Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by porcine reproductive and respiratory syndrome virus

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV), an enveloped, positive-stranded RNA virus that belongs to the family Arteriviridae (Snijder & Meulenberg, 1998). PRRSV has been recognized as one of the major aetiologic agents of porcine respiratory disease complex, which causes a serious health problem in the pig industry worldwide (Halbur, 1998). Although the mechanism(s) by which PRRSV undertake to invade the host immune system is unclear, several studies suggest that PRRSV may negatively modulate the host immune system (reviewed by Lager & Mengeling, 2000; Molitor et al., 1996).

PRRSV is, phenotypically, a highly variable virus. It generally causes a persistent infection and induces a wide range of secondary infections (Wardley et al., 1996). Following an infection, PRRSV persists in the infected pigs for up to 12 weeks and the infectious virus can be shed during this stage (Wills et al., 1997). Although PRRSV is highly contagious, virus replication appears to be limited mainly to phagocytic cell populations, including macrophages and activated monocytes (Molitor et al., 1996). However, proinflammatory cytokines were mostly undetectable or minimally increased following exposure to the virus (Van Reeth & Nauwynck, 2000). In most cases, there is a lack of correlation between the amount of viral antigen and the degree of pathological lesions, suggesting the possibility of immune-mediated pathogenesis rather than a direct effect of virus infection (reviewed by Lager & Mengeling, 2000).

Immune responses to PRRSV have been studied extensively and virus-specific cellular responses, including lymphocyte proliferation, delayed-type hypersensitivity, cytotoxic activity and cytokine production, have been demonstrated in PRRSV-infected pigs. However, there seems to be a delay in the onset of these responses, as compared to other pathogens. Cellular immune responses to PRRSV are not usually detected until 4 weeks after PRRSV infection (Bautista & Molitor, 1997; Lopez Fuertes et al., 1999). In contrast, the cellular immune response to other viruses, such as classical swine fever virus (CSFV), can be detected within a week following virus infection (Suradhat et al., 2001). Although PRRSV induces a strong antibody response within the first week post-infection, neutralizing antibodies are not detected until the fourth week after infection, long after the virus is cleared from circulation (Yoon et al., 1995). Thus, there appears to be a delay in the induction of cell-mediated and humoral immune responses in PRRSV-infected pigs.

Studies with regard to the role of porcine cytokines in immune regulation in pigs have been limited by the lack of porcine cytokine-specific immunological and biological assays. Recently, knowledge of the roles of cytokines in immunopathology and host–pathogen interactions has increased rapidly (Wood & Seow, 1996). RT-PCR has been shown to be a sensitive and effective method for
measuring cytokine mRNA expression in porcine samples (Dozois et al., 1997; Reddy et al., 2000; Thanawongnuwech et al., 2001). The relative levels of cytokine expression can be semi-quantitatively analysed by normalizing the amount of the target mRNA using that of a reference (housekeeping gene) mRNA. Multiplex PCR (MPCR), a variant of PCR in which two or more amplicons can be amplified in the same reaction, is a rapid and reliable way to study the level of cytokine gene expression. In this study, we report the use of MPCR for assessing porcine cytokine gene expression, in particular IFN-γ, IL-10, IL-2 and IL-4. The in vitro effects of PRRSV on cytokine gene expression in porcine peripheral blood mononuclear cells (PBMCs) were examined also.

METHODS

Viruses and cells. A wild-type strain of PRRSV (SVI-275) from the USA and the ALD strain of CSFV were kindly provided by S. Damrongwatanapokin at The National Institute of Animal Health, Bangkok, Thailand. PRRSV stocks were prepared in MARC-145 cells and virus titres were determined as described previously (Thanawongnuwech et al., 1998). CSFV was propagated in a swine kidney cell line, SK-6. Virus titres were determined as described previously (Suradhat et al., 2001). Stock viruses were kept at −80 °C until needed.

Heat inactivation of PRRSV was performed by placing a vial of the stock virus in a 56 °C water bath for at least 8 h, followed by determining infectivity to ensure complete inactivation.

Animals and immunization protocol. In the first experiment, blood samples were collected from three 5-week-old, non-vaccinated, crossbred pigs from a PRRSV-free commercial farm. In the second experiment, blood samples were collected from four crossbred pigs immunized with the lapinized yellow fever strain of PRRSV. Blood samples were collected at 5 and 7 weeks of age, according to the routine vaccination programme on the farm. Blood samples were collected when the pigs were approximately 16 weeks old.

Isolation and in vitro stimulation of porcine PBMCs. Porcine PBMCs were isolated from 10 ml of the heparinized blood samples using Isoprep separation medium (Robbins Scientific Cooperation), according to the manufacturer’s protocol. The freshly isolated PBMCs were resuspended in RPMI 1640 (GibcoBRL) supplemented with 10 % calf serum (Starrate), 2 mM L-glutamine (GibcoBRL), 100 μM non-essential amino acids (GibcoBRL), 1 mM sodium pyruvate (GibcoBRL), 50 μM 2-mercaptoethanol (Sigma), 100 U penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 0-25 μg amphotericin B ml⁻¹ (antibiotic/antimycotic solution was obtained from GibcoBRL). PBMCs were cultured at a concentration of 6×10⁶ cells ml⁻¹ per well of the 24-well plate in the presence of antigen in 5 % CO₂ at 37 °C for 24–48 h, as indicated in the text. The concentration of concanavalin A (ConA, Sigma) used for in vitro culture was 10 μg ml⁻¹. The titres of CSFV and PRRSV used for in vitro stimulation are indicated in the text. Following in vitro stimulation, cells were harvested and washed once with PBS. Cell pellets were kept at −20 °C in the presence of RNAlater (Ambion) until needed.

RNA extraction and reverse transcription. Total RNA was extracted from approximately 2×10⁶ cells using the Nucleospin RNA II kit (Macherey–Nagel), according to the manufacturer’s instructions. Contaminating DNA was removed using the DNase I treatment provided in the kit. At the final step, total RNA from each sample was eluted in 60 μl RNase-free water. Of the total RNA, 10 μl from each sample was reverse-transcribed using the Omniscript RT kit (Qiagen) in a total reaction volume of 20 μl. The RT reaction was carried out in the presence of 0.5 μg random hexamers (Promega) and 40 U ribonuclease inhibitor (RNaseOUT, Invitrogen) at 37 °C for 60 min, followed by heat inactivation at 95 °C for 5 min and rapid cooling on ice.

MPCR. All of the primers were designed specifically for MPCR based on the nearest neighbour analysis of possessing a melting temperature of 60 °C in order to minimize differences in amplification efficiency among the primers during MPCR. The sequences of the primer, the GenBank accession numbers of the reference sequences and the expected sizes of the PCR products are given in Table 1. MPCR was performed in a total reaction volume of 50 μl, consisting of 2 μl cDNA template, 10 μl primer mix (1 μl of each primer; see below), 10 mM dNTPs (Bio Basic), 2.5 U Taq DNA polymerase (HotStarTaq DNA polymerase, Qiagen) and 1-5× concentration of the PCR buffer provided with the enzyme. The amounts of each primer used in MPCR were optimized empirically according to the intensity of the band. The final concentration of the primers was 0.05 μM for GAPDH, 0.2 μM for IL-10 and 0.6 μM for IFN-γ, IL-2 and IL-4.

Cycling parameters were as follows: (1) ‘hot start’ at 95 °C for 15 min; (2) denaturing at 94 °C for 30 s; (3) annealing at 55 °C for 45 s; (4) extension at 72 °C for 45 s; and (5) final extension at 72 °C for 5 min. The number of PCR cycles was optimized to assure that none of the products reached a plateau phase during PCR amplification (data

Table 1. Oligonucleotide sequences designed for MPCR

<table>
<thead>
<tr>
<th>Gene specificity</th>
<th>Oligonucleotide sequences (5’→3’)</th>
<th>GenBank accession no.</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TCCACGCGCAGATTTCA (forward)</td>
<td>AF017079</td>
<td>576</td>
</tr>
<tr>
<td></td>
<td>GCGGTTAGTCCACAA (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>CTTCCGAAAATTTATTACAA (forward)</td>
<td>X53085</td>
<td>503</td>
</tr>
<tr>
<td></td>
<td>GCCTCTGGCCCTGA (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>AGGCACCTTAAGTCTGAGA (forward)</td>
<td>L20001</td>
<td>394</td>
</tr>
<tr>
<td></td>
<td>CGGCTCTGGGACCTTGAA (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>TCTGTGTTGATGATGCTAA (forward)</td>
<td>X56750</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>TGTTAGTGTTGACCTA (reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>GGAGCAAGTGCGACATA (forward)</td>
<td>X68330</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>GCAGTGTTGTTGCTGT (reverse)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
in this experiment, PCR amplification at 33 cycles was applied for further densitometric analysis. Following MPCR, 10 μl of the PCR product was subjected to agarose gel electrophoresis using 2.5 % agarose (Sigma) in 1× TBE buffer (GibcoBRL) in the presence of 0.5 μg ethidium bromide ml⁻¹ (Research Organics). A 100 bp DNA molecular mass marker (GibcoBRL) was run in every gel.

**Quantification of the PCR products.** Images of the MPCR products resolved in ethidium bromide-stained agarose gels were visualized using an UV illuminator and digitally saved by the Photo-print Photodocumentation system (Vilber Lourmat). The images were processed further for quantification of the band by densitometry using the SCION IMAGE software (Scion). The expression level of each product was determined by normalizing its expression against that of the housekeeping gene GAPDH. Results are expressed as the percentage of cytokine expression/GAPDH expression, referred to as percentage expression. When the PBMC populations were considered as a group, values were averaged and the SEM values were calculated and expressed as mean percentage expression ± SEM.

**Verification of the PCR product by sequence analysis.** Each cytokine and GAPDH PCR product was verified by sequencing. DNA fragments were cut from agarose gels and purified using the Nucleospin Extract kit (Macherey–Nagel), according to the manufacturer’s protocol. Purified DNA was used as a template for a cycle sequencing reaction (DNA Sequencing kit, Big Dye Terminator Cycle Sequencing, Applied Biosystems) and subsequently analysed on an ABI Prism 310 Genetic Analyser. The sequence identity was analysed using the NCBI BLAST software.

**Statistical analysis.** All statistical analyses were performed using the GRAPHPAD PRISM software, version 3.00, for Windows.

**RESULTS**

**Primer specificity and MPCR of cytokine mRNA transcripts**

The ability of ConA to induce cytokine expression in pigs has been demonstrated previously (Dozois et al., 1997). Thus, in the preliminary experiment, PBMCs from a naive pig were cultured in the presence of 10 μg ConA ml⁻¹ for 24 h and used as a positive sample for the establishment of MPCR. The newly designed primers were able to amplify a single band of the expected size of porcine GAPDH, IFN-γ, IL-10, IL-2 and IL-4 from the positive sample (Fig. 1a). The specificity of the primers was confirmed by sequence analysis of each amplified product (data not shown). Furthermore, the feasibility of MPCR was demonstrated when combining all the primer sets in a single-tube reaction (Fig. 1b). Increased levels of cytokine gene expression were observed in ConA-stimulated PBMCs, whereas the level of GAPDH mRNA expression remained comparable in both unstimulated and ConA-stimulated populations. It should be noted that in our system, unstimulated cells also produced a considerable amount of cytokine transcripts after the incubation period, as shown in Fig. 1. In addition, the levels of background cytokine expression were somewhat variable among the pigs. Therefore, in order to eliminate this individual variability, the results are expressed as: percentage cytokine gene expression from the stimulated population—percentage cytokine gene expression from the unstimulated (cell only) population.

**In vitro effect of PRRSV on porcine mRNA expression**

Following 24 h of in vitro cultivation with PRRSV, an increase in IL-10 gene expression was the most apparent among the tested cytokine genes. Mock-infected MARC-145 cell lysate did not induce any increase in cytokine gene expression. After 48 h of cultivation, IL-10 gene expression was significantly increased (P<0.05) in the sample cultured in the presence of PRRSV (m.o.i. of 0·01) but not at the lower concentration (m.o.i. of 0·001). In this system, PRRSV had minimal effects on IL-2 and IL-4 gene expression in porcine PBMCs (Fig. 2). Interestingly, heat-inactivated PRRSV also enhanced IL-10 gene expression in the PBMC population, following 24 h of incubation (P<0.05). The effect of heat-inactivated PRRSV on other cytokine expression was not observed (data not shown). The mean percentage of IL-10 expression induced by heat-inactivated PRRSV, at an equivalent volume as that of a dose of an m.o.i. of 0·01 at 24 h, was comparable to that of live PRRSV. However, enhanced IL-10 expression was not observed at 48 h (Fig. 3).

PRRSV titres following cultivation with porcine PBMCs were determined after a 24 and 48 h incubation period by immunoperoxidase monolayer assay. At 24 h, mean virus titres were reduced approximately 2 logs (data not shown) from the starting amount of approximately 6×10⁴ TCID₅₀ ml⁻¹ (m.o.i. of 0·01) and approximately 6×10³ TCID₅₀ ml⁻¹ (m.o.i. of 0·001). However, mean virus titres were elevated to a comparable level at 10⁵⁵ TCID₅₀ ml⁻¹ by 48 h.

The findings that PRRSV could enhance the level of IL-10 expression in porcine PBMCs raised the concern of whether this observation was truly the effect of PRRSV or a bias towards detection of the IL-10 gene by the newly established MPCR technique. In order to verify this, blood
samples from pigs that were primed with a CSFV vaccine (see Methods) were collected and used to assess the effect of PRRSV on antigen-specific immune responses. Following an incubation period of 24 h, the presence of PRRSV considerably increased the level of IL-10 gene expression ($P<0.05$) (Fig. 4a). In fact, the IL-10 gene was the most prominently expressed cytokine compared to others ($P<0.05$). This finding confirmed that the effect of PRRSV on IL-10 gene expression was reproducible even in pigs of different age and immune status. It should be noted that these pigs had never been exposed to or vaccinated with PRRSV; therefore, the enhanced IL-10 expression observed was not an antigen-specific immune response. In contrast, in the presence of recall antigen CSFV, IFN-$\gamma$ gene expression was prominently increased ($P<0.05$), indicating that our MPCR assay was not biased towards only the detection of IL-10 gene expression. Mock-infected SK-6 cell lysate did not induce any significant increase in cytokine gene expression.

Interestingly, when PBMCs from CSFV-primed animals were cultured in the presence of both CSFV and PRRSV, a significant reduction of IFN-$\gamma$ gene expression ($P<0.05$) and enhancement of IL-10 gene expression ($P<0.05$) were observed (Fig. 4a). These findings indicated that the presence of PRRSV in the culture could affect the recall antigen response to CSFV. Minimal effects of both viruses were observed on the levels of IL-2 and IL-4 gene expression (Fig. 4b), similar to the previous experiment.

**DISCUSSION**

In this study, we established the MPCR assay to assess the expression of four porcine cytokine genes and a housekeeping gene, simultaneously. RT-MPCR appeared to be
a rapid, inexpensive and powerful tool to explore the kinetics and expression levels of porcine cytokines from PBMC samples. Furthermore, the use of densitometric analysis allowed an affordable way to semi-quantitatively analyse the amount of mRNA transcripts. It should be noted that unstimulated cells also produced considerable amounts of the cytokine mRNA transcripts, in particular IL-10, following an in vitro culture. This finding was not unexpected, considering that several factors might influence cytokine production in the culture system. The presence of IL-10 transcripts in unstimulated cells had been observed previously (Dozois et al., 1997; Thanawongnuwech et al., 2001) and in some cases, the level was almost at the same level as that of the ConA-stimulated cells (Dozois et al., 1997). Despite the presence of background expression, results from our experiment clearly showed an induction of porcine IL-10 expression by PRRSV and an induction of porcine IFN-γ expression by the recall antigen, CSFV. Although an increase in IL-2 and IL-4 gene expression in porcine PBMCs after ConA stimulation was reported previously (Dozois et al., 1997), the changes in IL-2 and IL-4 gene expression were minimal in our study. Differences in primer sensitivity and the housekeeping gene used in the assay are likely to be the cause of this discrepancy. Nevertheless, our results were consistent with the previous observation, using quantitative RT-PCR, in which several mitogens, including lipopolysaccharide, phytohaemagglutinin, hen egg white lysozyme and purified protein derivative of tuberculin, did not induce any detectable level of IL-2 and IL-4 gene expression in porcine efferent lymph leukocytes. These results imply that pigs may not utilize these cytokines, as has been shown in humans and mice (Reddy et al., 2000).

Interestingly, both live and heat-inactivated PRRSV could significantly upregulate IL-10 gene expression in cultured PBMCs, in particular, at the dose of an m.o.i. of 0·01 (or equivalent), following 24 h of cultivation (Figs 2 and 3). The effect of heat-inactivated PRRSV reduced almost 10 times in PBMCs, cultured with one-tenth of the volume of stock virus that was equivalent to an m.o.i. of 0·01. In addition, the induction of IL-10 gene expression by heat-inactivated PRRSV was not seen at 48 h. These observations suggested that the increased IL-10 expression during the first 24 h of incubation was due to binding of virion protein(s) to the cells, regardless of virus infectivity. At 48 h, IL-10 gene expression in PBMCs cultured in the presence of live PRRSV continued to increase (Fig. 2). This finding was likely due to the ability of PRRSV to replicate in adherent monocytes. Although PRRSV has been reported to have limited cell tropism, several groups have reported that blood monocytes become permissive to PRRSV replication following 24 h of cultivation (Duan et al., 1997; Voicu et al., 1994). Following an incubation period of 48 h, the PRRSV titres obtained from both 0·01 and 0·001 m.o.i.-inoculated cultures were similar; however, enhanced IL-10 expression was only observed in PBMCs inoculated with live PRRSV (m.o.i. of 0·01). The reason for this is not clearly known. The comparable virus yield from both inoculated doses was likely due to the limited number of permissive cells. However, there might be more virion protein, as opposed to infectious virus particles, produced in the culture inoculated with the higher m.o.i., resulting in more signal for IL-10 expression.

The observation that PRRSV upregulated IL-10 expression in vitro is intriguing. There is at least one report describing an increase in IL-10 mRNA expression from PBMCs of piglets born from an infected sow (Feng et al., 2000). IL-10 has been known to be a potent cytokine capable of downregulating host immune responses. The exploitation of IL-10 appears to be the common mechanism of immunosuppression by several intracellular pathogens that specifically target macrophages for infection. Certain viruses induce IL-10 production, whereas others encode their own IL-10 to inhibit the host immune response and to hamper the process of virus clearance (reviewed by Fickenscher et al., 2002; Redpath et al., 2001). Considering the restricted tissue tropism of PRRSV, it is conceivable that PRRSV also uses IL-10 for suppressing the host immune response. Induction of IL-10 production at an early stage of infection may enhance virus survival within the host and delay the induction of protective immunity.

IL-10 is known to inhibit the production of several pro-inflammatory cytokines (Abbas et al., 2000). The inhibitory effects of IL-10 on IL-1 and TNF production are crucial for its anti-inflammatory activities (Moore et al., 2001). A series of reports on the kinetics of proinflammatory cytokine responses following PRRSV infection seems to support the role of IL-10 in PRRSV infection. Following PRRSV infection, production of TNF-α was almost undetectable (Van Reeth et al., 1999). Furthermore, the production of IFN-α was significantly suppressed in both PBMCs and alveolar macrophages. This inhibitory effect was not due to cell death (Albina et al., 1998). The poor cytokine response was consistent with the overall mild clinical course and minimal gross lung pathology following PRRSV infection (Van Reeth et al., 1999; Van Reeth & Nauwynck, 2000). Together, these findings support the notion that PRRSV may interfere with the overall function of the macrophage-like cells through the induction of immunosuppressive factors, such as IL-10, resulting in an alteration of the cascade of proinflammatory cytokine production. In addition, an increased incidence of secondary infections in the lung following an episode of PRRSV infection may also relate to the immunomodulatory effect of IL-10, apart from the direct effect of PRRSV on monocytes/macrophages. Moreover, an inhibitory effect of IL-10 on the functions of antigen-presenting cells (APCs) could contribute to the delayed induction of both arms of the protective immune response to PRRSV, which tend to develop long after the active phase of virus replication and viraemia.

In our previous work, strong, CSFV-specific IFN-γ production was detected from the PBMCs of primed animals...
by an ELISPOT assay (Suradhat & Damrongwatanapokin, 2000; Suradhat et al., 2001). Our observations that PRRSV could alter the cytokine profile in the recall antigen response supported the immunosuppressive role of PRRSV. It is well established that IL-10 strongly inhibits cytokine production and proliferation of CD4+ cells, particularly the T helper cell (Th1) population, resulting in an inhibition of cell-mediated immune responses (Moore et al., 2001). The observed reduction of IFN-γ gene expression was likely related to the effect of PRRSV-induced IL-10 on APCs and/or T cell functions. Consistent with our finding, alteration of the magnitude and delayed T cell responses to pseudorabies virus vaccine in PRRSV-infected pigs has been observed previously (De Bruin et al., 2000). Furthermore, infection or vaccination with PRRSV appears to decrease the efficacy of Mycoplasma hyopneumoniae bacterin in M. hyopneumoniae-challenged pigs (Thacker et al., 2000). Inhibition of the memory Th cell and/or effector cell functions by PRRSV-induced IL-10 could be one of the explanations, as cell-mediated immunity is believed to play a significant role in respiratory defence mechanisms (Dunkley et al., 1995). Together, these findings highlight the role of PRRSV in interfering with the cell-mediated immune response in the infected host.

In summary, our results imply that the induction of IL-10 production may be one of the strategies used by PRRSV to regulate the host immune system. It would be interesting to explore if this phenomenon occurs in vivo following PRRSV infection and what is the mechanism of virus-induced IL-10 production. Knowledge in regard to the effect of PRRSV on the host immune system would be crucial for the development of an effective control strategy and in designing a safe and effective PRRS vaccine in the future.

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