

**Preliminary characterization of protein binding factor for porcine reproductive and respiratory syndrome virus on the surface of permissive and non-permissive cells**

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**Summary.** In its natural host, porcine reproductive and respiratory syndrome virus (PRRSV) has been reported to have a restricted tropism for cells of the monocyte/macrophage lineage. To date, cloned monkey kidney cell lines, such as MARC-145 and CL2621 cells which have been established from MA-104 cells, are the only non-porcine cells known to support PRRSV replication. In the present study, a binding assay was set up to follow by flow cytometry the attachment of PRRSV on the surface of porcine and non-porcine cells. PRRSV was found to be able to bind permissive cells like porcine alveolar macrophages and MARC-145. Further binding assays with porcine peripheral blood leukocytes showed that only monocytes can attach the virus. By their lack of binding factor, lymphocytes appeared to be refractory to PRRSV infection. Pre-incubation of MARC-145 cells with chymotrypsin and pronase E, but not neuraminidase, blocked their binding activity for PRRSV. The binding activity of the protease-treated cells was regenerated 8 hours after treatment, but cells remained unable to bind PRRSV if maintained in the presence of cycloheximide, thus confirming the proteinic nature of the specific binding factor(s). Experiments conducted with cells that have been previously characterized as non-permissive to PRRSV infection showed that many of them were able to bind the virus. Data obtained suggest that interaction of PRRSV with a specific binding factor on the surface of some cells is not sufficient to lead to a productive infection, and that a second putative receptor or other phenomena are probably required to pursue later events.

**Introduction**

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of a new disease in swine, that has spread rapidly in most of the pig producing

countries. The disease is characterized by severe reproductive failure in sows and gilts, and respiratory problems in pigs of all ages [13]. PRRSV belongs to the recently recognized *Arteriviridae* family within the genus *Arterivirus*, order *Nidovirales*, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) [3, 4, 8]. These viruses share morphological and genomic similarities, can establish persistent infection in their natural host, and have a predilection for cells of the monocyte/macrophage lineage [38]. Mature PRRS virions are enveloped, approximately 60 to 70 nm in diameter, and possess an icosahedral capsid [2, 5]. The genome of the PRRSV is a positive single-stranded RNA molecule of approximately 15 kb in length, containing nine open reading frames (ORFs) that are transcribed in the infected cells as a nested set of subgenomic mRNAs [29, 40]. The ORF1a and ORF1b, located at the 5' end of the genome, represent nearly 75% of the viral genome and encode proteins with apparent replicase and polymerase activities [29, 38]. The ORFs 2 to 7, located at the 3' end of the genome, encode for major and minor structural proteins, except ORF3 of North American isolates which has been reported to encode a non-structural, but antigenic glycosylated protein, and the ORF2b which still need to be characterize as a structural protein in case of PRRSV [12, 25, 30, 40]. The three major structural proteins consist in a glycosylated envelope protein of 25 kDa (GP<sub>5</sub>), an unglycosylated membrane (M) protein of 19 kDa, and a 14 kDa nucleocapsid (N) protein, encoded by ORFs 5, 6 and 7, respectively [25, 26, 30]. Three other putative minor membrane-associated proteins, GP<sub>2</sub> (30 kDa), GP<sub>4</sub> (31 kDa) and E (ORF2b product, 7,4 kDa) have been also identified [31, 40, 43].

PRRSV has a restricted cell tropism in its host. The virus was first isolated on primary cultures of porcine alveolar macrophages (PAMs) and so far, these cells as well as blood monocytes, remain the only porcine cells that can effectively be used for viral propagation ex-vivo [44, 45]. In situ hybridization studies have established that the virus also infects macrophages in the spleen, liver, Peyer's patches, thymus, as well as microglial cells, but peritoneal macrophages and progenitor cells in the bone marrow are refractory [22, 41]. The testicular germ cells (spermatids) have been also shown to allow PRRSV replication [42]. This was the first report showing that PRRSV can also infect non-macrophage cells in its natural host. Two non-porcine permissive cell subclones, MARC-145 and CL2621 cells, both derived from the MA104 monkey kidney cell line are also routinely used for in vitro propagation of field and vaccine strains [1, 19]. It is difficult to extrapolate findings on the cellular susceptibility of PRRSV isolates from the European and North American continents, but data from routine diagnosis investigations, as well as several experimental studies, indicated that European strains of PRRSV are most successfully isolated in PAMs, whereas the great majority of North American strains can be initially isolated in the established monkey cell subpopulations [1, 27, 28]. The characteristics shared by these cell lines and PAMs allowing the infection by PRRSV are still unknown.

Factors implicated in the first stage of infection, which dictate the cell tropism, such as viral attachment protein(s) (VAP) and cellular receptor(s), have not been

yet identified for arteriviruses. Although monoclonal antibodies (MAbs) directed against GP<sub>4</sub> and GP<sub>5</sub> of PRRSV have been reported to neutralize virus infection, the mechanism of neutralization has not been elucidated, as well as whether it occurs prior or after viral attachment [32, 37]. Recently, it has been demonstrated that absence of PRRSV binding to cells might be one major determinant of PRRSV cell tropism [21]. It has been also reported that PRRSV enters in PAMs and in MARC-145 cells through a mechanism of receptor-mediated endocytosis, suggesting that specific yet unidentified receptors for PRRSV may be the key factors for the restricted tropism [20, 35]. Recently, two monoclonal antibodies directed against a 210 kDa protein have been produced which are able to block partially virus attachment to the plasma membrane of porcine alveolar macrophages and to inhibit infection [10]. Since a clear colocalization exists between attached virus and aggregates of this protein on the plasma membrane, it was concluded that these MAbs recognize either specifically the PRRSV receptor or a protein which is in close contact with it [10, 36]. Further research is needed to confirm the role of this protein as a specific receptor or a co-receptor for PRRSV. Other investigators have demonstrated a previously non-reported hemagglutinating activity (HA) for PRRSV, as it is also the case for EAV [16, 39]. They also reported that this HA activity, as well as the propagation of the virus, could be inhibited by heparin and suggest that a heparin-like molecule on the surface of susceptible cells could also play the role of cellular receptor for PRRSV [17]. The present study was designed to further characterize the mechanism that restricts PRRSV replication in a variety of porcine and non-porcine cell lines. A flow cytometry method was adapted to follow the binding of PRRSV to the surface of cells and to determine the phenotype of peripheral blood cells susceptible to PRRSV. The use of different enzymatic treatments was also investigated to characterize the biochemical properties of the specific binding factor(s) for PRRSV.

## Materials and methods

### *Cells and virus*

The Quebec cytopathic strain IAF-Klop of PRRSV was propagated for 15 to 20 passages in MARC-145 cells, a clone of MA-104 cells highly permissive to PRRSV [19, 23]. Peripheral blood cells (PBLs) were collected from 4- to 9-week-old pigs serologically negative for PRRSV, transmissible gastroenteritis virus, swine influenza virus, porcine parvovirus, *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis*. Blood was diluted with one volume of Eagle's minimum essential medium (MEM) and centrifuged onto Ficoll-hypaque gradient (Pharmacia, Biotech, Baie d'Urfé, PQ, Canada). Leukocytes were removed from the buffy coat, and cultured in Dulbecco's-MEM containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 0,1 mM non-essential amino acids, 50 U/ml penicillin-streptomycin, 60 µg/ml tylosin, 2,5 µg/ml amphotericin B, and 25 µg/ml gentamycin sulfate. Porcine alveolar macrophages (PAMs) were obtained by bronchoalveolar lavages and cultured, as previously described [45]. The MARC-145 cells were kindly provided to us by J. Kwang (U.S. Meat Animal Research Center, Clay Center, NE) and were grown as monolayers in MEM supplemented with 8% FBS, 10 mM HEPES, 2 mM L-glutamine and antibiotics. Continuous cell lines of African green monkey kidney (MA-104) cells, rabbit kidney cells (RK-13) and human carcinoma cells (293A) were cultured

in DMEM containing 10% FBS, 2 mM L-glutamine, and antibiotics. Baby hamster kidney (BHK-21) cells were cultured in DMEM containing 10% FBS, 10% tryptose broth, 2 mM L-glutamine, and antibiotics. Porcine testicular cells (PT) and porcine kidney cells (PK-15) were cultured in MEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotics, whereas U937 cells, a human monocyte/macrophage cell line, were cultured in RPMI medium with 10% FBS, 2 mM L-glutamine, and antibiotics. The cell cultures were infected at a multiplicity of infection (MOI) of 0,1 to 10 TCID<sub>50</sub> of virus per cell. Following an adsorption period of 90 min at room temperature, cells were washed twice in PBS and incubated with fresh medium for 48 h at 37 °C in an atmosphere of 5% CO<sub>2</sub>.

#### *RT-PCR detection of PRRSV infection*

At 48 hours post-infection (PI), cells were harvested and RNA was extracted with the Trizol reagent (Pharmacia, Baie d'Urfé, Pointe-Claire, Québec). Total RNA was resuspended in 20 µl of DEPC-treated water and processed for RT-PCR, as previously described [24]. The oligonucleotide primers 1010 PLS and 1011 PLR, designed to amplify a 434 bp DNA fragment encompassing the entire ORF7 gene and a portion of the 3' terminal non-coding region of PRRSV, were used [24].

#### *Indirect immunofluorescence*

At 24 or 48 h PI, cell culture medium was removed and cells were fixed with 80% cold acetone in phosphate buffered saline (PBS) for 20 min at 4 °C. Cells were first incubated with monoclonal antibodies (MAb) IAF-K8 directed against a well conserved epitope of the nucleocapsid protein of both North American and European strains of PRRSV [6], for a period of 45 min at 37 °C. After washing in PBS, cells were further incubated in the presence of a 1:50 dilution of FITC-conjugated anti-mouse IgG (Boehringer Mannheim, Laval, Québec, Canada) for a period of 45 min at 37 °C. Following final washings in PBS, cell monolayers were examined under a U.V. light microscope (Leitz DM IL, Wetzlar, Germany) for the presence of specific cytoplasmic fluorescence.

#### *Binding assay*

At confluency, cell monolayers were washed twice with PBS and cells were dispersed by incubation in the presence of PBS supplemented with 20 mM of ethylene glycol (b-aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA) for 30 min at 37 °C. Prior the binding assay, the cells were washed twice in PBS containing 1% BSA and 0.05% sodium azide. A total of  $5 \times 10^5$  cells in suspension were inoculated at a M.O.I. of 10 TCID<sub>50</sub> of virus per cell. Following an adsorption period of 1 h at 4 °C and two washing steps with PBS, cells were reincubated for 30 min at 4 °C in the presence of the biotinylated MAb IAF-K8. Free antibodies were removed by washing the cells twice in PBS. The binding of the virus to the cells was then determined by incubating the cells for 20 min at 4 °C in the presence of a 1/200 dilution of streptavidin-phycoerythrin (Gibco BRL, Burlington, Ontario). Following two final washes in PBS, cells were fixed in PBS containing 1% paraformaldehyde and kept at 4 °C. Fluorescence level on each cell was analyzed by flow cytometry using an Epics XL cytometer (Coulter Electronics, Hialeah, FL).

#### *Virus treatment with chloroform or 1,1,2- Trichlorotrifluoroethane*

MARC-145 cells were infected at a moi of 0.01 TCID<sub>50</sub> with the IAF-KLOP isolate, and left at 37 °C till the maximum of cytopathic effect was obtained. After being frozen and thawed two times, the supernatant was harvested and centrifuged at 6000 RPM for 30 min. The clarified

supernatant was put onto a solution of 30% (W/V) sucrose, and centrifuged at 100,000 g for 3 h at 4 °C. The pellets, containing the virus, were resuspended in a minimal volume of PBS solution. Further treatment of semi-purified virus was done at 4 °C. To one volume of the virus suspension was added one volume of chloroform or 1,1,2-Trichlorotrifluoroethane (Fisher Inc., Montreal, Canada). Virus were mixed for 1 min with a vortex and incubated 5 min on ice. After another centrifugation step, the upper phase was collected and used for the binding assay. For Western blot, aliquots of 15 µl of each of the virus suspension, were analysed using 12% SDS-polyacrylamide slab gels. Following electrotransfer of the virus proteins to nitrocellulose membrane and saturation of the binding sites with 5% of skin milk overnight at 4 °C, the membrane was incubated for 45 min with 1/50 dilution of polyclonal sera from PRRSV-infected convalescent pigs. The immune complexes were revealed following an incubation in the presence of goat anti-pig IgG antibody coupled with horseradish peroxidase (ICN, Montreal, Qc). Staining of the immune complexes was obtained by a final incubation of the nitrocellulose membrane in a chloronaphthol solution containing 3% of H<sub>2</sub>O<sub>2</sub>.

#### *Treatment of the cells with proteases and inhibitors*

Prior the binding assays, cells that have been dispersed as described above in Dulbecco's-MEM without FBS, were pre-treated for 30 min at 37 °C with different proteases including chymotrypsin, pronase E (Sigma) and neuraminidase (RDE or receptor destroying enzyme). In most experiments, proteolytic enzymes were added to the cells suspension at a concentration of 10 U/ml. In other experiments, cells were incubated at 37 °C for 8 h in presence of 5 µg/ml of cycloheximide (Sigma) prior the binding assays.

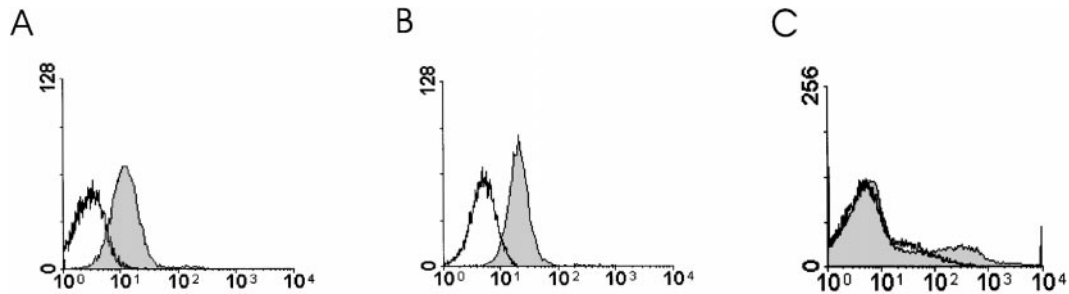
#### *Labelling with surface markers*

PBLs collected from 4 -to 9-week-old pigs were resuspended in PBS to a concentration of 10<sup>6</sup> cells/ml. Cells were labelled by incubation for 30 min at 4 °C with MAbs directed to different cell surface markers. The following MAbs were used for labelling the cells: My4, mouse anti-human CD14-phycoerythrin (Coulter); 74-12-4, mouse anti-porcine CD4 (VMRD, Pullman, WA); MIL12, mouse anti-porcine CD8 (Serotec, Raleigh, NC); and 74-22-15 or SWC3 mouse anti-porcine complement factor 3 (ATCC, Rockville, MD). Following two washing steps with PBS, binding of the primary antibody was determined by incubating the cells with either sheep anti-mouse IgG conjugated to phycoerythrin (Sigma) or sheep anti-mouse IgG conjugated to FITC (Boehringer Mannheim). Following two final washes in PBS, cells were fixed with PBS containing 1% paraformaldehyde at 4 °C, and then examined by flow cytometry.

## **Results**

### *Binding of PRRSV on permissive cells*

To investigate on the cellular tropism of PRRSV, a binding assay was set up to follow the virus attachment on susceptible and permissive cells, a step which is necessary to initiate the viral infection cycle. Preliminary experiments were focused on porcine alveolar macrophages (PAMs) and MARC-145 cells, two cell types known to be highly permissive to PRRSV infection. In the first step of the binding assay, the biotinylated MAb IAF-K8 was used to label viral particles attached at the surface of the cells. In the second step of the assay, immune complexes were revealed following incubation with phycoerythrin-streptavidin giving rise to a fluorescent signal that was analysed by flow cytometry. As illustrated in



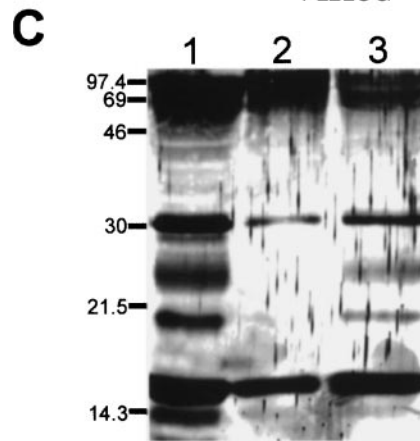
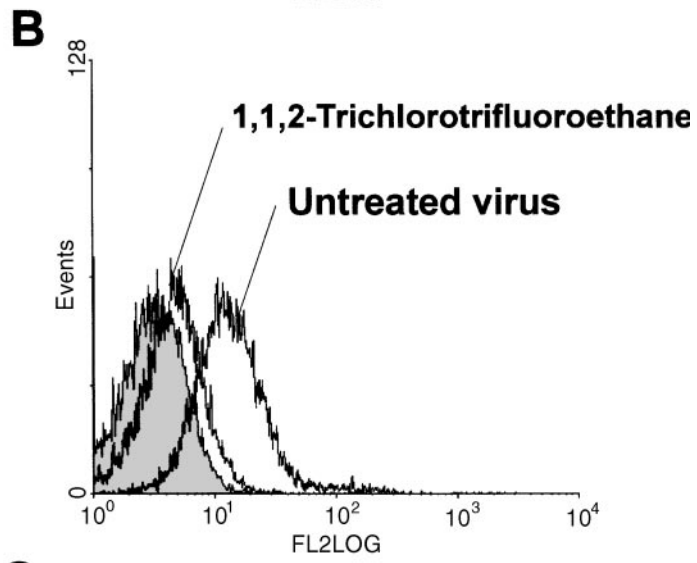
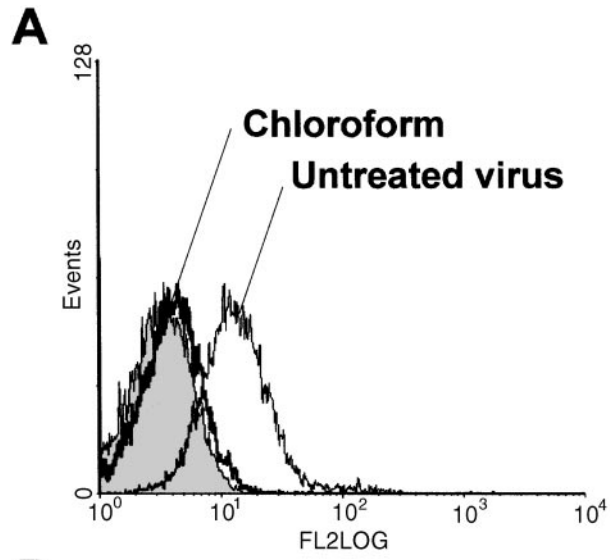
**Fig. 1.** Binding of PRRSV on permissive cell lines as followed by flow cytometry. Suspensions of PAMs (A), MARC-145 cells (B) and PBLs (C) were inoculated with the IAF-Klop strain of PRRSV at a MOI of 10 TCID<sub>50</sub> of virus per cell. Following an adsorption period of 1 h at 4 °C , infected cells were washed with PBS and incubated with biotinylated MAb IAF-K8 for 30 min at 37 °C. The surface bound immune complexes were labelled with streptavidin-phycoerythrin, and fluorescent level was analysed by flow cytometry. Un-filled patterns represent control cells incubated in presence of biotinylated MAb IAF-K8 and streptavidin-phycoerythrin

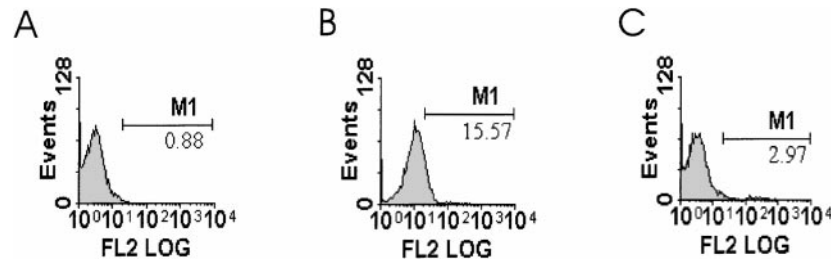
Fig. 1A, the majority of the cells in the enriched PAMs preparation depicted a shift in their fluorescence signal when exposed to the virus, at a MOI of 10 for 30 min at 4 °C, compared with the control. At this incubation temperature, internalization of PRRSV was prevented. Similar results were obtained with MARC-145 cells (Fig. 1B). The flow cytometric patterns of fluorescence observed with both cell cultures indicated that most of the cells possess a specific binding factor for PRRSV. On the other hand, a very small population of the porcine peripheral blood leukocytes (PBLs) appeared to bind the virus (Fig. 1C).

#### *Free N protein or empty capsids do not bind on permissive cells*

In preliminary studies on the sensitivity and specificity of the binding assay, MAbs directed to the GP<sub>5</sub> envelope protein of the homologous PRRSV strain [37] were first tested for their ability to detect viral particles attached at the surface of the permissive cells. Using a pool of neutralizing anti-GP<sub>5</sub> MAbs, only weak shifts of fluorescence could be revealed with both enriched PAMs and MARC-145 cells

**Fig. 2.** Treatments of PRRSV with chloroform and 1,1,2-Trichlorotrifluoroethane interfere with its attachment to MARC-145 cells. PRRSV was treated or not with chloroform (A) or 1,1,2-Trichlorotrifluoroethane (B) as described in Materials and methods, and were used for the binding assay. Following an adsorption period of 1 h at 4 °C , infected cells were washed with PBS and incubated with biotinylated MAb IAF-K8 for 30 min at 37 °C. The surface bound immune complexes were labelled with streptavidin-phycoerythrin, and fluorescent level was analysed by flow cytometry. Filled patterns represent control incubated without virus. C. Western blot analysis of concentrated extracellular virions (1), treated with chloroform (2) or with 1,1,2-Trichlorotrifluoroethane (3). The nitrocellulose membrane was incubated in the presence of serum from a PRRSV-infected convalescent pig. The immune complexes were revealed with goat anti-pig IgG antibody coupled with horsedish peroxidase





**Fig. 3.** Effect of pretreatment of MARC-145 cells with chymotrypsin on the binding of PRRSV. **A** Control MARC-145 cells were incubated only with biotinylated MAb IAF-K8 and streptavidin-phycoerythrin. MARC-145 cells were incubated in the absence (**B**) or presence (**C**) of 10 U of chymotrypsin for 30 min at 37 °C. After washing twice with PBS, cells were inoculated with the IAF-Klop strain of PRRSV at a MOI of 10 TCID<sub>50</sub> of virus per cell. Following an adsorption period of 1 h at 4 °C, infected cells were washed with PBS, then incubated with MAb IAF-K8 for 30 min at 37 °C. The immune complexes were after labelled with streptavidin-phycoerythrin and fluorescent level was analysed by flow cytometry. M1 range was used as a parameter to follow fluorescence shift

(data not shown) which could be attributed to the fact that these MAbs were all directed against linear antigenic determinants of the GP<sub>5</sub> [37], in addition to the intensity of the background that was observed with some cell types. On the other hand, more significant results were obtained using IAF-K8 MAb directed against the N protein, which is a sticky and basic protein [7], and this even if no special treatments were done to permeabilize the viral envelope. To assure that the shift of fluorescence observed in Fig. 1A and 1B was not due to the attachment of empty capsids, the binding assay was conducted with concentrated virus preparation that have been treated with chloroform or 1,1,2-Trichlorotrifluoroethane (Freon) prior to the binding assay. Chloroform was used as a lipidic solvent to solubilize the lipid bilayer surrounding the viral icosahedric nucleocapsid, whereas Freon was used to extract residual cellular or viral lipoproteins [11, 15]. As illustrated in Fig. 2A and 2B, following both treatments, virus binding on PAMs was almost completely abrogated to the level of the controls without virus, no shift in the fluorescence signals being observed. The presence of free empty capsids in the treated viral preparations was confirmed by SDS-PAGE and Western blot analysis. As depicted in Fig. 2C, treatment with chloroform resulted in the loss of the membrane-associated proteins, especially the GP<sub>5</sub> and the M proteins with estimated  $M_r$  of 25 and 19 kDa, in comparison to untreated purified virus. However, the N protein (15 kDa) remained present in amounts comparable to that observed with the untreated viral preparation, as well as the presence of a putative N dimer with estimated  $M_r$  of approximately 30 kDa. On the other hand, the M and GP<sub>5</sub> envelope proteins could still be revealed following treatment with Freon, suggesting that the virus had lost its binding ability despite the conservation of its envelope components.



**Table 1.** Effects of proteolytic enzymes and inhibitors on PRRSV attachment to MARC-145 cells

Treatments (10 U/ml)	Binding of PRRSV <sup>a</sup>
Pronase E	–
Chymotrypsin	–
RDE	+
Cycloheximide (5µg/ml) <sup>b</sup>	–

<sup>a</sup>Binding of PRRSV was considered negative when 80% of the fluorescence shift was inhibited

<sup>b</sup>Cells were treated with chymotrypsin 1 h at 37 °C, then rinsed and incubated for 8 h at 37 °C in culture medium supplemented with cycloheximide, prior the binding assay

#### *Effects of proteolytic enzymes and protein inhibitor on virus attachment*

The above findings indicated that the binding of PRRSV on permissive cells could be followed by flow cytometry. To further investigate on the chemical nature of the specific cellular factors involved in the virus-cell interactions, cells were pre-incubated with various enzymes prior to the binding of the virus to the cell surface. As shown in Fig. 3, incubation of MARC-145 cells in the presence of 10 U/mL chymotrypsin for 30 min at 37 °C inhibited 85% of the binding activity for PRRSV. Similar flow cytometric patterns were obtained following pre-incubation of cells in the presence of 10 U/ml of pronase E (Table 1). Fluorescence microscopy also showed that only a few scattered cells within confluent monolayers of the protease-treated MARC-145 cells could be detected by immunofluorescence with anti-N MAb after 48h PI at a MOI of 10 (data not shown). Data suggested that few number of cells were probably infected by residual virus, remaining after the washing steps that followed the adsorption period, when newly binding molecules were regenerated at their surface. In further experiments, it was possible to demonstrate that MARC-145 can regenerate all of their binding activity 8 h after being treated with the above proteases (data not shown). However, reestablishment of the binding capacity of MARC-145 cells could not be recovered in the presence of cycloheximide, a specific inhibitor of the protein synthesis (Table 1), thus confirming the proteinic nature of the specific binding factor(s). On the other hand, pre-incubation of MARC-145 cells in the presence of 10 U/mL of neuraminidase (RDE) did not seem to affect their binding capacity for PRRSV (Table 1).

#### *Binding of PRRSV on non-permissive cell lines*

To verify if the attachment on the cells, in case of PRRSV infection, is the only requirement for initiating a productive infection, the binding assay was conducted on cell lines known to be non-permissive to PRRSV infection. Within the cell lines tested by flow cytometry, continuous porcine cell lines such as PT and PK-15 cells

**Table 2.** Binding of PRRSV on permissive and non-permissive cells

Cell types	Binding of PRRSV <sup>a</sup>	Permissivity to PRRSV <sup>b</sup>
PAMs	+	+
MARC-145	+	+
MA-104	+	+
PBLs	+ <sup>c</sup>	+ <sup>c</sup>
RK-13	+	—
BHK-21	+	—
PK-15	+	—
PT	+	—
U937	—	—
293A	+	—

<sup>a</sup>Virus that bound to cells in suspension was labelled with biotinylated MAb IAF-K8 and streptavidin-phycoerythrin, then fluorescent level was analysed by flow cytometry. A shift in the fluorescence pattern was considered as positive

<sup>b</sup>Replication of PRRSV in the various cell lines was determined after 48 h post-infection by indirect immunofluorescence using MAb IAF-K8 and by RT-PCR

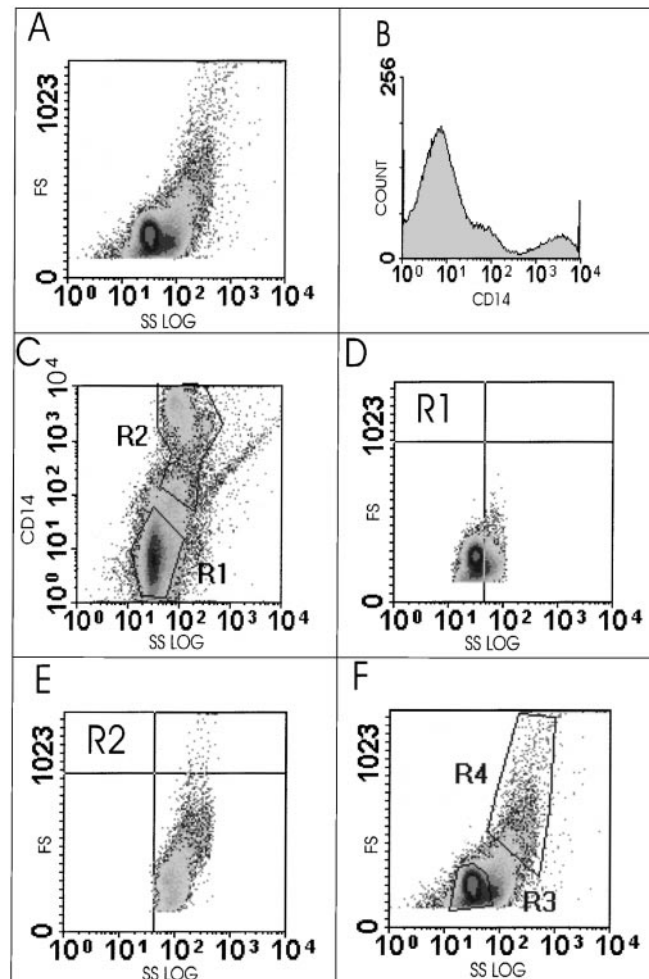
<sup>c</sup>Only few cells were able to bind or to be infected by PRRSV

were able to bind PRRSV with an efficiency comparable to that of MARC-145 cells and PAMs (Table 2). The MA-104 cells, from which derived the MARC-145 cell line, also bound the virus. These cells could also be infected by PRRSV, as suggested by the microscopic fluorescence patterns obtained following labelling with MAb IAF-K8, but with a lower efficiency. The BHK-21 and RK-13 cell lines, known for their permissivity to EAV infection [38], were also able to bind PRRSV, as well as human 293 fibroblastic cells. Based on immunofluorescence experiments, these three cell lines appeared to be non-permissive to PRRSV. In the case of the human U937 cells, they were found to lack a specific binding factor for PRRSV and were non-permissive to PRRSV infection.

#### *Interactions of PRRSV with PBLs*

Previous investigators have demonstrated that PRRSV can infect PBLs in vitro. However, only a small population of these cells seem to be infected, most probably monocytes [44]. To confirm the nature of PBLs infected by PRRSV in vitro, flow cytometry experiments using surface markers together with the virus binding assay were performed. Discrimination of monocytes-macrophages from lymphocytes was accomplished using forward versus side light scatter characterization,

as well as immune phenotyping using phycoerythrin-conjugated MAb to CD14 cell marker. CD14 is the major cell surface receptor for LPS highly expressed on monocytes/macrophages and neutrophils cell populations [14]. The CD14-specific MAb My4 has been previously reported to recognize the porcine CD14 receptor [18]. To determine which PBLs cell populations bind the PRRSV, a multiparametric approach based on size (FS), granularity (SS LOG), and surface



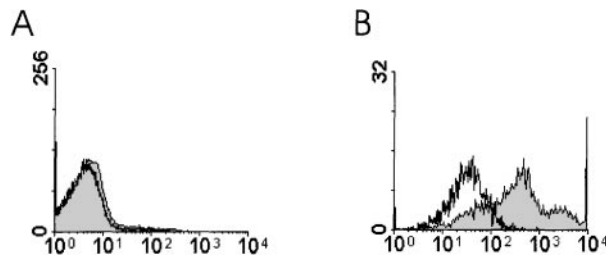
**Fig. 4.** Separation of porcine peripheral blood leucocytes by their size (FS), their granularity (SS LOG) and surface expression of the CD-14 marker by flow cytometry. **A** SS LOG×FS density plot graph of PBLs recovered by centrifugation onto Ficoll-hypaque gradient of peripheral blood from 4- to 9- week-old SPF pigs. **B** Fluorescent histogram of cells in A following labelling with MAb anti-CD14 (My4) coupled to phycoerhytrin. **C** SS LOG×CD14 density plot graph of cells in A. Regions R1 represent CD14<sup>-</sup> cells and R2 CD14<sup>+</sup> cells, respectively. **D** SS LOG×FS density plot graph of cells that segregated into the R1 region. **E** SS LOG×FS density plot graph of cells that segregated into the R2 region. **F** Two distinct subpopulations were further identified in R1 region on the basis of their intensity labelling with anti-CD14 MAb: R3 for CD14<sup>-</sup> cells and R4 for CD14<sup>+</sup> cells

**Table 3.** Cellular surface markers identified on CD14<sup>+</sup> and CD14<sup>-</sup> porcine PBLs

	CD14 <sup>-a</sup>	CD14 <sup>+b</sup>
CD4	+	-
CD8	+	-
SWC3	-	+

<sup>a</sup>Population CD14<sup>-</sup> represents cells that segregated into region R3 (Fig. 4F)

<sup>b</sup>Population CD14<sup>+</sup> represents cells that segregated into region R4 (Fig. 4F)



**Fig. 5.** Binding of PRRSV on PBLs as followed by flow cytometry. PBLs were enriched by centrifugation onto Ficoll-hypaque gradient of peripheral blood from 4- to 9- week-old SPF pigs. The cell suspension was then inoculated with the IAF-Klop strain of PRRSV at a MOI of 10 TCID<sub>50</sub> of virus per cell. Following an adsorption period of 1 h at 4 °C , infected cells were washed with PBS and incubated with MAb IAF-K8 for 30 min at 37 °C. The immune complexes were after labelled with streptavidin-phycoerythrin, and fluorescent level was analysed by flow cytometry. **A** Fluorescent histogram of CD14<sup>-</sup> cells that segregated in the pre-defined R3 region. **B** Fluorescent histogram of CD14<sup>+</sup> cells that segregated in the pre-defined R4 region. Unfilled patterns represent mock-infected cells

expression of CD14 was developed. PBLs collected from heparinized blood of SPF piglets were resuspended in PBS to a concentration of 10<sup>6</sup> cells/ml. When MAb anti-CD14 was incubated in presence of PBLs, three cell populations were observed: CD14<sup>-</sup>, CD14<sup>low</sup> and CD14<sup>high</sup> (Fig. 4B). As expected, most PBLs (over 90%) did not express CD14. When granularity and expression of CD14 were considered in a plot graphic (Fig. 4C), PBLs segregated in two distinct populations: one representing CD14<sup>-</sup> cells (R1) and the other defined by the CD14<sup>+</sup> cells (R2) (including CD14<sup>low</sup> and CD14<sup>high</sup>). Cells of each region were then plotted in a FS X SS LOG graphic (Fig. 4 D and E). The CD14<sup>-</sup> and CD14<sup>+</sup> cell populations were differed by their size and granularity, although an overlapping region was identified. According to the data obtained, two distinct subpopulations were defined; one comprised CD14<sup>-</sup> cells (R3) and the other CD14<sup>+</sup> cells (R4) (Fig. 4 F).

Surface markers CD4 and CD8 are usually present only in the T lymphocytes population, and MAbs directed against these molecules have been made for swine

[9]. Surface marker SWC3, also designated MAC-1 or CD11b/CD18, is mostly expressed on the surface of monocyte/macrophage cells and neutrophils [9]. Table 3 summarizes data on the phenotyping of the cells comprised in the pre-defined regions R3 and R4 for CD4, CD8, SWC3 and CD14 markers. Region R3 was shown to contain CD4<sup>+</sup> and CD8<sup>+</sup> cells, and these cells were CD14<sup>-</sup>, as defined above. On the other hand, region R4 contained cells that did not express CD4 or CD8 markers, but were SWC3<sup>+</sup> and CD14<sup>+</sup>. Accordingly, R3 was defined as a lymphocytes-enriched fraction, whereas monocytes segregated in the R4 region.

The PRRSV binding assay was then performed on PBLs and specific immunofluorescence patterns were analysed with parameters defined above for distinguishing between lymphocytes and monocytes (Fig. 5). In the case of lymphocytes, the fluorescence pattern observed in the presence of virus was similar to that observed with non-infected cells (Fig. 5A). In contrast, CD14<sup>+</sup> cells, corresponding to the monocytes-enriched population, were able to bind PRRSV (Fig. 5B).

### Discussion

The attachment of viral particles to specific receptors on the surface of permissive cells is a necessary step for a productive infection. Several studies confirmed that PRRSV has a strongly restricted tropism for cells of the monocyte/macrophage lineage both in vivo and in vitro [1, 5, 10, 22, 35, 44]. To further investigate on the cellular factors that determined the tropism of PRRSV, a specific binding assay was designed to follow the attachment of PRRSV on susceptible (that permit virus attachment) and permissive (that permit virus replication) cells. As expected, both MARC-145 cells and PAMs, were found to attach with high efficiency PRRSV on their surface and to allow completion of its replication cycle. Further experiments were made to confirm the specificity of the binding assay. The first objective was to eliminate the possibility that shift of fluorescence observed with PAMs and MARC-145 cells was due to the attachment of empty capsids reacting with the anti-N MAbs IAF-K8 used to label viral particles attached at the surface of the cells. The second objective was to demonstrate that bound viral particles detected by flow cytometry analysis were still infectious. Treatment with chloroform and freon removed or interfered with the chemical nature of viral components that appeared to be necessary for attachment on the surface of susceptible cells. The data obtained following treatment with chloroform confirmed that free N protein or empty capsids did not have the ability to bind *per se* on the surface of both permissive cell types and that the viral envelope was most probably permeable or sufficiently damaged to allow interaction of the IAF-K8 MAbs with virus-associated N protein. However, GP<sub>5</sub> and M proteins were still present following treatment of the PRRSV virion with freon. It may be possible that the latter treatment has affected the envelope integrity or has resulted in conformational changes somehow to interfere with attachment or interaction of virus binding protein (s) with a putative specific cellular receptor. Another possibility is that GP<sub>5</sub> and M are not the major components involved in the attachment of PRRSV on the surface of targeted cells. Minor envelope proteins, GP<sub>4</sub>, GP<sub>2</sub> and E, which are also

exposed at the surface of the virion, could also be implicated in the interactions with a specific binding protein for PRRSV on the surface of susceptible cells.

Treatment of cells with different proteases and cycloheximide, and subsequent analysis of binding of PRRSV in the assay indicated that the receptor has a proteinic nature. Since virus binding was not blocked following pre-treatment of cells with neuraminidase, sialic acid or NAMA is apparently not involved in PRRSV attachment to permissive cells. This study did not, however, allow to conclude whether the PRRSV-specific binding factor on PAMs and MARC-145 cells is the same. The MA104 cell line has been established from epithelial monkey kidney cells [19], so their physiological functions *in vivo* are quite different from those of PAMs. Consequently, one can speculate that the entry of PRRSV in MARC-145 cells is probably mediated by a different mechanism: through an evolutionary common ancestor receptor or a non-tissue-specific cell surface molecule. Since EAV and SHFV, but not LDV, have previously demonstrated their ability to infect MA-104 cells, from which MARC-145 cells have been established, one can speculate that PRRSV, EAV and SHFV enter by the same pathway in these epithelial cells via a common receptor [38]. However, the receptor on macrophages should be different since PAMs are not susceptible to EAV and LDV. Recently, a 210 kDa protein has been identified as a putative cellular receptor for PRRSV on PAMs, the latter being recognized by a specific MAb that blocks the infection [10]. Since this MAb does not recognise epitopes on the surface of porcine monocytes and also on MARC-145 cells, it seems that the pathway used by PRRSV in these cells is different than the one involved with PAMs [10]. In this regard, the hypothesis of a common binding factor and the possibility of a second receptor (such as the 210 kDa protein of PAMs) that would dictate specific tissue tropism is plausible.

As previously demonstrated by other investigators [21], data obtained in the present study also indicated that PRRSV does not only bind on permissive cells such as monocytes, PAMs and MARC-145, but that other cell lines known to be refractory to PRRSV infection can also bind the virus on their surface. Thus binding of PRRSV on the host cells does not necessarily lead to a productive infection, the basis for the restricted replication of PRRSV in certain cell types being found in the different subsequent steps that have to be passed through successfully before new viral particles are produced: internalization, release of the viral genome, transcription, translation and assembly [36].

In the present study, it was demonstrated that PRRSV can bind at the surface of BHK-21 and PK-15 cells, features which have not been demonstrated by others who have previously studied the susceptibility of those cell lines to PRRSV [21]. The discrepancy obtained on the binding capacity of these two cell lines can be attributed to the technical approaches that have been used, the flow cytometry method being most probably more sensitive for the detection of bound viral particles at the surface of the cells than the recovery of biotinylated viral proteins by SDS-PAGE analysis of lysates of PRRSV-infected cells [21]. Interestingly, the production of infectious virus has been previously observed in Vero cells following polyethylene glycol-mediated fusion of virus and in BHK-21 and vero cells following transfection experiments with full-length infectious cDNA clones

or infectious genomic RNA [21, 33]. In both cases, the cell membrane barrier was bypassed, therefore minimizing the importance of a specific surface cellular receptor for PRRSV. Thus, the restricted cell tropism in the case of PRRSV apparently also involved phenomena that occur following binding to the cell surface and releasing of the infectious RNA genome. The data previously obtained by others with Vero cells raise again the possibility that more than one cellular receptors is required for the internalization of PRRSV [21].

Several authors have reported that PRRSV can infect porcine blood leukocytes *in vivo* and *in vitro* [34, 44]. In this study, PRRSV infection was found to be restricted to CD14<sup>+</sup> cells, indicating that lymphocytes cannot be infected *in vitro* by PRRSV because they probably lack a specific binding factor on their surface. In contrast, monocytes were found to be able to bind the virus and support the virus growth, as previously reported by others [34, 44]. Recently, it has been demonstrated that PRRSV has a restricted tropism for only some sub-populations of porcine monocytes/macrophages and that some specific states of differentiation and activation of monocytes/macrophages considerably affect their susceptibility, bone marrow cells being not susceptible to PRRSV as an example of immature cells of the monocyte/macrophage lineage [34]. Such a restricted tropism for only some sub-populations of monocytes/macrophages is also observed for LDV, since in young mice only differentiated peritoneal macrophages can be infected by this murine arterivirus [38].

In the case of PRRSV-infected pigs, the immune response is delayed which favours the establishment of secondary bacterial infections [34]. The factors implied in this temporary immunodeficiency has not yet been identified. Data from experimental infection studies suggested that infection of the monocytes and macrophages at the early stages of the infection, compromises the first defense barrier of the upper respiratory tract and probably affects the presentation of antigens to cells of the lymph nodes, a step which is required to initiate a specific and effective immune response. The infection, and destruction of these cells, may perturb the regulatory mechanisms of synthesis or secretion of cytokines and chemokines, which are important for an effective immune response.

In conclusion, the binding assay used in the present study demonstrated that PRRSV does not only bind on permissive cell lines such as monocytes, PAMs and MARC-145. Other cell lines that were clearly demonstrated to be resistant to PRRSV infection were found to be able to bind the virus on their surface. Consequently, the binding of PRRSV on the host cell is not sufficient to allow a productive infection. The data obtained suggest that probably more than one cellular receptors is required for the internalization of PRRSV. The absence of such proteinic binding factor(s) at the surface of lymphocytes explains why they are refractory to PRRSV infection. Infection of PAMs and monocytes during the early stages of PRRSV infection certainly favours the establishment of secondary bacterial infections. The understanding of the restricted cellular tropism of PRRSV and that of other members of the *Arteriviridae* family, mainly the characterization of their specific cellular receptors, will be very helpful for better comprehension of their pathogenesis.

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