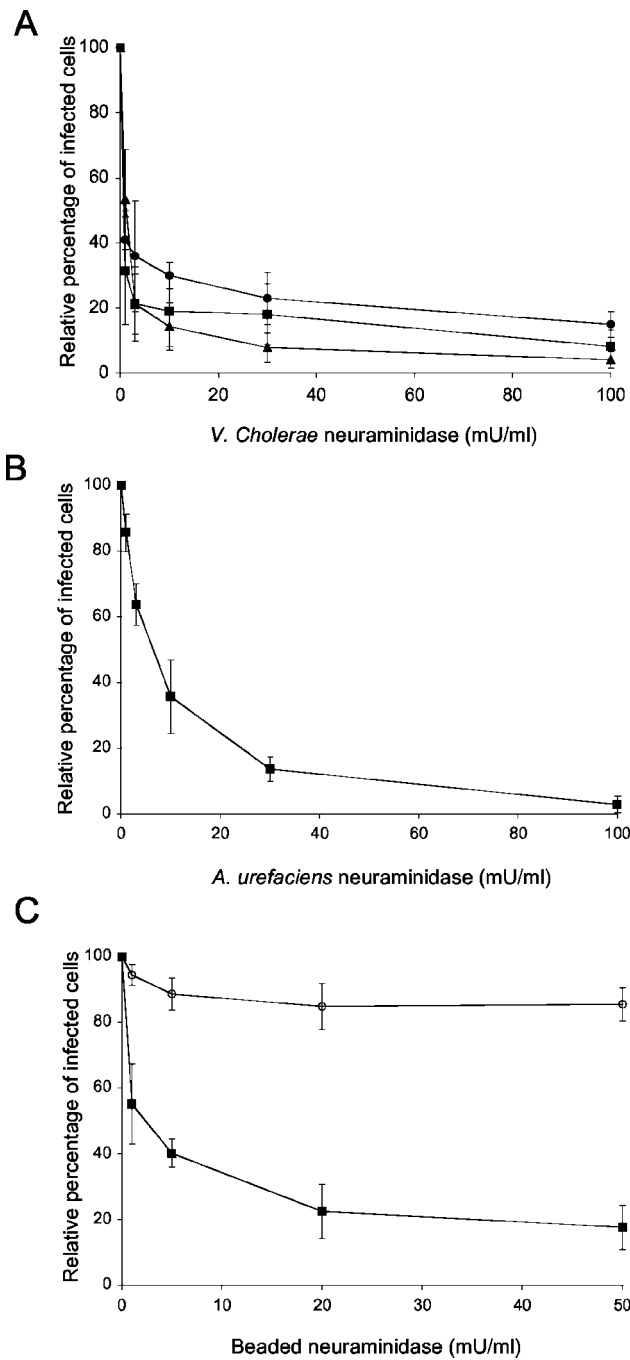


FIG. 2. Effect of neuraminidase treatment of PRRSV LV (■), 94V360 (▲), and VR-2332 (●) on infection of PAM. PRRSV was treated either with *V. cholerae* (A) or with *A. urefaciens* (B) neuraminidase, which remove sialic acids with  $\alpha$ 2-3,6,8 and  $\alpha$ 2-3,6,8,9 linkages, respectively. PAM were infected with the virus for 1 h at 37°C, washed to remove unbound virus and enzyme, and fixed 10 h postinoculation (C) PRRSV LV was incubated for 1 h at 37°C with beaded neuraminidase (■) or with heat-inactivated beaded neuraminidase (○). The beaded enzyme was removed by low-speed centrifugation, PAM were inoculated with the virus, washed to remove unbound virus after 1 h, and fixed 10 h postinoculation. Data represent means  $\pm$  standard deviation (error bars) of triplicate assays.

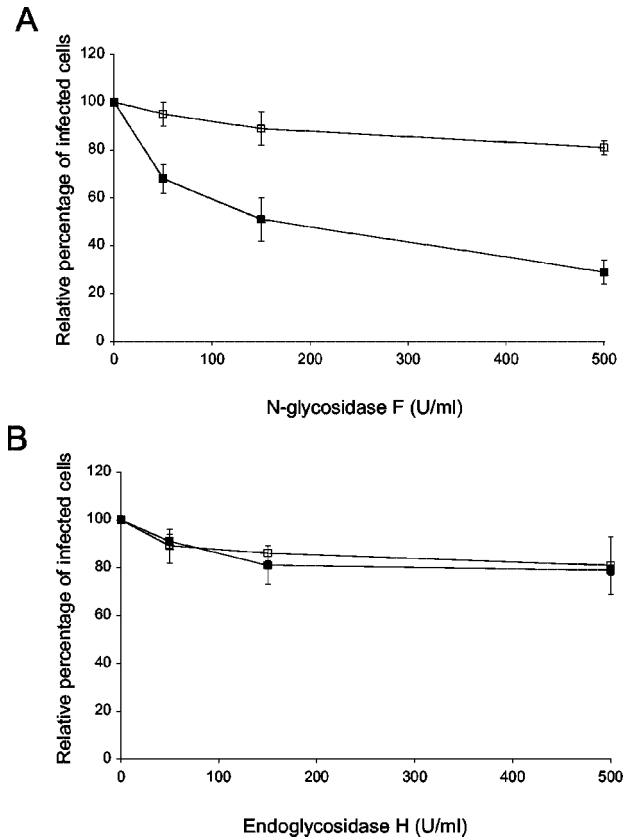


FIG. 3. Effect of N-linked glycan removal on PRRSV infection of PAM. PRRSV (■) or PAM (□) were treated with N-glycosidase F (A) or endoglycosidase H (B) before infection of PAM. After treatment, PAM were inoculated for 1 h at 37°C with PRRSV, washed to remove unbound virus, and cells were fixed 10 h postinoculation. Data represent means  $\pm$  standard deviation (error bars) of triplicate assays.

treatment and that sialic acids on PRRSV are not involved in infection of Marc-145 cells. As an extra control, we found that infection of PAM with pseudorabies virus could not be blocked by treating pseudorabies virus with neuraminidase (data not shown). Together, these results show that sialic acids on PRRSV are essential for infection of PAM but not for infection of Marc-145 cells.

**Removal of complex N-glycans from PRRSV reduces infectivity.** Sialic acids are terminal sugars that can be present on fully processed, complex N-linked glycans. High-mannose N-linked glycans, on the contrary, do not possess sialic acids. To investigate whether N-linked glycans containing sialic acids are involved in PRRSV infection, PRRSV was treated with N-glycosidase F, which removes all N-glycans, or with endoglycosidase H, which removes only high-mannose N-glycans.

N-Glycosidase F treatment of PRRSV strongly reduced PRRSV infection of PAM in a dose-dependent way up to 71%, while pretreatment of PAM with enzyme did not reduce infection (Fig. 3A). Treatment of virus or cells with endoglycosidase H had no effect on infection (Fig. 3B). Complex N-linked glycans are thus important for PRRSV infectivity, whereas N-linked glycans of the high-mannose type are not involved in PRRSV infection.



**Sialyllactose acid- and sialic acid-containing neoglycoconjugates and proteins reduce PRRSV infection of PAM.** PAM were infected in the presence of different concentrations of sialyllactose or lactose in order to compete with the virus for sialic acid-dependent attachment to pSn. Sialyllactose had a small but significant effect on PRRSV infection at the highest concentration used (1,000  $\mu\text{M}$ ), as determined with Student's *t* test. In contrast, lactose, the analogue lacking sialic acid, did not reduce the infection (Fig. 4A).

Similar experiments were performed with the sialic acid-containing neoglycoprotein 3'-*N*-acetylneuraminyl-*N*-acetyl-lactosamine-BSA and its sialic acid-deprived analogue *N*-acetyl-lactosamine-BSA. As shown in Fig. 4B, the sialic acid-containing neoglycoprotein reduced infection up to 53%, while the analogue without sialic acid had no effect.

Finally, infection was done in the presence of different concentrations of fetuin, a sialic acid-containing protein, and in the presence of asialofetuin, which differs from fetuin only by the lack of sialic acids. Fetuin strongly inhibited infection in a dose-dependent way up to 77%, while asialofetuin did not reduce the number of infected cells (Fig. 4C).

**Sialic acid-specific lectins reduce PRRSV infection of PAM.** Several lectins with different sugar binding specificities were tested for their ability to affect PRRSV infection of PAM. Incubation of virus with GNA lectin, specific for mannose, did not reduce PRRSV infection. Wheat germ agglutinin and *T. mobilensis* lectins, which react with a broad range of sialic acids with different linkages, both reduced PRRSV infection in a dose-dependent way (Fig. 5). MAA lectin, specific for  $\alpha$ 2-3-linked sialic acids, also strongly reduced PRRSV infection, while the  $\alpha$ 2-6-specific lectin SNA had only a minor effect on infection.

None of the lectins tested had a significant effect on infection when PAM were preincubated with lectin for 1 h and washed to remove unbound lectin prior to infection (data not shown).

**Sialic acids on PRRSV are involved in PRRSV attachment to PAM.** To investigate whether neuraminidase treatment of virus has an effect on attachment to PAM, biotinylated PRRSV was treated with different concentrations of *V. cholerae* neuraminidase for 90 min at 37°C. Afterwards, PAM were incubated with the biotinylated virus for 1 h at 4°C, allowing virus attachment but no internalization. After washing PAM to remove unbound virus and incubating with fluorescein isothiocyanate-labeled streptavidin to label the bound virus, mean fluorescence intensity was determined by flow cytometry. Neuraminidase treatment clearly reduced PRRSV attachment to PAM (Fig. 6). When PAM were treated with neuraminidase before incubation with biotinylated virus, no effect on attachment was observed (data not shown). Sialic acid present on the PRRSV virion is thus involved in attachment to PAM.

## DISCUSSION

Recently, it was shown that pSn mediates the attachment and internalization of PRRSV, both in macrophages and in recombinant pSn-expressing cells (42). The human and rodent sialoadhesins are sialic acid binding immunoglobulin-like lectins (Siglecs), and the structural features of this sialic acid binding capacity are conserved across species (10, 19, 42, 44).

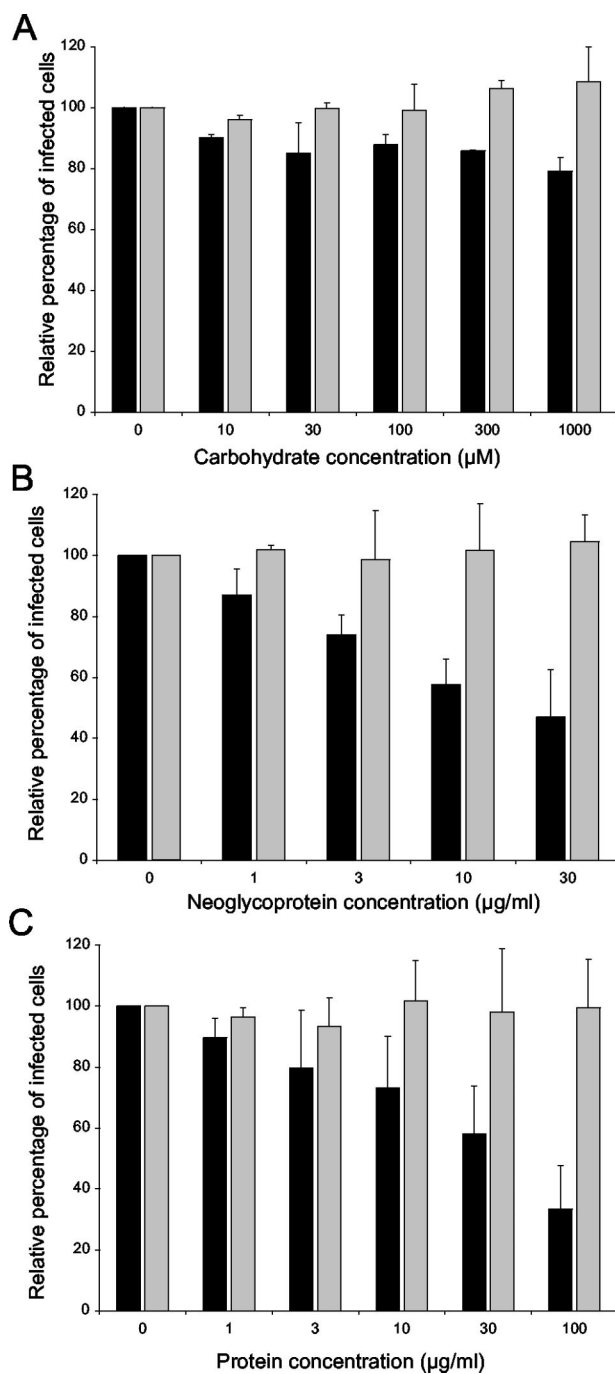


FIG. 4. Effect of sialic acid and sialylated (neo)glycoproteins on PRRSV infection. PAM were infected with PRRSV in the presence of (A) sialyllactose (black bars) or lactose (gray bars), (B) BSA neoglycoproteins containing sialyllactose (black bars) or lactose (gray bars), (C) the sialic acid-containing protein fetuin (black bars) or asialofetuin, which is devoid of sialic acid (gray bars). Cells were washed 1 h postinoculation to remove the inoculum and fixed 10 h postinoculation. Data represent means  $\pm$  standard deviation (error bars) of triplicate assays.

The capacity of pSn to bind sialic acid had not yet been demonstrated. Similar to what was shown for murine Sn (mSn) (10), we observed that sheep erythrocytes clearly bind to porcine macrophages expressing pSn, and removal of sialic acids

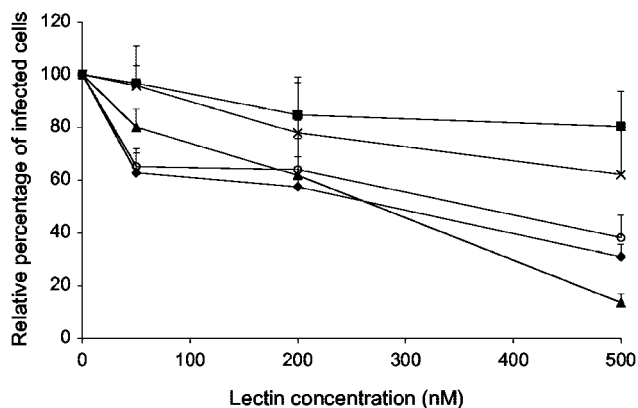


FIG. 5. Effect of lectins on PRRSV infection of PAM. PRRSV was incubated for 1 h at 37°C with wheat germ agglutinin (□) and *T. mobilensis* lectin (◻), which react with a broad range of sialic acids; MAA lectin (○), which recognizes  $\alpha$ 2-3-linked sialic acids; SNA lectin (◻), which recognizes  $\alpha$ 2-6-linked sialic acids; and GNA lectin (■), which does not react with sialic acids but does react with mannose residues. Virus-lectin mixtures were then inoculated on PAM. PAM were washed after 1 h of incubation at 37°C and fixed 10 h postinoculation. Data represent means  $\pm$  standard deviation (error bars) of triplicate assays.

from sheep erythrocytes but not from macrophages completely abolished agglutination. Interestingly, sheep erythrocyte attachment was also completely blocked by pSn-specific monoclonal antibody 41D3, even at low concentrations. The effect of monoclonal antibody 41D3 is unlikely to be nonspecific, since control monoclonal antibody 74-22-15, which abundantly binds to SWC3 on the cell surface of PAM, had no effect on sheep erythrocyte attachment. Previous experiments showed that two mSn-specific monoclonal antibodies (SER-4 and 3D6) that recognize a region vicinal to the sialic acid binding region of mSn, could only block sheep erythrocyte binding when used together (11, 27). These monoclonal antibodies can only partially reduce sheep erythrocyte binding to the mSn, although they bind to a region very close to the sialic acid binding domain. Monoclonal antibody 41D3, which can very efficiently block sheep erythrocyte attachment, may recognize a site even closer to the sialic acid binding region of pSn.

Several viruses, such as Theiler's murine encephalomyelitis virus (39), adenovirus type 37 (1), human polyomavirus JC virus (25), type 1 reovirus (21), the coronavirus transmissible gastroenteritis virus (38), and some rotaviruses (6), have been shown to use sialic acids on the cell surface for virus attachment. In the present study, we showed the reverse: sialic acids on the PRRSV surface are essential to mediate infection. Indeed, neuraminidase treatment of virus almost completely blocked infection of PAM, whereas pretreatment of macrophages with neuraminidase did not decrease but rather increased PRRSV infectivity. This slight enhancement is consistent with other studies with mSn where neuraminidase treatment of cells expressing sialoadhesin enhanced the binding of sialylated ligands to mSn (2). Experiments with neuraminidase covalently linked to beaded agarose, which was removed from the virus by low-speed centrifugation prior to inoculation of PAM, showed that the observed reduction in infection is the result of sialic acid removal from PRRSV.

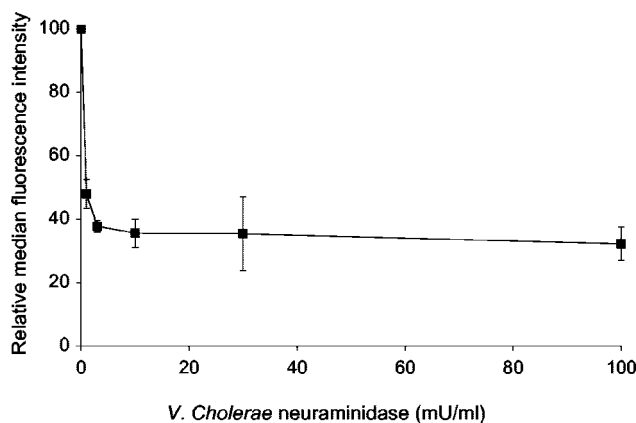


FIG. 6. Flow cytometric analysis of PRRSV attachment to PAM. Biotinylated PRRSV was incubated for 90 min at 37°C with different concentrations of *V. cholerae* neuraminidase. PAM were then incubated for 1 h at 4°C with enzyme-treated virus. After washing, bound virus particles were stained with fluorescein isothiocyanate-labeled streptavidin, and fluorescence intensity was analyzed by flow cytometry. Data represent means  $\pm$  standard deviation (error bars) of triplicate assays.

Neuraminidase treatment of PRRSV did not reduce infection of Marc-145 cells, which confirms that PRRSV uses different entry pathways to infect PAM and Marc-145 cells. Previously, we observed that both the pSn-specific monoclonal antibody 41D3 and a polyclonal monospecific anti-pSn serum generated by DNA immunization in mice both blocked PRRSV infection of PAM while having no effect on PRRSV infection of Marc-145 cells (17; Delputte et al., unpublished data).

Removal of complex N-linked glycans strongly reduced PRRSV infection, indicating that sialic acid-carrying N-linked glycans on the virus are involved in infection. However, a complete reduction of infection, as observed by neuraminidase treatment, was not observed. We therefore cannot rule out a role for other virion-bound sialylated ligands in infection of PAM. O-linked carbohydrates are a possible but unlikely candidate because these have not been reported to be present on structural PRRSV proteins. N-Glycosidase F is known not to remove all the N-glycan chains from nondenatured viral proteins. Since we treated virus with N-glycosidase F in nondenatured conditions, this might explain the incomplete reduction of infection.

The presence of monovalent sialoside during PRRSV inoculation only partially reduced infection, while sialic acid containing (neo)glycoproteins strongly reduced infection. Previous reports demonstrated that the affinity of monovalent sialoside Sn is low and that strong binding of sialylated ligands to the sialoadhesin is dependent on the ligand valency (20, 32). These findings could thus explain why monovalent free sialyllactose has a minimal effect on attachment of multivalent PRRSV particles to pSn, whereas multivalent (neo)glycoproteins could successfully compete with PRRSV for attachment to pSn.

Several structural PRRSV proteins (GP2a, GP3, GP4, and GP5) contain complex N-linked glycans that potentially may carry sialic acids (reviewed in reference 12). Both GP4 and GP5 have neutralizing epitopes and could thus be involved in

virus attachment to cellular receptors (29, 34, 36, 37, 45, 49). Since neutralizing antibodies directed against GP5 are more effective than anti-GP4 neutralizing antibodies (45), and since the neutralization epitope identified in the GP4 protein is not conserved between American and European strains, its possible role in attachment to cellular receptors is controversial (12, 29, 45). In contrast, a virus-neutralizing epitope that contains a highly conserved N-linked glycosylation site has been identified in GP5 of both European and American PRRSV strains and is thought to be involved in receptor attachment (34, 37). A similar virus neutralization site containing an N-linked glycan has been mapped to the ORF5 product of the mouse arterivirus lactate dehydrogenase-elevating virus (24). Furthermore, the lactate dehydrogenase-elevating virus neutralizing monoclonal antibody 159-18, which recognizes this epitope, also neutralizes both European type PRRSV Lelystad virus and the American type PRRSV VR-2332 (37).

In the present study, two neuraminidases with different specificities for the type of sialic acid linkage that they cleave were used. The *V. cholerae* neuraminidase cleaves  $\alpha$ 2-3,6,8 linkages, whereas the *A. urefaciens* enzyme cleaves  $\alpha$ 2-3,6,8,9 linkages. Both enzymes exhibited a similar effect on infection, indicating that  $\alpha$ 2-9 linkages are not involved in PRRSV infection, leaving a possible role for  $\alpha$ 2-3,6,8 linkages. *S. enterica* serovar Typhimurium neuraminidase, which preferentially cleaves  $\alpha$ 2-3- over  $\alpha$ 2-6-linked sialic acids, induced a clear reduction of infection which was lower than that achieved by the other neuraminidases used. Additional studies with lectins showed that lectins recognizing  $\alpha$ 2-3 linkages reduced PRRSV infection more than  $\alpha$ 2-6-specific lectins. Together, these data implicate the involvement of both  $\alpha$ 2-3- and  $\alpha$ 2-6-linked sialic acids on PRRSV in infection, with a preference for  $\alpha$ 2-3-linked sialic acids. These findings are in agreement with previous studies in which mouse sialoadhesin was shown to selectively interact with  $\alpha$ 2-3-linked sialic acids and to a lesser extent with  $\alpha$ 2-6-linked sialic acids (10).

Flow cytometric analysis showed that PRRSV attachment to PAM is partially dependent on the presence of sialic acids on the virus. Attachment could not be blocked completely even at high concentrations of neuraminidase that do block PRRSV infection. These results may be consistent with an earlier report that monoclonal antibody 41D3, directed against pSn, completely blocked PRRSV infection of PAM, whereas it only partially reduced PRRSV attachment (17). Thus, another receptor on PAM has to be involved in virus attachment. Recently, it was shown that heparan sulfate mediates PRRSV attachment to PAM but is not essential for infection (13).

The role of sialic acid in infection has been documented for respiratory syncytial virus, and treatment of virus with neuraminidase was shown to enhance infection and syncytium formation (3). For human immunodeficiency virus type 1, neuraminidase treatment enhances virus attachment, infection, and syncytium formation (18, 22, 41). In contrast, the need for sialic acids on the virion surface for attachment to cellular receptors and infectivity, as we show in the current report for PRRSV, has not been demonstrated before, to our knowledge.

#### ACKNOWLEDGMENTS

We thank C. Vanmaercke, D. Defever, and C. Boone for excellent technical assistance and H. Favoreel and G. Van Minnebruggen for

helpful comments and discussions. We are also grateful to G. Labarque for critical reading of the manuscript and for assistance in collecting alveolar macrophages and to N. Callewaert and W. Vervecken for assistance in the lectin experiments and for kindly providing sialyllactose. We thank T. Mettenleiter for providing the PrV strain Kaplan.

P. Delputte was supported by a grant from the Flemish Institute for the Promotion of Innovation by Science and Technology (I.W.T.-Flanders). Part of this research was financially supported by Intervet International (Akzo-Nobel) and the Belgian Federal Public Service of Health, Food Chain Safety and Environment.

#### REFERENCES

1. Arnberg, N., K. Edlund, A. H. Kidd, and G. Wadell. 2000. Adenovirus type 37 uses sialic acid as a cellular receptor. *J. Virol.* **74**:42–48.
2. Barnes, Y. C., T. P. Skelton, I. Stamenkovic, and D. C. Sgroi. 1999. Sialylation of the sialic acid binding lectin sialoadhesin regulates its ability to mediate cell adhesion. *Blood* **93**:1245–1252.
3. Barretto, N., L. K. Hallak, and M. E. Peeples. 2003. Neuraminidase treatment of respiratory syncytial virus-infected cells or virions, but not target cells, enhances cell-cell fusion and infection. *Virology* **313**:33–42.
4. Benfield, D. A., E. Nelson, J. E. Collins, L. Harris, S. M. Goyal, D. Robinson, W. T. Christianson, R. B. Morrison, D. Gorcyca, and D. Chladek. 1992. Characterisation of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J. Vet. Diagn. Investig.* **4**:127–133.
5. Cavanagh, D. 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. *Arch. Virol.* **142**:629–633.
6. Ciarlet, M., and M. K. Estes. 1999. Human and most animal rotavirus strains do not require the presence of sialic acid on the cell surface for efficient infectivity. *J. Gen. Virol.* **80**:943–948.
7. Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. C. Hennings, D. P. Shaw, S. M. Goyal, S. McCullough, R. B. Morrison, H. S. Joo, D. Gorcyca, and D. Chladek. 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J. Vet. Diagn. Investig.* **4**:117–126.
8. Crocker, P. R., and S. Gordon. 1986. Properties and distribution of a lectin-like hemagglutinin differentially expressed by murine stromal tissue macrophages. *J. Exp. Med.* **164**:1862–1875.
9. Crocker, P. R., and S. Gordon. 1989. Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. *J. Exp. Med.* **169**:1333–1346.
10. Crocker, P. R., S. Kelm, C. Dubois, B. Martin, A. S. McWilliam, D. M. Shotton, J. C. Paulson, and S. Gordon. 1991. Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages. *EMBO J.* **10**:1661–1669.
11. Crocker, P. R., S. Mucklow, V. Bouckson, A. McWilliam, A. C. Willis, S. Gordon, G. Milon, S. Kelm, and P. Bradfield. 1994. Sialoadhesin, a macrophage sialic acid binding receptor for haematopoietic cells with 17 immunoglobulin-like domains. *EMBO J.* **13**:4490–4503.
12. Dea, S., C. A. Gagnon, H. Mardassi, B. Pirzadeh, and D. Rogan. 2000. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and the European isolates. *Arch. Virol.* **145**:659–688.
13. Delputte, P. L., N. Vanderheijden, H. J. Nauwynck, and M. B. Pensaert. 2002. Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparan sulfate receptor on porcine alveolar macrophages. *J. Virol.* **76**:4312–4320.
14. Dobbe, J. C., Y. van der Meer, W. J. Spaan, and E. J. Snijder. 2001. Construction of chimeric arteriviruses reveals that the ectodomain of the major glycoprotein is not the main determinant of equine arteritis virus tropism in cell culture. *Virology* **288**:283–294.
15. Duan, X., H. J. Nauwynck, and M. B. Pensaert. 1997. Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to PRRSV. *Arch. Virol.* **142**:2483–2497.
16. Duan, X., H. J. Nauwynck, and M. B. Pensaert. 1997. Virus quantitation and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with PRRSV. *Vet. Microbiol.* **56**: 9–19.
17. Duan, X., H. J. Nauwynck, H. W. Favoreel, and M. B. Pensaert. 1998. Identification of a putative receptor for porcine reproductive and respiratory syndrome virus on porcine alveolar macrophages. *J. Virol.* **72**:4520–4523.
18. Hart, M. L., M. Saifuddin, and G. T. Spear. 2003. Glycosylation inhibitors and neuraminidase enhance human immunodeficiency virus type 1 binding and neutralization by mannose-binding lectin. *J. Gen. Virol.* **84**:353–360.
19. Hartnell, A., J. Steel, H. Turley, M. Jones, D. Jackson, and P. R. Crocker. 2001. Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. *Blood* **97**:288–296.
20. Hashimoto, Y., M. Suzuki, P. R. Crocker, and A. Suzuki. 1998. A streptavidin-based neoglycoprotein carrying more than 140 GT1b oligosaccharides:

- quantitative estimation of the binding specificity of murine sialoadhesin expressed on CHO cells. *J. Biochem.* **123**:468–478.
21. Helander, A., K. J. Silvey, N. J. Mantis, A. B. Hutchings, K. Chandran, W. T. Lucas, M. L. Nibert, and M. R. Neutra. 2003. The viral  $\sigma 1$  protein and glycoconjugates containing  $\alpha 2$ -3-linked sialic acid are involved in type 1 reovirus adherence to M-cell apical surfaces. *J. Virol.* **77**:7964–7977.
  22. Hu, H., T. Shioda, C. Moriya, X. Xin, M. K. Hasan, K. Miyake, T. Shimada, and Y. Nagai. 1996. Infectivities of human and other primate lentiviruses are activated by desialylation of the virion surface. *J. Virol.* **70**:7462–7470.
  23. Kim, H. S., J. Kwang, I. J. Yoon, H. S. Joo, and M. L. Frey. 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch. Virol.* **133**:477–483.
  24. Li, K., Z. Chen, and P. Plagemann. 1998. The neutralization epitope of lactate dehydrogenase-elevating virus is located on the short ectodomain of the primary envelope glycoprotein. *Virology* **242**:239–245.
  25. Liu, C. K., G. Wei, and W. J. Atwood. 1998. Infection of glial cells by the human polyomavirus JC is mediated by an N-linked glycoprotein containing terminal  $\alpha 2$ -6-linked sialic acids. *J. Virol.* **72**:4643–4649.
  26. Mardassi, H., B. Massie, and S. Dea. 1996. Intracellular synthesis, processing and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. *Virology* **221**:98–112.
  27. May, A. P., R. C. Robinson, M. Vinson, P. R. Crocker, and E. Y. Jones. 1998. Crystal structure of the N-terminal domain of sialoadhesin in complex with 3'sialyllactose at 1.85 Å resolution. *Mol. Cell* **1**:719–728.
  28. Meulenber, J. J. M., A. Petersen-den Besten, E. P. De Kluyver, R. J. Moormann, W. M. Schaaper, and G. Wensvoort. 1995. Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. *Virology* **206**:155–163.
  29. Meulenber, J. J. M., A. P. van Nieuwstadt, A. van Essen-Zandbergen, and J. P. M. Langeveld. 1997. Post-translational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. *J. Virol.* **71**:6061–6067.
  30. Meulenber, J. J. M., and A. Petersen-den Besten. 1996. Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP2 encoded by ORF2 is incorporated in virus particles. *Virology* **225**:44–51.
  31. Meulenber, J. J. M., M. M. Hulst, E. J. de Meijer, P. L. Moonen, A. Petersen-den Besten, E. P. de Kluyver, G. Wensvoort, and R. J. Moormann. 1994. Lelystad virus belongs to a new virus family, comprising lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus. *Arch. Virol. Suppl.* **9**:441–448.
  32. Munday, J., H. Floyd, and P. R. Crocker. 1999. Sialic acid binding receptors (Siglecs) expressed by macrophages. *J. Leukoc. Biol.* **66**:705–711.
  33. Nauwynck, H. J., and M. B. Pensaert. 1995. Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. *Arch. Virol.* **140**:1137–1146.
  34. Ostrowski, M., J. A. Galeota, A. M. Jar, K. B. Platt, F. A. Osorio, and O. J. Lopez. 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J. Virol.* **76**:4241–4250.
  35. Pescovitz, M. D., J. K. Lunney, and D. H. Sachs. 1984. Preparation and characterisation of monoclonal antibodies reactive with porcine PBL. *J. Immunol.* **133**:368–375.
  36. Pirzadeh, D., and S. Dea. 1997. Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. *J. Gen. Virol.* **78**:1867–1873.
  37. Plagemann, P. G., R. R. Rowland, and K. S. Faaberg. 2002. The primary neutralization epitope of porcine reproductive and respiratory syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. *Arch. Virol.* **147**:2327–2347.
  38. Schwegmann-Weßels, C., G. Zimmer, H. Laude, L. Enjuanes, and G. Herler. 2002. Binding of transmissible gastroenteritis coronavirus to cell surface sialoglycoproteins. *J. Virol.* **76**:6037–6043.
  39. Shah, A. H., and H. L. Lipton. 2002. Low-neurovirulence Theiler's viruses use sialic acid moieties on N-linked oligosaccharide structures for attachment. *Virology* **304**:443–450.
  40. Snijder, E. J., and J. J. Meulenber. 1998. The molecular biology of arteriviruses. *J. Gen. Virol.* **79**:961–979.
  41. Sun, J., B. Barbeau, S. Sato, and M. J. Tremblay. 2001. Neuraminidase from a bacterial source enhances both HIV-1 mediated syncytium formation and the virus binding/entry process. *Virology* **284**:26–36.
  42. Vanderheijden, N., P. L. Delputte, H. W. Favoreel, J. Vandekerckhove, J. Van Damme, P. A. van Woensel, and H. J. Nauwynck. 2003. Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into alveolar macrophages. *J. Virol.* **77**:8207–8215.
  43. Van Nieuwstadt, A. P., J. J. M. Meulenber, A. Van Essen-Zandbergen, A. P. Den Besten, R. J. Bende, R. J. M. Moorman, and G. Wensvoort. 1996. Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (Arteriviridae) are structural proteins of the virion. *J. Gen. Virol.* **70**:4767–4772.
  44. Vinson, M., P. A. van der Merwe, S. Kelm, A. May, E. Y. Jones, and P. R. Crocker. 1996. Characterization of the sialic acid-binding site in sialoadhesin by site-directed mutagenesis. *J. Biol. Chem.* **271**:9267–9272.
  45. Weiland, E., M. Wiczorek-Krohmer, D. Kohl, K. K. Conzelmann, and F. Weiland. 1999. Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to GP4. *Vet. Microbiol.* **66**:171–186.
  46. Wensvoort, G., C. Terpstra, J. M. A. Pol, E. A. ter Laak, M. Bloemraad, E. P. de Kluyver, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, J. M. Broekhuijsen, P. L. J. M. Moonen, T. Zetstra, E. A. de Boer, H. J. Tibben, M. F. de Jong, P. van't Veld, G. J. R. Groenland, J. A. van Gennepe, M. Th. Voets, J. H. M. Verheijden, and J. Braamskamp. 1991. Mystery swine disease in the Netherlands: the isolation of the Lelystad virus. *Vet. Q.* **13**:121–130.
  47. Wensvoort, G., E. P. de Kluyver, J. M. A. Pol, R. J. M. Moorman, M. M. Hulst, R. Bloemraad, A. den Besten, T. Zetstra, and C. Terpstra. 1992. Lelystad virus, the cause of porcine epidemic abortion and respiratory syndrome: a review of mystery swine disease research at Lelystad. *Vet. Microbiol.* **33**:185–193.
  48. Wiczorek-Krohmer, M., F. Weiland, K. Conzelmann, D. Kohl, N. Visser, P. Van Woensel, H. J. Thiel, and E. Weiland. 1996. Porcine reproductive and respiratory syndrome virus (PRRSV): monoclonal antibodies detect common epitopes on two viral proteins of European and U. S. isolates. *Vet. Microbiol.* **51**:257–266.
  49. Wissink, E. H. J., H. A. R. van Wijk, M. V. Kroese, E. Weiland, J. J. M. Meulenber, P. J. M. Rottier, and P. A. van Rijn. 2003. The major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome virus contains a neutralization epitope in its N-terminal ectodomain. *J. Gen. Virol.* **84**:1535–1543.