รายงานสั้น

CYTOKINE PROFILES FOLLOWING MYCOPLASMA HYOPNEUMONIAE AND PRRSV CO-INFECTION IN PIGS

Roongroje Thanawongnuwech[1*] Eileen L. Thacker[2]

Abstract

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Bronchoalveolar cells collected by lung lavage 28 days post infection (DPI) of negative controls, *Mycoplasma hyopneumoniae (M. hyo)*-infected, Porcine Respiratory and Reproductive Syndrome virus (PRRSV)-infected and dual-infected pigs, were evaluated for various cytokine mRNA (IL1α, IL1β, IL6, IL8, IL10, IL12, and TNFα). Semiquantitative RT-PCR revealed that IL1β, IL6, IL10 and the IL12 mRNA expression was significantly increased in dual-infected pigs, compared with the negative controls. PRRSV-infected pigs had significantly increased levels of IL10 and IL12 mRNA. The increased IL10 gene expression that was observed in the dual infected pigs may play a role in the potentiation of PRRSV-induced pneumonia by *M. hyo*. These results suggest that increased mRNA levels of proinflammatory cytokine gene expression may be related to the increased severity of clinical disease and pneumonia, especially in the dual infected pigs and that the reciprocal regulation of IL10 and IL12 genes may explain, in part, the prolonged observed persistence of PRRSV infection in pigs that are simultaneously infected with *M. hyo*.

Keywords : *Mycoplasma hyopneumoniae*, PRRSV, macrophages, cytokines, pigs

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บทคัดย่อ

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รูปแบบไซโตไคน์หลังจากได้รับเชื้อมัยโคพลาสม่า ร่วมกับ ไวรัส พี อาร์ อาร์ เอส ในสุกร

ทำการเก็บเซลล์จากการล้างปอดของสุกรควบคุม และสุกรที่ได้รับเชื้อมัยโคพลาสม่า ไฮโอนิวโมนิอี้ ไวรัสพี อาร์ อาร์ เอส หรือทั้งสองชนิดที่ 28 วันหลังได้รับเชื้อเพื่อทำการทดสอบหาปริมาณmRNAของไซโตไคน์ต่อไปนี้ IL1α, IL1β, IL6, IL8, IL10, IL12 และTNFα โดยวิธี semiquantitative RT-PCR พบว่าปริมาณ mRNA ของ IL1β, IL6, IL10 และ IL12 เพิ่มขึ้นอย่างมีนัยสำคัญในสุกรที่รับเชื้อสองชนิดเทียบกับสุกรในกลุ่มควบคุม สุกรที่รับเชื้อไวรัสพี อาร์ อาร์ เอสพบปริมาณ mRNA ของ IL10 และ IL12 เพิ่มขึ้นอย่างมีนัยสำคัญ การเพิ่มการสร้าง mRNA ของ IL10 ในสุกรที่ได้รับเชื้อสองชนิดอาจมีผลทำให้รอยโรคที่ถูกเหนี่ยวนำโดยไวรัสพี อาร์ อาร์ เอส รุนแรงขึ้นจากการติดเชื้อมัยโคพลาสม่า ซึ่งผลจากการศึกษาชี้นำว่าการเพิ่มระดับ mRNA ของ proinflammatory cytokines อาจมีผลเกี่ยวข้องกับการเพิ่มความรุนแรงทางคลินิก และรอยโรคปอด โดยเฉพาะในกลุ่มสุกรที่ได้รับเชื้อทั้งสองชนิด การออกฤทธิ์ในทางตรงกันข้ามของ IL10 และ IL12 สามารถอธิบายการติดเชื้อแบบเรื้อรังที่พบในโรคพี อาร์ อาร์ เอส โดยเฉพาะในสุกรที่ติดเชื้อร่วมกับมัยโคพลาสม่า

คำสำคัญ : มัยโคพลาสม่า ไฮโอนิวโมนิอี้ ไวรัสพี อาร์ อาร์ เอส แมคโครฟาจ ไซโตไคน์ สุกร

Introduction

Cytokines participate in many physiological processes including the regulation of immune and inflammatory responses. Cytokines produced by bronchoalveolar cells, particularly pulmonary alveolar macrophages(PAMs) are particularly important for controlling the amplitude and duration of inflammatory responses in the lung. PAMs uniquely interact with infectious and non-infectious agents that enter the lung. Conventionally reared pigs are commonly exposed to a wide variety of infectious agents including *Mycoplasma hyopneumoniae* (*M. hyo*), Porcine Reproductive and Respiratory Syndrome virus (PRRSV) and Swine Influenza virus (SIV), which are important pathogens of the Porcine Respiratory Disease Complex (PRDC). Proinflammatory cytokines have been found to be important mediators of viral respiratory diseases (Van Reeth et al., 1999). Interferon (IFN) α, interleukin (IL) 1 and tumor necrosis factor (TNF) α are the first cytokines to increase following infection with Swine Influenza virus (Van Reeth et al., 1999). PRRSV has been reported to elevate IL1 levels (Van Reeth et al., 1999) and alter macrophage gene expression including an ubiquitinated protein degradation pathway, which is thought to play an important role in clinical PRRSV infection (Zhang et al., 1999). *M. hyo* has been reported to induce the production of several proinflammatory cytokines including IL1, TNFα (Asai et al., 1993) and IL6 (Asai et al., 1994). Our *M. hyo*-PRRSV coinfection model demonstrated that infection with *M. hyo* potentiated both the severity and the duration of PRRSV-induced pneumonia in pigs (Thacker et al., 1999). However, the mechanisms by which the two organisms induce proinflammatory cytokines and the roles these cytokines play in the potentiation of PRRSV-induced pneumonia remain to be defined.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) has been used to compare cytokine mRNA expression from pulmonary alveolar mac-

rophages (PAMs) in pigs infected with *M. hyo*, PRRSV or both, as previously described (Thanawongnuwech et al., 2001). The objectives of this study were to investigate the potential involvement of selected cytokines (IL1α, IL1β, IL6, IL8, IL10, IL12 and TNFα) in the generation of inflammatory and immune responses following *M. hyo*-PRRSV coinfection.

Materials and Methods

Experimental animals

PRRSV- and *M. hyo*-free crossbred pigs were obtained from a commercial gilt-multiplier herd when 10-12 days of age. Pigs were randomly assigned to 4 groups (n = 6 /group) consisting of negative controls, *M. hyo*-infected, PRRSV-infected or dual-infected pigs. The pigs were 5-weeks-old when inoculated intranasally with a 10^5 tissue culture infective dose $(TCID)_{50}$ /5 ml of a high virulent PRRSV(VR-2385) or with 5 ml of *M. hyo* strain 232, a derivative of strain 11 (10^5 color changing units/ml) or both in the appropriate groups, on the same day. Pigs were examined daily and euthanized 28 days postinoculation when lung scoring for PRRSV-induced pneumonia or *M. hyo*-induced pneumonia was performed, as previously described (Thacker et al., 1999). Bronchoalveolar cells, mainly PAMs (over 85%), were harvested using bronchoalveolar lavage and kept in RNAlater™ (Ambion, Austin, TX) until tested.

Total RNA isolation and RT-PCR quantification

Total RNA was isolated from $1x10^7$ BAL cells/pig using E.Z.N.A.® Total RNA kit (Omega Biotek, Doraville, GA) according to the companies procedures. The quality and quantity of the total RNA yield was measured as previously described (Thanawongnuwech et al., 2001) and kept at -20°C until needed. RT-PCR was performed to measure IL1α, IL1β, IL6, IL8, IL10, IL12, TNFα and cyclophilin (a housekeeping gene) using the Access RT-PCR System (Promega, Madison, WI). The procedure and the primers used were similar to a previous study (Thanawongnuwech et al., 2001). RT-PCR product intensity, as a percent of cyclophilin intensity, was quantitated by AlphaEase™ spot densitometry (Alpha Innotech Corp., San Leandro, CA).

Statistical analysis

Data were subjected to an analysis of variance (ANOVA). If the p value from the ANOVA was less than or equal to 0.05, pairwise comparisons of the different treatment groups were performed by calculating the least significant difference at the $p<0.05$ rejection level.

Results and discussion

Dyspnea and fever were observed in the PRRSV-infected pigs. Macroscopic and microscopic examination confirmed the presence of PRRSV-induced pneumonia. The percentage of lung with macroscopic lesions, attributable to *M. hyo*- and PRRSV, was greater in dual-infected pigs than in the single infection pigs (Table 1), as previously reported (Thacker et al., 1999). Levels of mRNA expression from the PAMs are summarized in Figure 1. Pigs infected with both *M. hyo* and PRRSV showed significantly increased mRNA expression of IL1β, IL6, IL10, and IL12 at 28 DPI (p <0.05). Pigs infected with PRRSV had significantly increased levels of IL12 compared to control pigs (p <0.05). Interestingly, the IL6 and IL10 mRNA expression in PRRSV-infected pigs tended to be increased, although not significantly so conpared with the control group. Levels of the IL10 and IL12 were significantly higher in PRRSV-infected and dual infected pigs, compared to *M. hyo*-infected pigs. There was no significant difference in IL1α, IL8, and TNFα mRNA expression between the groups, although, these cytokines tended to increase in all infected groups.

In the study reported here, the mRNA expression of IL1β and IL6 was significantly increased in the dual-infected pigs. PRRSV infection has been reported to induce the production of IL1 but not TNFα or IFNα at 10 DPI (Van Reeth et al., 1999). IL1 (is known to be a potent inducer of IL6 production (Zoja et al., 1991). In previous studies of pneumonia and disease induced by *Actinobacillus*

pleuropneumoniae (APP), the appearance of IL1, TNFα (Huang et al., 1999) and IL6 (Fossum et al., 1998) coincided with the onset of clinical signs and increased body temperatures. When treated with antibiotics, APP-infected pigs recovered and systemic IL6 levels returned to normal (Fossum et al., 1998). These significantly increased levels of the proinflammatory cytokines in the *M. hyo*-PRRSV-infected pigs, acting in concert with inflammatory mediators, may be responsible for the increased and prolonged pulmonary lesions and the clinical signs repeatedly observed in several experiments conducted in our laboratory.

In contrast to previous reports (Asai et al., 1993; Asai et al., 1994), the upregulation of TNFα mRNA expression was not significantly increased in *M. Hyo*-only, infected pigs. It has been demonstrated that TNFα is detectable only at the onset of acute manifestation of LPS stimulation both *in vivo* (Michie et al., 1988) and *in vitro* (Vezina et al., 1995). The increased level of TNFα may have passed the peak level when tested at 28 DPI.

Levels of IL10 and IL12 mRNA were significantly increased in dual infected pigs. An *in vitro* study conducted in our laboratory (Thanawongnuwech et al., 2001) demonstrated significantly increased mRNA expression of IL1α, IL1β, IL8, and TNFα, but not IL10 or IL12, from the bronchoalveolar cells cultured with the two organisms 24 hours post infection. Unlike the *in vitro* study, the microenvironment *in vivo* consists of many cell types and mediators, which may effect cytokine production by PAMs or other bronchoalveolar cells and may influence the level of PRRSV replication.

During a normal immune response, both the Th1 and Th2 cell types are involved in immunoregulation (Visser et al., 1998). IL12 regulates cell-mediated immune responses by a positive feedback mechanism, mediated by Th1 cells through IFNα or by a negative feedback through Th2 cells secreting IL10 (Aste-Amezaga et al., 1998; Visser et al., 1998). IL10 inhibits the activation of macrophages, T cells and NK cells and the production of many proinflammatory cytokines(Moore et al., 1993). The induction of both IL10 and IL12 mRNA may serve to balance the inflammatory and immune response in PRRSV-infected pigs. Alterations in the balance of IL10 and IL12 production may influence both accessory and effector macrophage functions.

Apoptosis induced by acute PRRSV infection (Sirinarumitr et al., 1998) is believed to be a defense mechanism used by host cells to reduce viral replication. IL10 has been reported to rescue cells from apotosis (Visser et al., 1998), thus providing susceptible macrophages for PRRSV persistency.

The resolution of PRRSV-induced lesions may be due to the increased production of IL12 and suppression of the further production of proinflammatory cytokines by IL10. Increased IL10 production observed in the dual-infected pigs may play a role in PRRSV persistence as mentioned above. Understanding the reciprocal regulation of IL12 and IL10 may provide insight into the regulation of cellular and humoral immune responses and form the basis for future PRRSV vaccination strategies and therapeutic interventions. In conclusion, these results suggest that increased mRNA levels of proinflammatory cytokines may be related to the increased severity and duration of the clinical signs and pneumonic lesions in the dual-infected pigs, while the reciprocal relationship between IL10 and IL12, may provide partial answers to PRRSV resolution and persistence.

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Table 1 Percent of gross lung lesions in pigs necropsied 28 DPI*

	Treatment Groups			
Gross lung lesions	**Control**	***M. hyo***	**PRRSV**	**Dual**
M. hyo-induced	0.24 ± 0.34[a]	5.03 ± 3.31b	0.01 ± 0.02[a]	11.98 ± 9.44[b]
PRRSV-induced	0 ± 0a	1 ± 1.67[a]	6.67 ± 12.82[b]	23.33 ± 12.66[c]

*Data presented as the mean ± SD, six pigs per group

[a,b,c]Within each row, values with different superscripts are significantly different ($p < 0.05$)

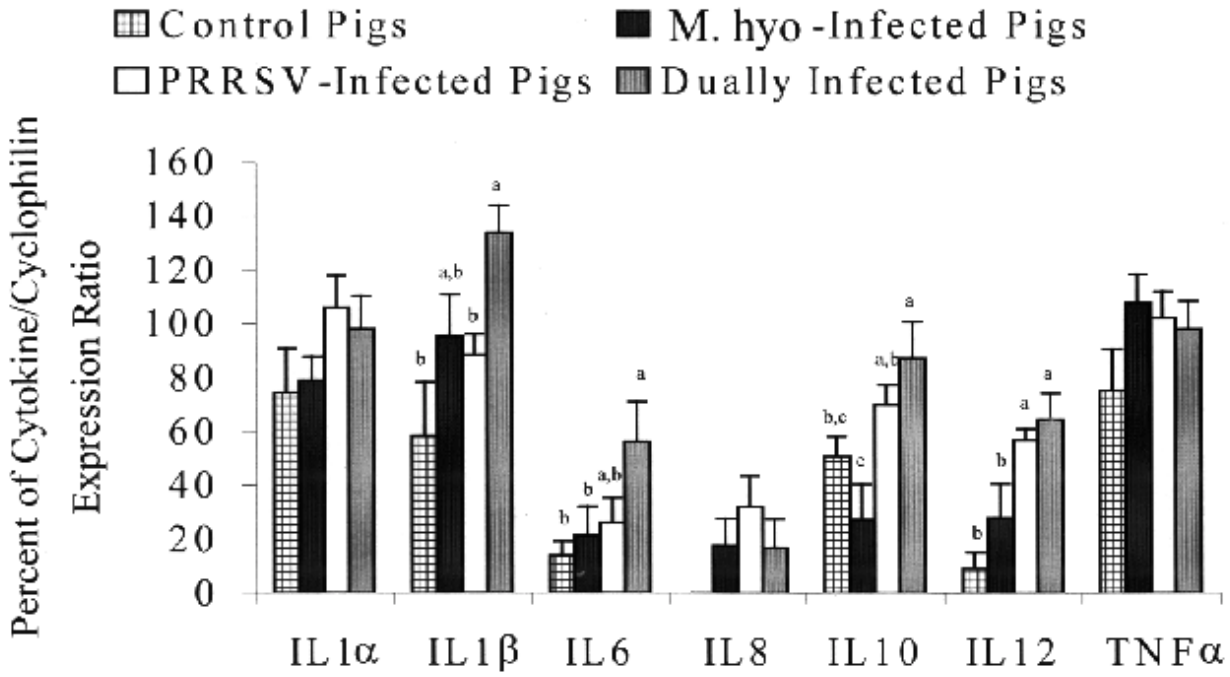

FIGURE 1 Relative mRNA levels from PAMs are presented as a percent of the ratio of cytokine/Cyclophilin RT-PCR product band intensity. Data are the means ± SEM from 6 pigs.

[a,b,c] Within each cytokine, values with different superscripts are significantly different ($p < 0.05$)

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