Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV)

X. Duan, H. J. Nauwynck, and **M. B. Pensaert**

Laboratory of Veterinary Virology, University of Gent, Merelbeke, Belgium

Summary. In this study, the susceptibility of porcine peripheral blood monocytes (BMo), peritoneal macrophages (PMϕ) and alveolar macrophages (AMϕ) to PRRSV was examined. To test the effect of differentiation and activation on their susceptibility, AMϕ and BMo were aged, cultivated in either adhesion or suspension and treated with bacterial lipopolysaccharide (LPS) and phorbol myristate acetate (PMA). It was found that freshly isolated PMϕ and BMo were non-permissive to PRRSV. PMϕ remained refractory but a few BMo became susceptible after 1 day cultivation. AMϕ were permissive with a significant increase of their susceptibility after one day cultivation. In a binding assay, it was demonstrated that the attachment of biotinylated PRRSV to AMϕ is much more efficient than to PMϕ and BMo. Two monoclonal antibodies (Mabs) 41D3 and 41D5 which block PRRSV infection of AMϕ and are directed against a candidate receptor for PRRSV only reacted with the cell membrane of AMϕ. PMA treatment of AMϕ blocked PRRSV replication in the cells in a dose-dependent manner. The blocking effect of PMA decreased after 9 h continuous pre-treatment and diminished after 24 h continuous pre-treatment. PMA treatment did not affect the binding of PRRSV and MAb 41D3 and 41D5 to AMϕ. Direct or indirect treatment of AMϕ and BMo with LPS or cultivation in suspension did not significantly affect their susceptibility. These results provide clear evidence that PRRSV has a strongly 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.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) resembles lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV), three other members of the arterivirus

group with regard to morphology, genetic organisation and structural proteins [9, 19]. One peculiar common characteristic of these viruses is that they have a strong tropism for monocytes/macrophages.

Of many procine cell systems tested, only porcine alveolar macrophages (AMϕ) support replication of PRRSV [2, 8, 31, 34]. Replication of PRRSV in some cultivated porcine peripheral blood monocytes (BMo) has also been reported [30]. Unexpectedly it was also found that PRRSV replicate in two non-porcine cell lines: an established cell line from monkey kidney, MARC-145 [15], and a proprietary cell line, CL2621 [18]. Similarly, the replication of LDV and SHFV is also highly restricted to primary cultures of host macrophages in vitro. EAV forms an exception as it replicates in many different cell types [25].

PRRSV also shows a strict cell specificity "in vivo". Cells of the macrophage lineage have previously been identified as the predominately and consistently infected cell type in PRRSV infected pigs [11, 14, 26, 27]. Furthermore, several observations indicated that PRRSV only replicates in some sub-populations of monocytes/macrophages. It was found that AMϕ even during the period of the highest virus titres in the lungs after natural or experimental infection, only a low percentage of levaged AMϕ carries PRRSV antigens [11, 18]. Also, immature macrophages and macrophages progenitor stem cells are not or less susceptible to a PRRSV infection. This was particularly evident for bone marrow cells, where replication of PRRSV was not detected in experimentally PRRSV infected pigs [11]. Similar evidence comes from "in vitro" experiments in which different sub-populations of alveolar macrophages fractionated by density gradient centrifugation were shown to have different susceptibilities to a PRRSV infection [6]. Such heterogeneity may be a reflection of the sate of differentiation and/or activation of the macrophages [28]. A number of factors such as ageing, some cytokines and a few chemical products have been found to be able to differentiate and activate mononuclear phagocytes [28]. Bacterial lipopolysaccharide (LPS) and phorbol myristate acetate (PMA) are two frequently used stimulants. Both LPS and PMA have a strong, rapid and easily reproducible activating capacity. Their structure and sites of activation in the cells have been extensively studied [7, 5]. LPS is a structural component of the outer membrane of Gram-negative bacteria. It activates monocytes and macrophages and stimulates them to produce certain factors, including TNF-α, IL-1, and prostaglandin E_2 [5, 29]. Studying the effect of LPS treatment on the susceptibility of AMϕ to PRRSV may give new insights in the pathogenesis of dual infection with PRRSV and bacteria. PMA has effects on monocyte/macrophages, including dramatic changes in cell shape, spreading, endocytosis, and release of lytic mediators and regulators [5, 7, 20]. PMA also stimulate premonocytic cells of some continuous cell lines to differentiate into a more mature monocyte/macrophage phenotype [20]. Because of those remarkable properties, PMA has been used to investigate the relationship between cell activation/differentiation and viral replication with some viruses [13, 33].

In this study, it was examined if porcine monocyte/macrophage lineage cells isolated from peritoneal cavity, lungs and peripheral blood are susceptible to PRRSV and if their susceptibility was related to the degree of virus attachment. Furthermore, the effect of differentiation and activation of monocytes/macrophages by ageing, adhesion and treatment with LPS and PMA on their susceptibility to PRRSV was evaluated.

Materials and methods

PRRS virus

Two PRRSV isolates were used: the Lelystad strain of PRRSV (kindly provided by Dr. Wensvoort) and a Belgian isolate of PRRSV designated 94V360. A Lelystad virus stock of the thirteenth passage grown in porcine AMφ with a titre of $10^{5.3}$ TCID$_{50}$/ml was used in this study. The 94V360 was adapted to MARC-145 cells, purified and biotinylated as earlier described [12]. Briefly, a fifth passage of 94V360 was first clarified by centrifugation at $4000 \times \mathbf{g}$ for 20 min, then precipitated at $75\,000 \times \mathbf{g}$ for 3 h in a Beckman T35 rotor at $4\,°C$. The pellets were resuspended in 1/100 of original volume in TNE buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4) and centrifuged on a 0.5 to 1.5 M discontinuous sucrose gradient in a SW41 rotor at $110\,000 \times \mathbf{g}$ for 16 h. After centrifugation, the virus band was harvested and its purity was determined with a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The titre of the resulting virus preparation was 2 to 4×10^7 TCID$_{50}$/mg as determined on MARC-145 cells. The number of virus particles, as determined by negative staining electron microscopy, was 1 to 2×10^{10}/mg virus protein. Purified PRRSV was labelled with biotin by using a protein biotinylation kit (Amersham, Buckinghamshire, UK). The virus was pelleted and re-suspended in biotinylation buffer (40 mM Na$_2$CO$_3$, pH 8.6) at a protein concentration of 1 mg/ml. After a brief sonication 40 μl biotin reagent was added per mg of viral 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 purifying on a Sephadex G-25 column and diluted in PBS at a concentration of 0.2 mg/ml. After biotinylation, the titre of PRRSV was reduced by 50%. Biotinylated virions were stored at $-70\,°C$.

Antibodies

A swine myeloid cell specific monoclonal antibody (MAb), 74.22.15, was used to determine the percentage of porcine monocyte/macrophage cells [24]. MAbs against PRRSV nucleocapsid protein, WBE1 and WBE4–6 were used for immunofluorescecnece [10]. Two monoclonal antibodies (MAb), 41D3 and 41D5, which have been raised against AMφ and which are able to block PRRSV infection of AMφ [12], were used for membrane immunofluorescence staining to test their reactivity with various cells. Isotype matched irrelevant MAbs 18E8 and 41G3 directed against suid herpesvirus type 1 [23], were used as negative controls.

Isolation of porcine monocyte/macrophage lineage cells

Porcine alveolar macrophages (AMφ)

AMφ were obtained from 4- to 6-weeks old conventional Belgian Landrace pigs from a PRRSV negative herd according to the method previously described by Wensvoort et al.

[31]. Briefly, the lungs were lavaged with 200 ml cold phosphate buffered saline solution without calcium and magnesium (PBS). The lavaged cells were collected by centrifugation at $500 \times \mathbf{g}$ for 15 min at 4 °C. After two washings with cold PBS, a cell smear was made and stained with Hemacolar reagents (Diagnostica Merck, Darmstadt, Germany) and the percentage of neutrophils was determined. The cells were stained with 74.22.15 by membrane immunofluorescence. The percentage of cells from the monocytes/macrophages lineage was estimated by subtracting the percentage of neutrophils from the 74.22.15 positive cells. By doing so, more than 90% of lung lavage cells were found to be monocyte/ macrophage lineage cells.

Porcine peritoneal macrophages (PMφ)

PMφ were isolated by lavaging the peritoneal cavity of four 6- to 10-weeks old conventional Belgian Landrace pigs originating from a PRRSV negative herd with 100 ml cold PBS. After centrifugation at $500 \times \mathbf{g}$ for 15 min at 4 °C and two washings with PBS, the cells were collected and the percentage of monocyte/macrophage lineage cells was determined with the technique described above. More than 90% of lavaged peritoneal cells were characterised as monocytes/macrophages.

Porcine blood monocytes (BMo)

BMo were separated and cultivated as previously described [22]. Briefly, peripheral blood was obtained from four 10 to 20 weeks old conventional Belgian Landrace pigs originating from a PRRSV negative herd and peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation according to the method recommended by the manufacturer. 5×10^7 PBMC were layered on a polystyrene cell culture dish (Corning glass works, Corning, NY, USA) which was coated with 1 ml autologous plasma. After 1 h incubation at 37 °C, non-adherent cells were removed by three washings with PBS. Adherent cells representing enriched monocytes were harvested by gently flushing, the number of cells were counted and the percentage of monocytes was estimated with MAb 74.22.15. More than 95% of cells were found to be monocyte/macrophage lineage cells.

The viability of all cells used was > 95% as assessed by 2% nigrosin staining. AMφ, PMφ and BMo were brought in 24-well cell culture plates (Nalge Nunc international, Roskilde, Denmark) at a concentration of 10^6 cells per well. The medium used for cultivation was RPMI supplemented with 5% of foetal calf serum.

Inoculation of AMφ, BMo and PMφ with PRRSV

AMφ, BMo and PMφ were inoculated by replacing the medium with 1 ml stock solution containing $10^{5.3}$ TCID$_{50}$ PRRSV. After incubation at 37 °C for 1 h, three washings with PBS were performed. Then, the cells were refed with medium and further incubated at 37 °C with 5% CO$_2$.

Effect of ageing

To evaluate the effect of maturation of monocytes/macrophages on their susceptibility to PRRSV, freshly isolated AMφ, PMφ and BMo from five donors were seeded in 24-well tissue culture plates at a concentration of 10^6 cells/ml/well and further incubated in RPMI medium plus 5% of foetal bovine serum at 37 °C with 5% CO$_2$. After 1 and 24 h incubation,

the cells were inoculated with PRRSV. Extracellular virus titre and the percentage of viral antigen positive cells were determined at 0, 24 and 48 h after inoculation.

Effect of adhesion

To test if the adhesion of BMO and AMφ affect their susceptibility to PRRSV, freshly isolated AMφ and BMo from five donors were cultivated either in suspension in Teflon inserts (Poly Labo, Strasbourg, France) or attached to polystyrene in 24-well cell culture plates at a concentration of 10^6 cells/well in RPMI medium plus 5% of foetal bovine serum at 37 °C with 5% CO_2. After 24 h cultivation, the cells were inoculated with PRRSV. Extracellular virus titre and the percentage of viral antigen positive cells were determined at 0, 24 and 48 hours after inoculation.

Effect of PMA and LPS treatment

The effect of PMA treatment on PRRSV replication in BMo was examined by treating one day cultivated BMo with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Vertriebs GmbH, Deisenbofen, Germany) for 1 h before PRRSV inoculation. After PMA treatment, the cells were washed three times, then inoculated with PRRSV. Extracellular virus titre and the percentage of viral antigen positive cells were determined at 0, 24 and 48 h after inoculation.

In order to examine the effect of duration of PMA pre-treatment of AMφ on PRRSV replication, AMφ were treated with 10 ng/ml PMA for different time periods prior to PRRSV inoculation. After PMA treatment, the cells were washed three times then inoculated with PRRSV. Intracellular virus titre was determined at 12 h after inoculation. To test the effect of PMA treatment during virus replication in AMφ, the AMφ were treated with 10 ng/ml PMA for 1 h at different times after PRRSV inoculation. Intracellular virus titre was determined at 12 h after inoculation. To estimate the dose dependent effect of PMA treatment of AMφ on the PRRSV replication, one day cultivated AMφ grown in 96-well-plates were treated with different concentrations of PMA for 1 h after 1 h PRRSV inoculation. The PRRSV positive wells were determined by fixing and staining the plate using an immunoperoxidase monolayer assay (IMPA) as earlier described [31] after 48 h inoculation.

Direct and indirect LPS treatments were performed to evaluate the effect induced directly by LPS or indirectly by LPS-induced cytokines. For direct treatment, bacto lipopolysaccharide (LPS) of *E. coli* 0111:B4 (Difco Laboratories, Detroit, Michigen, USA) was used at a concentration of 10 µg/ml medium. For indirect treatment, 10% of the supernatant from 24 h LPS treated porcine AMφ was used. After 1 and 24 h treatment, the cells were washed three times with PBS and inoculated with PRRSV. Extracellular virus titre and the percentage of viral antigen positive cells were determined at 0, 24 and 48 h after inoculation.

PMA and LPS had no negative effect on the viability of the treated cells as assessed by nigrosin staining after each treatment. A cytotoxicity bioassay with PK15 cells for detecting TNF [9] and a proliferation assay on D10(N4)M cells for determining IL-1 concentrations [15] were performed to asses the efficacy of the LPS treatment.

Virus titrations

For determining the titre of extracellular virus, medium from PRRSV infected cells was collected and centrifuged at $9000 \times \mathbf{g}$ for 15 min. 100 µl of tenfold dilutions of the

supernatants were inoculated in 96-well microtiter plates (Nalge Nunc, Roskilde, Denmark) previously seeded with AMφ. For determining the titre of intracellular virus, AMφ, PMφ and BMo were harvested by flushing, washing, washed twice with PBS and lysed by freeze-thawing. 100 μl of tenfold dilutions were inoculated in 96-well microtiter plates previously seeded with cultivated AMφ. Inoculated 96-well plates were fixed 48 h after inoculation and stained using an immunoperoxidase monolayer assay (IPMA) as earlier described [31].

Immunofluorescence

Macrophages and monocytes were detached by thorough flushing of the bottom of polystyrene dishes and washed once with PBS. Cell smears were made using a cytocentrifuge at $140 \times$ **g** for 5 min. The smears were fixed in acetone for 20 min at $-20\,^{\circ}$C. A streptavidin-biotin immunofluorescence technique was performed. Briefly, the smears were pre-incubated with 1:20 diluted sheep serum to block non-specific staining. The smears were first incubated with a mixture of MAbs WBE1 and WBE4–6, then with biotinylated sheep-anti-mouse immunoglobulin and finally with streptavidin-fluorescein isothiocyanate (FITC)-conjugate (Amersham, Buckinghamshire, UK). The smears were washed three times with PBS between each incubation. To confirm the specificity of the staining, two mouse ascites fluids containing irrelevant MAbs against suid herpesvirus type 1 of the same isotype as WBE1 and WBE4–6 were used as negative controls [23]. Positive cells were counted using a Leica DM RBE fluorescence microscope.

Flow cytometry analysis

The membrane reactivity of biotinylated PRRSV, MAbs 41D3 and 41D5 to PBMC, BMo, PMφ and PMA-treated (10 ng/ml PMA for 1 h) and -untreated AMφ were evaluated by a flow cytometer FACSCalibur (Becton Dickinson) flow cytometer. 1×10^6 cells were washed three times with cold PBS and incubated with 100 μl of 4% paraformaldehyde at room temperature for 10 min. After washing once with cold PBS, the fixed cells were first incubated with biotinylated PRRSV (5 μg) or MAbs (15 ng/ml of 41D3, 41D5 or 18E8), then 1:50 diluted streptavidin-fluorescein isothiocyanate (FITC)-conjugate or goat anti-mouse IgG FITC (Amersham, Buckinghamshire, UK) for 1 h on ice. The cells were washed three times after each incubation. The mean fluorescence intensity of each sample was measured on the flow cytometer. Relative mean fluorescence measurements were corrected for autofluorescence of control cells.

Statistical analysis

All data were statistically analysed by the Student's t-test.

Results

PRRSV replication in AMφ

PRRSV productively replicated in AMφ. Table 1 shows the virus titres and percentage of PRRSV positive cells in freshly isolated and one day cultivated AMφ at 1, 24 and 48 h after PRRSV inoculation. The virus titres and percentage of viral antigen positive cells in freshly isolated porcine AMφ at 24 and 48 h after inoculation were respectively 1 to 2 $\log_{10}$TCID$_{50}$ and 5 to 10 times

Table 1. Replication of PRRSV in porcine alveolar macrophages, freshly isolated and after one day cultivation

AMϕ	Hours after inoculation	% viral antigen positive cells[a]	Virus titre ($\log_{10}$TCID$_{50}$)	
			extracellular (per ml)	intracellular (per 10^6 cells)
Freshly isolated	1	0	1.2 ± 0.3	1.5 ± 0.5
	24	0.1 ± 0.1	1.8 ± 0.2	2.0 ± 0.4
	48	12.7 ± 8.9	2.9 ± 0.6	3.3 ± 0.5
24 h cultivated	1	0	1.3 ± 0.5	1.8 ± 0.3
	24	12.3 ± 2.5	3.6 ± 0.7	3.7 ± 0.6
	48	52.3 ± 8.5	5.3 ± 0.5	5.3 ± 0.4

[a] Mean value of three experiments $\pm$ standard deviation

Table 2. Replication of PRRSV in porcine alveolar macrophages and blood monocytes cultivated in suspension or attached to polystyrene

Cell type	Grown on	Extracellular virus titre ($\log_{10}$TCID$_{50}$)		% viral antigen positive cells[a]	
		24[b]	48[b]	24[b]	48[b]
Porcine alveolar macrophages	teflon	4.0 ± 0.5	5.3 ± 0.6	10.0 ± 5.3	45.3 ± 8.2
	polystyrene	4.2 ± 0.7	5.2 ± 0.5	9.5 ± 2.6	49.0 ± 6.6
Blood monocytes	teflon	1.0 ± 0.4	2.0 ± 0.4	0.1 ± 0.1	0.3 ± 0.2
	polystyrene	0.8 ± 0.5	2.3 ± 0.6	0.1 ± 0.1	0.3 ± 0.2

[a] Mean value of three experiments $\pm$ standard deviation
[b] Hours PI

lower than those of one day cultivated ones, which is significantly different ($P < 0.01$).

PRRSV replication in BMo and PMϕ

A productive replication of PRRSV was not detected in freshly isolated BMo obtained from four pigs. However, when the BMo were inoculated after one day of cultivation, $10^{1.0}$ to $10^{2.3}$ TCID$_{50}$ per 10^6 cells virus and 0.1 to 0.5% viral antigen positive cells were detected at 24 hours and 48 hours post inoculation, respectively (Table 2).

Productive replication of PRRSV was not found in freshly isolated PMϕ obtained from five pigs. After one day cultivation, PMϕ remained refractory to PRRSV infection.

Effect of adhesion

As shown in Table 2, no statistically significant differences in both viral antigen expression and virus titres were observed between AMφ and BMo cultivated in suspension in Teflon inserts and those attached to polystyrene dishes.

Effect of LPS treatment

The efficacy of activation after LPS treatment was shown by the presence of TNF-α (titre: 270 biological units per ml for the supernatant of AMφ and 140 biological units per ml for that of BMo) and IL-1 (titre: 1700 biological units per ml for the supernatant of AMφ and 1500 biological units per ml for that of BMo) in the supernatant of AMφ and BMo after 24 h treatment with LPS. However, no significant differences in both viral antigen expression and virus titres were found between untreated and the directly or indirectly LPS treated AMφ (Table 3).

No virus replication was detected in the BMo directly or indirectly treated with LPS (data not shown).

Effect of PMA treatment

No virus replication was detected in the BMo treated with PMA.

When AMφ were pre-treated with PMA for a short time (1 to 6 h), they became resistant to PRRSV infection (Fig. 1). After 9 h continuous exposure to PMA, AMφ gradually regained their susceptibility to PRRSV and after 24 h continuous pre-treatment, virus production reached the level similar to that of untreated cultures.
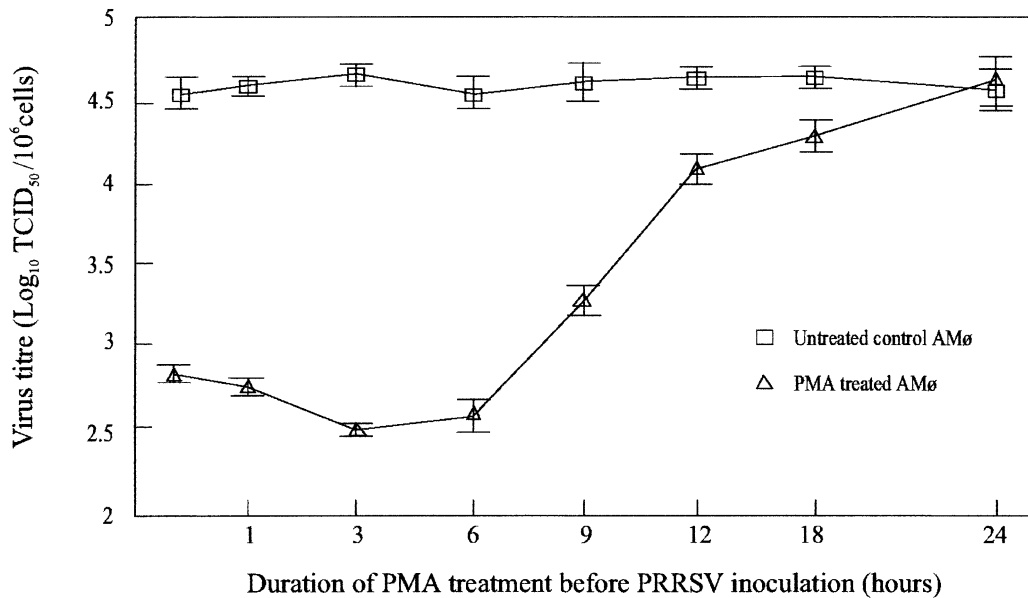
Table 3. Replication of PRRSV in porcine alveolar macrophages treated with bacterial lipopolysaccharide (LPS)

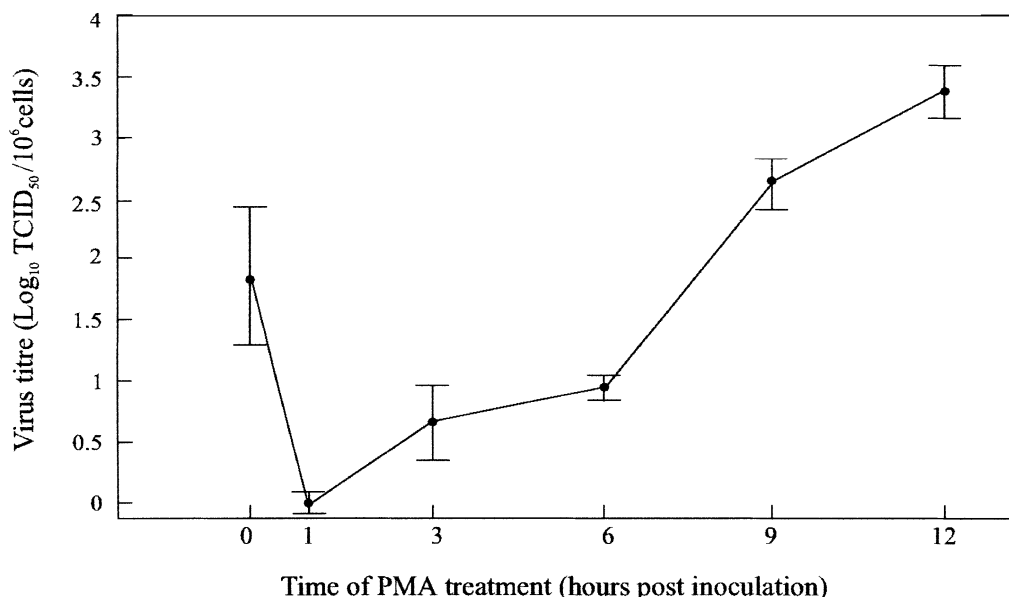| Treatment	Time (h)	Extracellular virus titre ($\log_{10}$TCID$_{50}$)		% viral antigen positive cells[a]	
		24[b]	48[b]	24[b]	48[b]
LPS	1	4.3 ± 0.3	5.4 ± 0.2	9.0 ± 2.6	44.3 ± 1.2
(10 µg/ml)	24	4.3 ± 0.3	5.1 ± 0.2	9.5 ± 1.3	51.0 ± 4.2
Supernatant of LPS	1	4.4 ± 0.4	5.3 ± 0.4	8.3 ± 3.1	46.7 ± 5.7
treated macrophages	24	4.3 ± 0.5	5.3 ± 0.4	10.5 ± 3.9	51.0 ± 12.7
None	1	4.3 ± 0.7	5.3 ± 0.5	9.5 ± 3.3	47.0 ± 5.6
(Control)	24	4.2 ± 0.5	5.1 ± 0.4	10.2 ± 2.6	50.0 ± 10.5

[a] Mean value of three experiments $\pm$ standard deviation
[b] Hours PI

Fig. 1. The effect of the duration of the PMA treatment of porcine alveolar macrophages (AMϕ) before PRRSV inoculation on the virus replication afterwards. One day cultivated AMϕ were treated with 10 ng/ml PMA for different hours as indicated before inoculation with PRRSV. Intracellular virus titre was determined at 12 h post inoculation. The mean value and standard error are shown using the AMϕ from three different donors

Fig. 2. The effect of 1 h PMA treatment of porcine alveolar macrophages (AMϕ) during PRRSV replication. One day cultivated AMø were treated with 10 ng/ml PMA for 1 h at different time periods after PRRSV inoculation as indicated. Intracellular virus titre was determined at 12 h post inoculation. The mean value and standard error are shown using the AMϕ from three different donors
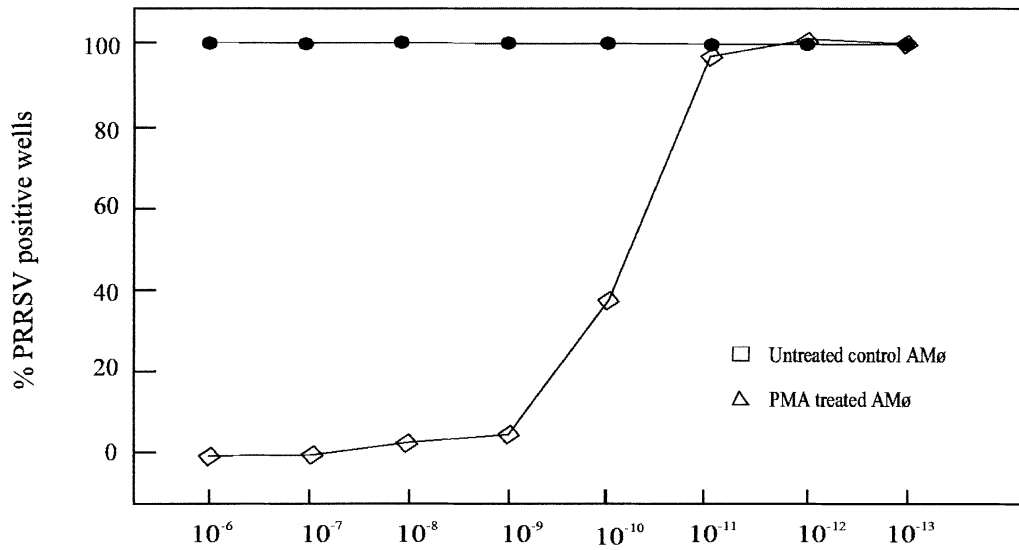
Fig. 3. The effect of different concentrations of PMA on the PRRSV replication in porcine alveolar macrophages after 1 h treatment
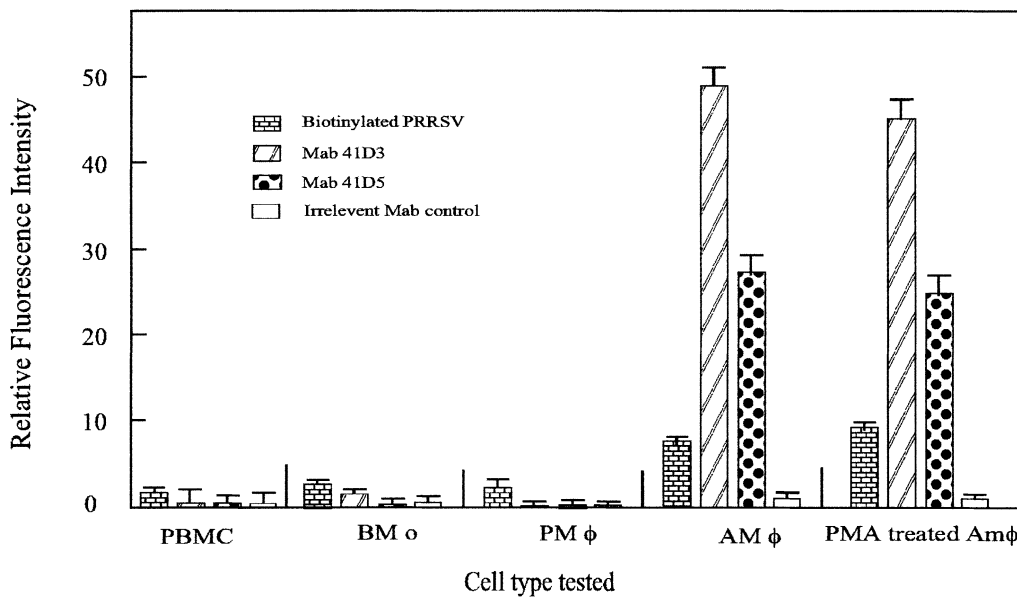
Fig. 4. The membrane reactivity of different porcine monocyte/macrophage lineage cells with biotinylated PRRSV and anti-porcine alveolar macrophage monoclonal antibodies Mabs 41D3 and 41D5 which both block PRRSV infection of AMϕ and are directed against a candidate PRRSV receptor. The mean value and standard error are expressed with the data of the cells from three donors

The effect of a 1 h treatment of AMϕ with PMA at different time points after a PRRSV inoculation on the virus replication is shown in Fig. 2. Simultaneous virus inoculation and PMA treatment for 1 h reduced the virus production to a level of $10^{2.0}$ TCID$_{50}$ per 10^6 cells. Replication of PRRSV was completely blocked when AMϕ had been inoculated for 1 h before PMA treatment. The longer the interval between inoculation and treatment, the higher the virus titre.

The blocking effect was concentration dependent as shown in Fig. 3. Concentrations as low as 1×10^{-8} µg/ml PMA completely inhibited PRRSV infection.

The membrane reactivity of BMo, AMϕ and PMϕ to biotinylated PRRSV and anti-AMϕ monoclonal antibodies 41D3 and 41D5

As shown in Fig. 4, the binding of biotinylated PRRSV to BMo, AMϕ and PMϕ was demonstrated by flow cytometry with a significantly higher fluorescence intensity in AMϕ. The binding of biotinylated PRRSV to the AMϕ was not affected by PMA treatment.

After staining with anti-porcine AMϕ monoclonal antibodies 41D5 and 41D3, the membrane fluorescence was detected only on AMϕ and not on PBMC, BMo, and PMϕ (Fig. 4).

Discussion

The results of this study show that only some subsets of cells from the porcine monocyte/macrophage lineage are susceptible to PRRSV and that the specific differentiation and activation state may considerably affect their susceptibility to PRRSV infection in vitro.

Cells of the porcine monocyte/macrophage lineage from different anatomic locations are clearly heterogeneous in their permissiveness for PRRSV infection. PRRSV replication was detected in porcine AMϕ, while freshly isolated BMo and PMϕ were completely resistant. These results are in agreement with earlier ''in vivo'' experiments, in which the replication of PRRSV was found in alveolar macrophages but not in peripheral blood mononuclear cells [11].

In the present study, very low virus titres and few infected cells were detected in cultivated BMo. This is in contrast with the observations of Voicu et al. [30], who found much higher virus titres in cultivated BMo. This variation may be due to differences in the genotype/phenotype of the donor animals, differences in PRRSV isolates and differences in techniques for the isolation of BMo. Genetic, antigenic and pathogenic variations among PRRSV isolates in the USA and Europe have been reported [17, 32]. Even with AMϕ and CL2621, two commonly used cell systems for PRRSV isolation, nearly one third of American PRRSV isolates grown in one cell type failed to grow in the other one [1].

The experiments also revealed that AMϕ show some restriction to a PRRSV infection when they were freshly isolated, even though they are relatively the

most sensitive cell type for PRRSV isolation [1, 31]. The susceptibility clearly increases after one day cultivation. A similar increase of permissiveness to PRRSV infection was observed in a very small number of cultivated BMo. These results suggest that the state of monocyte/macrophage differentiation plays an important role in determining their susceptibilty to PRRSV. Similar enhancing effects of differentiation on virus replication have been observed with other viruses such as the respiratory syncytial virus (RSV), parainfluenza virus-3, African swine fever virus, cytomegalovirus or herpes simplex virus (HSV), human immunodeficiency virus type 1 (HIV-1), Visna-maedi virus and pseudorabies virus in monocytes/macrophages [13, 21]. Since BMo are precursors of tissue macrophages and readily mature into macrophages when cultivated in vitro [28], it is logical to predict that some BMo may mature into susceptible macrophages similar to alveolar macrophages by cultivation in vitro. Resident PMϕ, which represent another population of well-differentiated tissue macrophages from a restricted lineage, were completely resistant to a PRRSV infection.

In this study, treatment of AMϕ with PMA was associated with a clear reduction of the replication of PRRSV and the blocking effect of PMA was transitory. When AMϕ were continuously pre-treated for 1 to 9 h, the virus replication was almost completely blocked. The cells regained full susceptibility after 24 h of continuous treatment. This rather peculiar finding can be explained by the fact that, while the activating capacity of monocytes and macrophages by PMA reaches a maximum after 1 h exposure, a prolonged exposure to PMA for more than 6 h causes macrophages and monocytes to become refractory to PMA stimulation [7]. When monocyte/macrophage lineage cells are treated with PMA, both enhanced or decreased viral replication has been observed with other viruses. For example, an increased virus replication with herpes simplex virus has been noticed upon cell differentiation [33] whereas a down-regulation of viral replication was observed with feline immunodeficiency virus [4].

The mechanism through which PMA inhibits PRRSV infection of AMϕ is unclear. In contrast to the PMA treatment, LPS treatment of porcine AMϕ and BMo did not influence the susceptibility to PRRSV infection. The biological properties of PMA are generally considered to be due to the activation of protein kinase C, an enzyme involved in many cellular responses. Although the activation of protein kinase C is also an essential process for LPS induced activation of the macrophage, the signal pathways used by PMA and LPS in macrophages are quite different [29]. Therefore, it seems that PMA modulated inhibition of PRRSV infection might be mediated by a specific PMA-signalling pathway in porcine AMϕ. Recent observations suggest that PRRSV enters AMϕ through a process of receptor-mediated endocytosis (unpubl. data). PMA treatment does not change the binding activity of PRRSV and monoclonal antibody against the putative PRRSV receptor(s) to AMϕ. Therefore, the blocking effect of PMA does not act through down-regulating the expression of the virus receptor(s) on the cells.

Mononuclear phagocyte differentiation is a process of normal cellular development and homeostasis and reflects a permanently altered expression of a cell's genetic potential, while macrophage activation is the process that causes reversible changes in macrophage phenotype and functions [28]. The differentiation may induce some factors that are essential for virus replication, such as receptors or transcription factors, while activation may up- or down-regulate the expression of these factors in cells. Inefficient binding of biotinylated PRRSV to PMφ, BMo and PBMC suggests that these cells lack the more specific binding sites that are expressed on the membrane of AMφ. Different binding activity of MAb 41D3 and 41D5, which block PRRSV infection of AMφ and recognise a candidate receptor on the cell, to different monocytes/macrophages provide further evidence that the restriction of PRRSV replication in some subsets of monocytes/macrophages is due to a reduced expression of virus receptor(s).

Acknowledgements

References

1. Bautista EM, Goyal SM, Yoon IJ, Joo HS, Collins JE (1993) Comparison of porcine alveolar macrophages and CL 2621 for the detection of porcine reproductive and respiratory syndrome (PRRS) virus and anti-PRRS antibody. J Vet Diagn Invest 5: 163–165

2. Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, Christianson WT, Morrison RB, Gorcyca D, Chladek D (1992) Characterisation of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest 4: 127–133

3. Bertoni G, Kuhnert P, Peterhans E, Pauli U (1993) Improved bioassay for the detection of porcine tumor necrosis factor using a homologous cell line: PK(15). J Immunol Methods 160: 267–271

4. Brunner D, Pedersen NC (1989) Infection of peritoneal macrophages in vitro and in vivo with feline immunodeficiency virus. J Virol 63: 5 483–5 488

5. Chen TY, Lei MG, Suzuki T, Morrison DC (1992) Lipopolysaccharide receptors and signal transduction pathways in mononuclear phagocytes. Curr Topics Microbiol Immunol 181: 169–187

6. Choi C, Gustafson K, Chinsakchai S, Hill H, Molitor TW (1994) Heterogeneity of porcine alveolar macrophages sub-population: immune functions and susceptibility to PEARS virus. In: Proceedings of the 13th Congress of the International Pig Veterinary Society (IPVS), Bangkok, Thailand, June, pp 67

7. Chung T, Kim YB (1988) Two distinct cytolytic mechanisms of macrophages and monocytes activated by phorbol myristate acetate. J Leukoc Biol 44: 329–336

8. Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS, Gorcyca D, Chladek D (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 Invest 4: 117–126

9. Conzelmann KK, Visser N, Van Woensel P, Thiel HJ (1993) Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology 193: 329–339

10. Drew TW, Meulenberg JJ, Sands JJ, Paton DJ (1995) Production, characterization and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome virus. J Gen Virol 76: 1 361–1 369

11. Duan X, Nauwynck HJ, Pensaert MB (1997) Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Vet Microbiol 56: 9–19

12. Duan X, Nauwynck HJ, Favoreel H, Pensaert MB (1997) Porcine reproductive and respiratory syndrome virus infection of alveolar macrophages can be blocked by monoclonal antibodies against cell surface surface antigens. In: Enjuanes L, Siddell S, Spaan W (eds) Coronaviruses and Arteriviruses: Proceedings of the VIIth International Symposium on Coronaviruses and Arteriviruses. Plenum Press, New York (in press)

13. Gendelman HE, Morahan PS (1992) Macrophages in viral infections. In: Lewis CE, McGee JOD (eds) The macrophages. IRL Press, New York, pp 157–213

14. Halbur PG, Miller LD, Paul PS, Meng XJ, Huffman EL, Andrews JJ (1995) Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Vet Pathol 32: 200–204

15. Hopkins SJ, Humphreys M (1989) Simple, sensitive and specific bioassay of interleukin-1. J Immunol Methods 120: 271–276

16. Kim HS, Kwang J, Yoon IJ, Joo HS, Frey ML (1993) Enhanced replication of porcine reproductive syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch Virol 133: 477–483

17. Meng XJ, Paul PS, Halbur PG, Lum MA (1995) Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch Virol 140: 745–755

18. Mengeling WL, Lager KM, Vorwald AC (1995) Diagnosis of porcine reproductive and respiratory syndrome. J Vet Diagn Invest 7: 3–16

19. Meulenberg JJ, Hulst MM, de Meijer EJ, Moonen PL, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJ (1993) Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192: 62–72

20. Minta JO, Pambrun L (1985) In vitro induction of cytologic functional differentiation of the immature human monocyte cell line U-937 with phorbol myristate acetate. Am J Pathol 119: 111–126

21. Nauwynck HJ, Pensaert MB, Goddeeris B (1992) Replication of virulent Aujeszky's disease virus (ADV) in different blood mononuclear cell subpopulations. In: Proceedings of the 12th Congress of the International Pig Veterinary Society (IPVS), The Hague, The Netherlands, August, pp 63

22. Nauwynck HJ, Pensaert MB (1994) Virus production and viral antigen expression in porcine blood monocytes inoculated with pseudorabies virus. Arch Virol 137: 69–79

23. Nauwynck HJ, Pensaert MB (1995) Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. Arch Virol 140: 1 137–1 146

24. Pescovitz MD, Lunney JK, Sachs DH (1984) Preparation and characterization of monoclonal antibodies reactive with porcine PBL. J Immunol 13: 368–375
25. Plagemann PG, Moennig V (1992) Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv Virus Res 41: 99–192
26. Pol JM, van Dijk JE, Wensvoort G, Terpstra C (1991) Pathological, ultrastructural, and immunohistochemical changes by Lelystad virus in experimentally induced infections of mystery swine disease (synonym: porcine epidemic abortion and respiratory syndrome (PEARS)). Vet Q 13: 137–143
27. Rossow KD, Benfield DA, Goyal SM, Nelson EA, Christopher Hennings J, Collins JE (1996) Chronological immunohistochemical detection and localization of porcine reproductive and respiratory syndrome virus in gnotobiotic pigs. Vet Pathol 33: 551–556
28. Rutherfold MS, Witsell A, Schook LB (1993) Mechanism generating functionally heterogeneous macrophages: chaos revisited. J Leukoc Biol 53: 602–618
29. Shinji H, Akagawa KS, Yoshida T (1994) LPS induces selective translocation of protein kinase C-beta in LPS-responsive mouse macrophages, but not in LPS-non-responsive mouse macrophages. J Immunol 153: 5 760–5 771
30. Voicu IL, Silim A, Morin M, Elazhary MASY (1994) Interaction of porcine reproductive and respiratory syndrome virus with swine monocytes. Vet Rec 134: 422–423
31. Wensvoort G, Terpstra C, Pol JM, ter Laak EA, Bloemraad M, de Kluyver EP, Kragten C, van Buiten L, den Besten A, Wagenaar F, Broekhuijsen JM, Moonen PLJM, Zetstra T, de Boer EA, Tibben HJ, de Jong MF, van Veld P, Groenland GJR, van Gennep JA, Voets MT, Verheijden JHM, Braamskamp J (1991) Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet Q 13: 121–130
32. Wensvoort G, de Kluyver EP, Luijtze EA, den Besten A, Harris L, Collins JE, Christianson WT, Chladek D (1992) Antigenic comparison of Lelystad virus and swine infertility and respiratory syndrome (SIRS) virus. J Vet Diagn Invest 4: 134–138
33. Wu L, Morahan PS, Leary K (1990) Mechanism of intrinsic macrophage-virus interaction "in vitro". Microb Pathog 9: 293–301
34. Yoon IJ, Joo HS, Christianson WT, Morrison RB, Dial GD (1992) Isolation of a cytopathic virus from weak pigs on farms with a history of swine infertility and respiratory syndrome. J Vet Diagn Invest 4: 139–143

Authors' address: Dr. M. B. Pensaert, Laboratory of Veterinary Virology, Faculty of Veterinary Medicine, University of Gent, Salisburylaan 133, B-9820 Merelbeke, Belgium.