Increased Production of Proinflammatory Cytokines following Infection with Porcine Reproductive and Respiratory Syndrome Virus and Mycoplasma hyopneumoniae

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Received 3 December 2003/Returned for modification 6 April 2004/Accepted 2 June 2004

Porcine respiratory disease complex (PRDC) remains an important economic problem for swine producers worldwide. PRDC is characterized by slow growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea in finishing pigs. PRDC is multifactorial, as several viral and bacterial pathogens are typically detected. Porcine reproductive and respiratory syndrome virus (PRRSV) and/or Mycoplasma hyopneumoniae are commonly isolated from pigs with clinical signs consistent with PRDC, and despite the use of intervention strategies that include vaccines and antibiotics, control of PRDC is inconsistent at best.

Proinflammatory cytokines are believed to play an important role in porcine respiratory disease by coordinating and activating the adaptive immune response, which enables the host to eliminate offending pathogens (14, 21, 33, 34). However, if cytokine levels become excessive, tissue damage and even death of the host can occur. Therefore, determination of both the presence and quantity of proinflammatory cytokines can lead to an increased understanding of the pathogenesis of disease and the corresponding host’s immune response.

Production of proinflammatory cytokines has been shown to be associated with the development of M. hyopneumoniae-induced pneumonia. Increased levels of interleukin-1 (IL-1) (α and β), IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor alpha (TNF-α) in bronchoalveolar lavage (BAL) fluid have been reported in M. hyopneumoniae-infected pigs (1, 2, 30). Respiratory viruses also induce proinflammatory cytokine production, although cytokine levels and the clinical manifestations of pneumonia can vary by virus (33). For example, the levels of IL-1 and TNF-α were lower in BAL fluid from pigs infected with PRRSV than in BAL fluid from pigs infected with swine influenza virus (33). This finding may provide a possible mechanism for the prolonged persistence of PRRSV following infection (38).

Previously, we demonstrated (27) that the presence of M. hyopneumoniae significantly increased the severity and duration of pneumonia, consistent with PRRSV infection in pigs. The mechanism used by M. hyopneumoniae to enhance the viral pneumonia is unknown; however, the induction of proinflammatory cytokines by M. hyopneumoniae may play an important role. The cytokine response to pathogens in pigs has been poorly defined and has been limited by the lack of immunological and biological assays for porcine cytokines. The availability of reagents has increased dramatically in the past several years, and a number of studies have been performed to...
identify the cytokines produced by swine macrophages and monocytes (10, 35).

The goals of the study reported here were to investigate the levels of mRNA for select proinflammatory cytokines to begin to obtain a further understanding of the pathogenesis of the disease induced by these problem pathogens. Previous studies have demonstrated that each pathogen induces the production of the proinflammatory cytokines in different ways. The purpose of this study was to attempt to begin to investigate the interaction between these two pathogens at the molecular level by assessing their impacts on proinflammatory cytokines individually and together by using both in vitro and in vivo models. The cytokines measured in pigs experimentally inoculated with PRRSV and/or M. hyopneumoniae and necropsied at 10, 28, and 42 days postinfection (dpi) included IL-1 (α and β), IL-6, IL-8, IL-10, IL-12, and TNF-α. The levels of the IL-1β, IL-8, IL-10, and TNF-α proteins were also measured. In addition, the same proinflammatory cytokines produced by pulmonary alveolar macrophages (PAMs) infected or not infected with PRRSV and incubated with tracheal explants with or without M. hyopneumoniae infection were measured at 6 and 15 h postinfection (hpi) by an in vitro assay. Both the in vivo and the in vitro models described in this study have previously proven useful in investigating the proinflammatory cytokine responses by PAMs to infection with PRRSV and/or M. hyopneumoniae (31, 32). In an earlier study that measured in vitro cytokine production by PAMs at 24 and 48 hpi in pigs infected with PRRSV and/or M. hyopneumoniae, the differences in various proinflammatory cytokine levels were observed at 24 hpi. However, by 48 hpi the viabilities of PRRSV-infected PAMs were low, making accurate measurement of cytokine levels difficult.

Infection with both pathogens significantly increased the levels of the majority of the cytokines in both the in vivo and the in vitro models, suggesting that inflammation is important for inducing the lung lesions observed following coinfection with these two pathogens. Specifically, we found enhanced production of IL-10, a cytokine associated with a T-helper 2 (Th2) type of immune response, which may provide an explanation for the persistence of PRRSV in the presence of M. hyopneumoniae. Interestingly, the levels of IL-12, a cytokine known to induce the production of gamma interferon (IFN-γ), remained elevated up to 42 dpi in the in vivo trial and throughout the in vitro sampling time period. M. hyopneumoniae infection both in vitro and in vivo appeared to stimulate the production of more inflammatory cytokines than PRRSV. However, cytokine levels were often further increased in the presence of both pathogens. The results of this study provide additional information on the temporal production of proinflammatory cytokines by the lungs in response to respiratory pathogens by using both in vivo and in vitro models. This information will aid in understanding the pathogenesis of M. hyopneumoniae and PRRSV and their effects on the respiratory immune system.

MATERIALS AND METHODS

Animals and experimental design. For the in vivo study, 70 crossbred pigs (landrace, large white, and duroc; age, 10 to 12 days) serologically negative for PRRSV and M. hyopneumoniae were obtained from a commercial herd. The pigs were randomly assigned to four treatment groups with stratification by their weight on arrival. The treatment groups consisted of an uninoculated negative control group, M. hyopneumoniae-inoculated pigs, PRRSV-inoculated pigs, and pigs inoculated with both M. hyopneumoniae and PRRSV. Pigs within each group were randomly assigned to one of three necropsy dates at 10, 28, and 42 dpi. An additional six pigs (age, 7 weeks) from the same herd were used as donors for the tracheal explant study. Pigs were provided with commercial diets and water ad libitum throughout the study. All animal-related procedures were conducted in accordance with the guidelines of the Iowa State University Institutional Committee on Animal Care and Use.

Challenge inocula. For the in vivo study, pigs in the appropriate groups were inoculated intranasally with 10³ 50% tissue culture infective doses (TCID₅₀) of a hog epidemic PRRSV strain (ATCC VR-238) per 5 ml and/or M. hyopneumoniae strain 232, a derivative of strain 11, per 10 ml (at 10³ color-changing units/ml) on the same day at 6 weeks of age, as described previously (27).

M. hyopneumoniae strain 91.3 (5 × 10³ color changing units/ml), a highly adherent strain originally cloned from strain 232, was used to inoculate the tracheal rings in the in vitro study (39). In the in vitro study, PAMs from the appropriate groups were inoculated at a multiplicity of infection of 0.01 with the same PRRSV isolate used in the in vitro study.

Clinical evaluation and pathological examination. The pigs were evaluated daily for a minimum of 15 min for signs of clinical disease, including appetite, cough, increased respiratory rate, dyspnea, or behavioral changes. Rectal temperatures and a clinical respiratory score were obtained daily from 0 to 10 dpi and every other day from 11 to 20 dpi, as described previously (12). Pigs from each group were necropsied at 10, 28, or 42 dpi, as described previously (27). Bronchoalveolar lavages were collected aseptically from the left lobe for the isolation of M. hyopneumoniae and other bacterial pathogens. Macroscopic lesions consistent with mycoplasmal pneumonia (dark red to purple consolidated areas) were sketched on a standard lung diagram, and the proportion of the lung surface with lesions was calculated from the diagram by using a Zeiss SEM-IPS image-analyzing system (24). Pneumonia consistent with PRRSV was characterized by parenchyma that was mottled tan and rubbery in consistency and failure to collapse and was scored by a previously developed system based on the approximate volume that each lobe contributes to the entire lung: the right cranial lobe, the right middle lobe, the cranial part of the left cranial lobe, and the caudal part of the left cranial lobe each contributes 10% of the total lung volume; the accessory lobe contributes 5%; and the right and left caudal lobes each contributes 27.5% (12). These scores were then used to determine the total lung lesion score on the basis of the relative contribution of each lobe.

BAL was performed to collect epithelial lining fluid and bronchoalveolar cells, primarily consisting of PAMs (more than 85%), as described previously (20). BAL fluid and cells were collected by flushing the lungs with phosphate-buffered saline (PBS) containing 75 U of penicillin per ml and 75 μg of streptomycin (both from Sigma, St. Louis, Mo.) per ml. The BAL fluid was centrifuged, and the pelleted cells were washed twice with PBS. The pellet, which primarily consisted of PAMs, was resuspended in complete medium consisting of RPMI 1640 (Sigma) containing 5% fetal bovine serum, 75 U of penicillin per ml, and 75 μg of streptomycin per ml. The cells were counted, and the viability was determined with trypan blue dye. PAMs were kept in RNAlater (Ambion, Austin, Tex.) until they were tested.

Experimental design of in vitro study with tracheal rings and PAMs. The tracheas were removed aseptically from the six additional pigs and submerged in sterile chilled PBS. The tracheas were washed, and transverse sections (thickness, approximately 0.5 cm) were made by making an incision between the tracheal rings. Each tracheal ring was placed into a 30-mm culture plate insert (Millipore, Bedford, Mass.) containing 3 ml of complete medium. PAMs (approximately 5 × 10⁶ cells) were reconstituted in 3 ml of complete medium and placed in a six-well plate for 2 h at 37°C in 5% CO₂ prior to inoculation with PRRSV. PAMs were collected, and assays were performed at 6 and 15 hpi with PRRSV. All tissue culture media and supplements were confirmed to be endotoxin free by standard assays. The tracheal explants and PAMs were cultured overnight at 37°C in 5% CO₂ and experiments were performed on the next day.

The experimental design is summarized in Table 1 and consisted of the same treatments described previously (32). Cells and tracheal explants from each of the donor pigs were subjected to all treatments. M. hyopneumoniae was inoculated onto the tracheal rings of the appropriate groups 6 h prior to their addition to the PAMs. PAMs from the PRRSV-infected groups were inoculated with PRRSV 1 h prior to the addition of the tracheal rings. The tracheal ring inserts and their supernatants were transferred to the cultured PAMs, and the mixture was incubated at 37°C in 5% CO₂ for 6 or 15 hpi prior to harvesting of the PAMs and the supernatants. The supernatants from the tracheal cultures were decanted through the insert over the PAMs so no cell-to-cell contact between the tracheal rings and PAMs occurred. PAMs harvested at 6 and 15 hpi were placed in
TABLE 1. Experimental design of in vitro experiment

| Group                      | PAMs | Tracheal explant | M. hyopneumoniae |
|----------------------------|------|------------------|-----------------|
| Control                    | +    | +                | -               |
| M. hyopneumoniae           | +    | +                | +               |
| PRRSV                      | +    | +                | -               |
| M. hyopneumoniae and PRRSV | +    | +                | +               |

RNA Later (Ambion), according to the directions of the manufacturer, and were stored at 4°C until they were assayed.

Total RNA isolation, RT-PCR, and densitometric quantification of RT-PCR products. Total RNA isolation, the primers used, and reverse transcription-PCR (RT-PCR) procedures were performed with the PAMs collected by BAL from the in vivo study and the cultured PAMs from the in vitro study, as described previously (31, 32). RT-PCR products were run in parallel with each assay, according to the instructions of the manufacturer.

TABLE 2. Summary of macroscopic lung lesions from pigs necropsied at 10, 28, and 42 dpi with either M. hyopneumoniae or PRRSV

| Source of pneumonia and dpi | % of lung with visible lesions on necropsy at indicated dpi |
|----------------------------|---------------------------------------------------------|
| M. hyopneumoniae 10        | 0.02 ± 0.05A                                           |
| M. hyopneumoniae 28        | 0.0 ± 0.0A                                              |
| M. hyopneumoniae 42        | 0.17 ± 0.24A                                            |
| M. hyopneumoniae and PRRSV 10 | 0.0 ± 0.0A                                           |
| M. hyopneumoniae and PRRSV 28 | 0.0 ± 0.0A                                            |
| M. hyopneumoniae and PRRSV 42 | 0.0 ± 0.0A                                            |

As estimated by visual observation.
M. hyopneumoniae.

between inoculation groups. Values in rows with different letters (A, B, or C) are signifi-
cantly different (P < 0.001). M. hyo,
M. hyopneumoniae.

mRNA for all proinflammatory cytokines measured except
IL-8 were significantly increased in pigs inoculated with either
or both pathogens. IL-8 mRNA levels were no longer elevated
in the pigs infected only with PRRSV. The levels of mRNA for
IL-1β, IL-10, and IL-12 were significantly higher in both
groups of pigs infected with M. hyopneumoniae than in the pigs
infected only with PRRSV. No significant difference in the
levels of IL-1α, IL-6, and TNF-α mRNA expression between
the infected groups was observed at 28 dpi. By 42 dpi, the levels
of mRNA for IL-8 and IL-12 remained elevated in the group
infected with both pathogens. Interestingly, the levels of
mRNA for IL-10 and IL-12 remained increased in the group
infected only with PRRSV. Otherwise, the levels of mRNA for
all other cytokines had returned to those in the control pigs.

The background levels of all cytokines in all groups appeared
to be increased at 28 dpi, as shown in Table 3. Since all assays
were performed at the same time, it is unknown if this is a
normal occurrence associated with the maturation of pigs.

Effect of in vitro infection with M. hyopneumoniae and PRRSV
on cytokine RT-PCR products. PAMs harvested from BAL
fluid yielded more than 90% macrophages with 98% viability
following collection and centrifugation. At 6 hpi, PAMs from
the infected group with both M. hyopneumoniae and PRRSV
expressed significantly higher levels of mRNA for IL-1α, IL-
1β, IL-12, and TNF-α than the noninfected control group
(Table 4). Only data for the cytokines with levels that were
significantly different between groups are shown in Table 4.

The PAMs infected with only PRRSV had signifi-
cantly different between inoculation groups. Values in rows with different letters (A, B, or C) are significantly different (P < 0.001). M. hyo, M. hyopneumoniae.

| mRNA expression between inoculation groups | Significance |
|------------------------------------------|--------------|
| Controls compared to infected groups      | (P < 0.001)  |

**TABLE 3. Average mRNA levels from PAMs from in vivo study of pigs at 10, 28, and 42 dpi with PRRSV and/or M. hyopneumoniae**

| dpi and cytokine | Control | M. hyo | PRRSV | PRRSV and M. hyo |
|-----------------|---------|--------|-------|------------------|
| **10 dpi**      |         |        |       |                  |
| IL-1β           | 27.54 ± 17.34A | 55.50 ± 30.07B,C | 39.66 ± 10.88A,B | 67.42 ± 20.56C |
| IL-8            | 48.60 ± 38.89A | 56.49 ± 29.31A | 30.76 ± 35.87A | 101.17 ± 40.49B |
| IL-10           | 0.0 ± 0.0A | 0.0 ± 0.0A | 20.12 ± 17.55B | 34.99 ± 23.40B |
| IL-12           | 5.77 ± 14.13A | 9.63 ± 10.58B | 28.39 ± 16.99B | 47.52 ± 15.58C |
| TNF-α           | 47.94 ± 22.54A,B | 30.17 ± 20.87A | 41.24 ± 22.79A | 65.93 ± 10.43B |
| **28 dpi**      |         |        |       |                  |
| IL-1α           | 27.60 ± 14.55A | 100.11 ± 22.99B | 79.51 ± 7.07B | 90.46 ± 21.96B |
| IL-1β           | 41.16 ± 9.14A | 91.92 ± 28.75B,C | 76.52 ± 10.07B | 102.59 ± 12.90C |
| IL-6            | 0.0 ± 0.0A | 21.56 ± 11.45B | 22.46 ± 8.21B | 29.11 ± 16.31B |
| IL-8            | 18.80 ± 10.37A | 57.79 ± 22.88B | 28.95 ± 11.35A | 63.09 ± 8.57B |
| IL-10           | 0.0 ± 0.0A | 46.93 ± 16.81B,C | 30.20 ± 14.34B | 58.59 ± 20.50C |
| IL-12           | 9.56 ± 10.93A | 41.80 ± 15.82B,C | 29.30 ± 10.20B | 55.95 ± 18.94C |
| TNF-α           | 30.16 ± 7.53A | 86.95 ± 13.97B | 86.70 ± 8.80B | 89.69 ± 19.35B |
| **42 dpi**      |         |        |       |                  |
| IL-8            | 34.70 ± 19.27A | 38.75 ± 9.35A | 38.88 ± 55.73A | 68.62 ± 16.05B |
| IL-10           | 35.44 ± 12.39A | 22.05 ± 22.27A | 71.54 ± 40.33B | 50.27 ± 10.20A,B |
| IL-12           | 21.64 ± 19.37A | 36.37 ± 8.35A,B | 47.02 ± 13.08B,C | 59.65 ± 20.18C |

*The mRNA levels are presented as the ratio of cytokine/cyclophilin RT-PCR product band intensity. Data are shown only for cytokines with the significant differences between inoculation groups. Values in rows with different letters (A, B, or C) are significantly different (P < 0.001). M. hyo, M. hyopneumoniae.

**TABLE 4. Relative mRNA levels from PAMs in vitro at 6 and 15 hpi with PRRSV**

| hpi and cytokine | Control | M. hyo | PRRSV | PRRSV and M. hyo |
|-----------------|---------|--------|-------|------------------|
| **6 hpi**       |         |        |       |                  |
| IL-1α           | 77.72 ± 16.34A | 101.93 ± 25.08A,B | 119.55 ± 45.87B | 120.50 ± 45.60B |
| IL-1β           | 105.11 ± 17.77A | 121.86 ± 17.27A | 121.89 ± 63.2A | 170.88 ± 43.65B |
| IL-12           | 22.73 ± 18.94A | 31.28 ± 10.66A,B | 12.20 ± 19.57A | 49.87 ± 22.72B |
| TNF-α           | 96.18 ± 12.19A | 130.16 ± 40.56A,B | 154.56 ± 32.10C | 136.94 ± 28.23B |
| **15 hpi**      |         |        |       |                  |
| IL-1β           | 95.59 ± 27.00A | 136.60 ± 29.28B | 67.47 ± 14.54A | 87.20 ± 25.79A |
| IL-8            | 77.40 ± 26.18A | 131.17 ± 19.14B | 106.08 ± 39.66A,B | 100.90 ± 27.80A,B |
| IL-12           | 0.0 ± 0.0A | 17.11 ± 19.62B | 0.0 ± 0.0A | 8.09 ± 19.82B |
| TNF-α           | 46.53 ± 20.59A | 87.36 ± 20.04B,C | 64.96 ± 9.39A,B | 93.87 ± 29.48C |

*The mRNA levels are presented as the ratio of cytokine/cyclophilin RT-PCR product band intensity. Data are shown only for cytokines with significant differences between inoculation groups. Values in rows with different letters (A, B, or C) are significantly different (P < 0.001). M. hyo, M. hyopneumoniae.
Increased levels of mRNA for IL-1β were significantly in-
creased in both of the M. hyopneumoniae-infected groups at 28
dpi, with pigs coinfected with PRRSV having significantly higher
levels than pigs infected with M. hyopneumoniae alone. The levels of
the IL-8 protein in BAL fluid were very high and
may have been beyond the operating range of the assay, making
statistical differences difficult to interpret. Pigs infected
with both pathogens also had significantly increased levels of
IL-10 and TNF-α proteins in their BAL fluid at that time. By
42 dpi, only IL-1β and IL-8 protein levels were increased in the
BAL fluid of the group infected with both pathogens.

The in vitro assay found significantly increased levels of
IL-10 protein in the supernatants of M. hyopneumoniae-infected
tracheal ring cultures with or without PRRSV at 6 hpi. IL-8 protein
levels were significantly increased in the supernatants of the
group inoculated only with M. hyopneumoniae. There were no changes in the cytokine protein levels in the
PAMs infected with only PRRSV at either 6 or 15 hpi. At 15
hpi, the levels of proteins of all four cytokines were signi-
ficantly increased in the superna-
tants of the group inoculated only with M. hyopneumoniae.

**PRRSV and M. hyopneumoniae isolation and serology.** To
evaluate the relationship of the cytokine levels to viral clear-
ance, PRRSV was isolated from the BAL fluid of infected pigs.

### Table 6. Cytokine concentrations determined from supernatants of cocultured tracheal rings and PAMs at 6 and 15 hpi with PRRSV in vitro

| hpi and cytokine | Control | M. hyo | PRRSV | PRRSV and M. hyo |
|------------------|---------|--------|-------|-----------------|
| 6 hpi            |         |        |       |                 |
| IL-1β            | 61.00 ± 50.19 | 104.33 ± 50.58 | 6.17 ± 10.68 | 37.00 ± 64.09 |
| IL-8             | 1.18 ± 0.12A | 1.57 ± 0.52B | 0.95 ± 0.21A | 1.28 ± 0.33A,B |
| IL-10            | 0.125 ± 0.0A | 11.69 ± 4.54B | 0.33 ± 0.16A | 18.36 ± 10.47B |
| TNF-α            | 24.36 ± 3.79 | 29.08 ± 9.61 | 17.42 ± 3.56 | 26.17 ± 17.17 |
| 15 hpi           |         |        |       |                 |
| IL-1β            | 49.33 ± 18.09A | 396.83 ± 337.86B | 76.00 ± 34.37A | 359.33 ± 197.05A,B |
| IL-8             | 3.00 ± 0.82A | 13.05 ± 6.35B | 2.72 ± 0.59A | 10.79 ± 8.74A,B |
| IL-10            | 1.69 ± 1.56A | 18.56 ± 2.86B | 1.17 ± 1.80A | 18.45 ± 16.00B |
| TNF-α            | 9.78 ± 5.01A,B | 14.50 ± 7.95B | 7.14 ± 6.13A | 12.28 ± 5.09B |

| Cytokine concentrations were measured by a commercial ELISA. Values in rows with different letters (A, B, or C) are significantly different (P < 0.001). M. hyo, M. hyopneumoniae. |
In addition, the levels of virus in the PAM culture supernatants were determined by titration. Virus was isolated from all pigs inoculated with PRRSV at 10 dpi. At 28 dpi, 33% (two of six) of pigs infected only with PRRSV remained positive for virus, whereas 80% (four of five) of the pigs inoculated with both pathogens remained positive for virus. No virus was isolated from the BAL fluid of any pig at 42 dpi. *M. hyopneumoniae* was isolated from all *M. hyopneumoniae*-inoculated pigs at all necropsy times. No *M. hyopneumoniae* isolates were found in either the control group or the PRRSV-inoculated group at any necropsy date.

In vitro, although no significant differences were detected between the treatment groups, at 6 and 15 hpi, PAMs in the cultures inoculated only with PRRSV tended to yield higher viral titers ($10^{2.37} \pm 10^{0.24}$ and $10^{0.12} \pm 10^{0.27}$ TCID$_{50}$/ml, respectively) than PAMs in the group inoculated with both pathogens ($10^{2.21} \pm 10^{0.19}$ and $10^{2.75} \pm 10^{0.43}$ TCID$_{50}$/ml, respectively).

All pigs inoculated with PRRSV developed serum antibodies to PRRSV by 10 dpi and remained seropositive for the remainder of the trial. Only two pigs from either *M. hyopneumoniae*-infected group seroconverted to *M. hyopneumoniae* antibody positivity by 42 dpi.

**DISCUSSION**

Induction of an innate immune response is important for the control and elimination of invading pathogens. Proinflammatory cytokines produced by macrophages have a broad spectrum of effects that contribute to the host’s defense against pathogens. However, while the presence of proinflammatory cytokines can be beneficial in the control of pathogens, significant tissue damage can occur if their levels become excessive. In addition, many cytokines produced by macrophages, such as IL-10 and IL-12, are associated with the induction and implementation of either Th1 or Th2 types of immune responses. Development of a Th1 type of immune response can be crucial for host resistance and the control and elimination of intracellular pathogens such as viruses. The severity of respiratory disease associated with bacterial or viral infections in murine models has been shown to be influenced by these cytokine responses (5, 15).

The study reported here investigated the induction of a number of proinflammatory cytokines produced by macrophages in response to infection with PRRSV and/or *M. hyopneumoniae*, two common swine respiratory pathogens, by using both in vitro and in vivo models. These models enabled us to evaluate the effects of these pathogens in the respiratory tract in vivo, while the in vitro model enabled us to assess more directly the impacts of the pathogens on PAMs independently of other cells of the respiratory immune system. Other cell types, including epithelial cells, were present in the tracheal explants. Accordingly, future studies to assess the direct impact of *M. hyopneumoniae* on epithelial cells will provide further independent analysis of that specific interaction that was not assessed in this study. Together the results of this study suggest that these two pathogens have a powerful effect on cytokine production, which may play a role in the induction of disease and the persistence of the organisms in the host.

Previous studies have demonstrated that infection with *M. hyopneumoniae* induces a number of proinflammatory cytokines in vivo (1, 2, 30). The results of the study presented here confirm that *M. hyopneumoniae* appears to induce greater levels of the proinflammatory cytokines than PRRSV. In contrast, an earlier in vivo study showed that PRRSV did not induce proinflammatory cytokine production as effectively as swine influenza virus (34). PRRSV infection has been reported to induce minimal TNF-α at 10 dpi (33), which was confirmed in this study. This finding is interesting, as the maximal pathology associated with PRRSV infection occurs at this time. It has been hypothesized that PRRSV may inhibit TNF-α production by either unknown inhibitory factors or posttranscriptional control, as TNF-α has been shown to have an antiviral effect (16). In contrast, by 28 dpi, all groups of pigs infected with PRRSV and/or *M. hyopneumoniae* had significantly increased TNF-α levels, which may have been associated with the clearance of PRRSV from the respiratory tract.

IL-1β is known to induce IL-6 production (40). In previous studies of pneumonia and disease induced by *Actinobacillus pleuropneumoniae*, the appearance of IL-1, TNF-α, and IL-6 coincided with the onset of clinical respiratory disease and increased body temperature (11, 14). It is interesting that IL-6 levels were not elevated in the PRRSV-infected group at 10 dpi, when maximal pneumonia and clinical disease, including fever, occurred, but were increased at 28 dpi, when minimal pneumonia or clinical symptoms were present. It has previously been demonstrated that *M. hyopneumoniae* induces the production of IL-6, although at 42 dpi, the IL-6 levels in both *M. hyopneumoniae*-infected groups had returned to the same level as that in the negative control group, even though pneumonia remained (2). It is difficult to interpret the significance of either IL-1-β or IL-6 in this study.

IL-8 is a neutrophil chemotactic factor produced by both macrophages and epithelial cells (17). Increased levels of IL-8 mRNA were found in the pigs infected with both pathogens at all three necropsy times. PRRSV infection alone appeared to have a minimal impact on IL-8. The soluble IL-8 levels in BAL fluid detected by ELISA were extremely high and there was considerable variability in the assay results, so the data should be interpreted with caution. Despite the significant increase in IL-8 production in conjunction with *M. hyopneumoniae* infection observed in this study, large numbers of neutrophils are not associated with mycoplasmal pneumonia. *M. hyopneumoniae* has been reported to have a suppressive effect on neutrophil function (3, 8). This suppression of neutrophil function may contribute to the exacerbation of secondary bacterial infections associated with *M. hyopneumoniae* infection. However, IL-8 has also been reported to be chemotactic for T cells, which may contribute to the lymphocyte proliferation associated with mycoplasmal pneumonia (9).

IL-10, which is produced by Th2-type T cells and activated macrophages, is a potent inhibitor of macrophage function and is a potent regulatory cytokine that decreases inflammatory responses and T-cell stimulation (4). Constitutive production of IL-10 by bronchial epithelial cells in the lung may help modulate the inflammatory response to environmental irritants by suppressing macrophage function (7). In this study, the two pathogens increased the level of IL-10 production dramatically. One possible outcome that could result from the increased IL-10 levels associated with PRRSV and that could
possibly be enhanced by *M. hyopneumoniae* infection is the potential shift of the host immune response away from a Th1-type response, which would potentially be more effective in controlling PRRSV, toward a predominantly Th2-type response. A number of viruses, such as human respiratory syncytial virus and murine cytomegalovirus, weaken the host immune response by inducing IL-10 production (22, 23). A possible mechanism by which IL-10 may increase the severity of viral pneumonia is by the impact of IL-10 on macrophages. PRRSV infection has been demonstrated to induce apoptosis in bystander PAMs (26). However, IL-10 has been demonstrated to protect against apoptosis (25). The enhanced production of IL-10 by *M. hyopneumoniae* may inhibit the apoptosis of PAMs in response to PRRSV infection, thus increasing the number of macrophages susceptible to infection by the virus. In this model, PRRSV persists longer in the respiratory tract of pigs infected with both pathogens.

IL-12 induces the production of IFN-γ and is associated with a Th1 type of cytokine profile and the production of IFN-γ. The biologically active form of IL-12 is a heterodimeric cytokine composed of two disulfide-linked protein subunits. Macrophages and dendritic cells produce the subunits that form the active subunit IL12p70, made up of subunit IL12p35 and IL12p40 molecules. Other cell types, including respiratory epithelial cells, produce only the IL12p40 subunit, which often forms a homodimer that can bind to the IL-12 receptor, blocking binding and activation by the IL12p70 molecule (37). In this study we measured the level of only the IL12p40 molecule and not that of IL12p35. Thus, while IL-12 levels may appear to be elevated, if only IL12p40 is produced, the formation of IL12p80 could occur, which would potentially delay the production of IFN-γ, as has been reported from a study that used systemic lymphocytes and an enzyme-linked immunospot assay (18). This may be an important mechanism for viral persistence; however, we did not measure IFN-γ levels in this study.

The in vitro assay system enabled us to investigate the cytokines produced by PRRSV-infected PAMs without the impact of the entire respiratory immune system. The results of this study confirm that the colonization of ciliated epithelial cells by *M. hyopneumoniae* may influence the types of cytokines produced by PAMs. Our study did not assess the effects of *M. hyopneumoniae* on tracheal epithelial cells. It should be noted that various cells in the tracheal rings could produce cytokines. In addition, chemokines may have produced some of the cytokines measured in this study and may be important for the induction of inflammation and an immune response.

Similar to the in vivo study, when *M. hyopneumoniae*-inoculated tracheas were incubated with PRRSV-infected PAMs, they induced proinflammatory cytokines at levels higher than the levels detected in tracheas infected with either agent alone. In the in vitro model, the levels of mRNA for TNF-α were significantly increased in both PRRSV-infected groups at 6 and 15 hpi, and the soluble levels of TNF-α were also increased at 15 hpi in both groups infected with *M. hyopneumoniae*. The presence of increased levels of TNF-α mRNA in PAMs infected with PRRSV in association with low levels of soluble TNF-α in the culture supernatant and BAL fluid either may have been due to the short half-life of TNF-α in culture or may suggest a posttranslational impact of PRRSV on TNF-α production. This could be an explanation for the low levels of TNF-α observed during PRRSV infection (33).

Other differences in the levels of production (compared to those measured in vivo) of cytokines produced in response to infection of the tracheal implants and/or PAMs were observed. The increase in IL-1α and IL-1β levels was less consistent in vitro, and no increase in IL-6 levels was observed any time point, including 24 hpi, in the earlier study (32). However, IL-8 levels were increased in vitro in noninfected PAMs cultured with *M. hyopneumoniae*-inoculated tracheas at 15 hpi, and increased levels of IL-8 protein were present in the supernatant at 6 and 15 hpi. These results suggest that the production of IL-8 may be an important mechanism associated with *M. hyopneumoniae*. PAMs infected with only PRRSV exhibited no increase in IL-8 levels.

Interestingly, no increase in IL-10 mRNA levels in PAMs was found in vitro either in this study or in a previous study (32). It may be that the source of IL-10 is not from PAMs and that *M. hyopneumoniae*-infected tracheal epithelial cells may have been the source of the IL-10 protein detected in the cultured medium. Normal human bronchial epithelial cells have been found to produce IL-10; however, less is known about the response of swine epithelial cells (7). IL-12 mRNA levels were upregulated in both PRRSV-infected and noninfected PAMs incubated with the *M. hyopneumoniae*-infected trachea. The previous in vitro study did not detect increased IL-12 levels in any of the treatment groups at 24 hpi (32). Since we measured only the subunit IL12p40 molecule, it is possible that *M. hyopneumoniae* induces macrophages and, possibly, epithelial cells to produce IL12p40. Further investigations should assess the specific form of IL-12 produced in response to infection with PRRSV and *M. hyopneumoniae*. Although IL-12 was produced both in vitro and in vivo in this study, the increased levels of production of IL-10 may have suppressed the formation of both IL12p40 and IL12p35 gene upregulation at the transcriptional level (4).

The specific mechanisms, cellular or subcellular, by which *M. hyopneumoniae* potentiates PRRSV-induced pneumonia remain unidentified. Using in vitro and in vivo models, we investigated the role played by proinflammatory cytokines produced in response to each of the pathogens alone or together. Both models confirmed our earlier findings, which documented that *M. hyopneumoniae* induces significant levels of proinflammatory cytokines. Further in vitro and in vivo studies that would include blocking of the effects of these cytokines as well as measurement of the impacts of the pathogens at the transcriptional level will assist in providing a further understanding of how these two pathogens continue to induce disease and persist in pigs.

ACKNOWLEDGMENTS

We thank Theresa F. Young, Shan Yu, Raye Taylor, Nancy Upchurch, and Barb Erickson for technical assistance. The study was funded by USDA-NRI.

REFERENCES

1. Asai, T., M. Okada, M. Ono, T. Irisawa, Y. Mori, Y. Yokomizo, and S. Sato. 1993. Increased levels of tumor necrosis factor and interleukin 1 in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*. Vet. Immunol. Immunopathol. 38:253–260.

2. Asai, T., M. Okada, M. Ono, Y. Mori, Y. Yokomizo, and S. Sato. 1994. Detection of interleukin-6 and prostaglandin E2 in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*. Vet. Immunol. Immunopathol. 38:253–260.

3. Asai, T., M. Okada, M. Ono, T. Irisawa, Y. Mori, Y. Yokomizo, and S. Sato. 1996. Detection of interleukin-6 and prostaglandin E2 in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*. Vet. Immunol. Immunopathol. 38:253–260.
