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Porcine reproductive–respiratory syndrome virus infection predisposes pigs for respiratory signs upon exposure to bacterial lipopolysaccharide

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Abstract

This study examined whether an infection with porcine reproductive and respiratory syndrome virus (PRRSV) potentiates respiratory signs upon exposure to bacterial lipopolysaccharides (LPS). Five-week-old conventional pigs were inoculated intratracheally with the Lelystad strain of PRRSV and received 5 days later one or two intratracheal LPS administrations. The necessary controls were included. After LPS administration, pigs were intensively monitored for clinical signs. Additionally, some pigs were euthanatized after a second LPS administration for broncho-alveolar cell analysis and virological examinations of the lungs. Broncho-alveolar lavage (BAL) cells were counted and differentiated. Lung suspensions and BAL fluids were titrated for PRRSV. Exposure of pigs to PRRSV only resulted in a fever for time periods ranging from 1 to 5 days and slight respiratory signs. Exposure of pigs to LPS only resulted in general signs, characterized by fever and depression, but respiratory signs were slight or absent. PRRSV–LPS exposed pigs, on the other hand, developed severe respiratory signs upon LPS exposure, characterized by tachypnoea, abdominal breathing and dyspnoea. Besides respiratory signs, these pigs also showed enhanced general signs, such as fever and depression. Lung neutrophil infiltration was similar in non-infected and PRRSV-infected pigs upon LPS exposure. PRRSV quantities were similar in lungs and BAL fluids of pigs infected with PRRSV only and PRRSV–LPS exposed pigs. These data show a clear synergism between PRRSV and LPS in the induction of respiratory signs in conventional pigs. The

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synergism was observed in 87% of the pigs. So, it can be considered as reproducible and may be used to test the efficacy of preventive and therapeutic measures.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus, causes infections in pigs worldwide. The virus replicates highly in the respiratory tract and shows a distinct tropism for broncho-alveolar macrophages (Duan et al., 1997). However, a single PRRSV infection, particularly under experimental circumstances and with European isolates, fails to induce overt respiratory disease (Done and Paton, 1995). Also under field circumstances, most pigs become infected with PRRSV at growing age without respiratory disease. Still, the frequency and severity of respiratory disease have increased since the enzootic occurrence of PRRSV (Done and Paton, 1995). This has stimulated research into the combined effects of PRRSV and other infectious agents. Consequently, experimental dual infections have been performed with PRRSV followed by various bacteria such as Haemophilus parasuis, Pasteurella multocida, Streptococcus suis, Bordetella bronchiseptica, and Salmonella choleraesuis (Cooper et al., 1995; Galina et al., 1994; Brockmeier et al., 2000; Wills et al., 2000). We ourselves have performed dual infections with PRRSV followed by enzootic viruses, notably porcine respiratory coronavirus (PRCV) or swine influenza virus (SIV) (Van Reeth et al., 1996). The clinical effects of these combinations were extremely severe in some cases, but almost completely subclinical in other. Most important, none of the dual infections mentioned provides a reliable model to study pathogenetic features or to test control measures. We hypothesized therefore that the clinical outcomes of dual inoculations with two infectious agents are influenced by factors that are too difficult to control, such as the stage of replication and the viral or bacterial load.

Bacterial lipopolysaccharides (LPS) or endotoxins, a major constituent of the cell wall of gram-negative bacteria, are released in high concentrations in the lungs upon infection with gram-negative bacteria (Lamp et al., 1996; Kadurugamuwa and Beveridge, 1997) and these endotoxins are present in varying concentrations in dust in swine buildings (Rylander, 1994; Zejda et al., 1994). The release of LPS by gram-negative bacteria, such as H. parasuis, P. multocida, B. bronchiseptica, and S. choleraesuis may explain the more severe disease in the experimental dual infections with PRRSV and these bacteria (Cooper et al., 1995; Brockmeier et al., 2000; Wills et al., 2000). Van Reeth et al. (2000) recently demonstrated that dual inoculations with PRCV followed by bacterial LPS seriously aggravate respiratory signs in gnotobiotic pigs, while the respective single inoculations were subclinical. Therefore, we wanted to examine if exposure of PRRSV-infected pigs to LPS similarly enhances respiratory signs. PRRSV may lend itself excellently as a predisposing agent for synergism with LPS, because all pigs become infected at ages varying from 4 weeks to fattening age (Albina et al., 1994; Houben et al., 1995). Also, PRRSV persists in the lungs for 40 (Labarque et al., 2000) to 49 (Mengeling
et al., 1995) days. We have examined the clinical course of inoculations with PRRSV followed by LPS, and the effect of the timing and frequency of LPS administrations. Additionally, some preliminary investigations of cellular and virological aspects in the lungs were performed.

2. Materials and methods

2.1. Virus and LPS

A fifth passage on pulmonary alveolar macrophages (PAMs) of the Lelystad strain of PRRSV (Wensvoort et al., 1991) was used in this study. The inoculation dose was $10^{6.0}$ TCID$_{50}$/pig.

*Escherichia coli* LPS (O111:B4) was obtained from Difco Laboratories and used at a dose of 20 $\mu$g/kg body weight. This dose was based on data from previous experiments in gnotobiotic pigs, and selected to cause no respiratory signs (Van Reeth et al., 2000).

2.2. Pigs and experimental design

Forty-six conventional pigs, originating from 12 PRRSV-negative sows, were used. Pigs were weaned at 4 weeks of age and placed in isolation. They were allowed to acclimatize during 7 days before initiation of the experiments. PRRSV inoculations and LPS administrations occurred intratracheal as described by Van Reeth et al. (2000). Briefly, the pigs were held in vertical position with their neck extended. A needle was inserted through the skin cranial to the sternum and the inoculum was injected. The intratracheal administration was chosen to ensure that all the pigs received exactly the same dose in the lungs. Three experiments were performed.

In the first experiment, 15 pigs were inoculated with PRRSV and received one LPS administration 5 days later. Seven pigs were inoculated with PRRSV only. Eight pigs, not previously inoculated with PRRSV, received one LPS administration. Clinical monitoring was performed daily during 5 consecutive days after PRRSV inoculation and every 2 h during the first 12 h after LPS administration.

In the second experiment, eight pigs were inoculated with PRRSV and, 5 days later they received two LPS administrations with a 3 h interval. Four pigs, not previously inoculated with PRRSV, received two LPS administrations with a 3 h interval. Clinical monitoring was performed daily during 5 consecutive days after PRRSV inoculation and at 1, 3, 5, 7 and 9 h after the second LPS administration.

In the third experiment, 11 out of the 15 PRRSV–LPS exposed pigs, described in the first experiment, received a second LPS administration, 24 h after the first one. These pigs were divided in two subgroups. One subgroup of six pigs was again monitored for clinical signs every 2 h until 12 h after the second LPS administration. One subgroup of five pigs was euthanatized between 5 and 7 h after the second LPS administration for study of the broncho-alveolar lavage (BAL) cell population and for virological and bacteriological examinations of the lungs. From the seven PRRSV control pigs, described in the first experiment, four pigs were again monitored for clinical signs every 2 h for 12 h at day 6.
after PRRSV inoculation. The remaining three pigs were euthanatized at time points corresponding to those of the PRRSV–LPS exposed pigs and served as controls for the broncho-alveolar cell and virological examinations. All eight LPS exposed pigs, described in the first experiment, received a second LPS administration, 24 h after the first one. Four pigs were again monitored for clinical signs every 2 h until 12 h after the second LPS administration and four pigs were euthanatized between 5 and 7 h after the second LPS administration for broncho-alveolar cell and virological examinations. Four non-inoculated pigs were euthanatized for the same purpose.

2.3. Clinical monitoring

Pigs were monitored for general signs, notably fever and depression, and for respiratory signs, notably tachypnoea, abdominal breathing and dyspnoea. Scores were given for these five clinical parameters. Body temperatures ≤39.9 °C were scored as 0, temperatures between ≥40.0 and ≤40.9 °C were scored as 1 and temperatures ≥41.0 °C were scored as 2. Respiration rates ≤45 were scored as 0, rates between ≥46 and ≤59 were scored as 1 and rates ≥60 were scored as 2. Depression, abdominal breathing and dyspnoea were scored as 0 (absent) or 1 (present). Scores were added up and a mean of the cumulative general and respiratory scores per group was calculated.

2.4. Broncho-alveolar cell examinations

At necropsy, the lungs were removed. The right lung was used for broncho-alveolar cell examination after BAL using the method described by Van Reeth et al. (1998). The BAL fluid was centrifuged (400 × g, 10 min, 4 °C) to separate the cells and the cell-free lavage fluid. Aliquots of the cell-free lavage fluid were stored at −70 °C until virus titration on PAMs. BAL cells were counted in a Türk chamber and cytocentrifuge preparations were stained with DiffQuik (Baxter, Düdingen, Switzerland) to determine the percentage of mononuclear cells and neutrophils.

2.5. Virological and bacteriological examinations

The left lung was used for virological and bacteriological examinations. Twenty percent suspensions of lung lobes were made in a phosphate-buffered saline, clarified by centrifugation and the supernatant was used for PRRSV titration. Virus titration of lung suspensions or BAL fluids was performed on PAMs, as described by Labarque et al. (2000). For bacteriology, samples of lung tissue were plated on bovine blood agar and cultured aerobically. A nurse colony of coagulase-positive *Staphylococcus* species was streaked diagonally on each plate. Plates were inspected for bacterial growth after 48 and 72 h. Colonies were then identified by standard techniques.

2.6. Statistical analysis

Non-parametric tests were used, because of lack of normality in the data. Standard two-sample Mann–Whitney tests were used to compare general and respiratory clinical scores
between groups. $P < 0.05$ was taken as the level of statistical significance. Statistical analyses were performed using SPSS 6.1.

3. Results

3.1. Clinical signs after PRRSV only

Twenty-six of the total of 30 PRRSV-infected pigs developed fever for time periods ranging from 1 to 5 days. Respiratory signs were absent, except for two pigs, which showed tachypnoea and abdominal breathing.

In experiment 1, six of the seven PRRSV control pigs showed fever until the end of the monitoring period. Respiratory signs were slight in one pig and absent in the other pigs. The mean respiratory score was 1.0 (Table 1).

In experiment 3, all four PRRSV control pigs showed fever until the end of the monitoring period. Respiratory signs, characterized by increased respiration rates, were observed in one of the four pigs. The mean respiratory score was 2.5 (Table 1).

3.2. Clinical signs after one LPS administration

In non-infected pigs, a single LPS administration induced transient general signs (Fig. 1). Respiratory signs were slight or absent and the mean respiratory score was only 0.6 (Table 1). In PRRSV-infected pigs, however, LPS induced severe clinical signs with fever

| Table 1 |
|---|
| Mean general and respiratory scores after the last LPS administration in PRRSV–LPS exposed pigs and their controls |

| Experiment | Exposure | Number of pigs | Mean of the cumulative clinical scores | General | Respiratory |
|---|---|---|---|---|---|
| 1 | PRRSV–5d–LPS | 15 | 10.0 | 11.8 | |
| | PRRSV | 7 | 6.0 B | 1.0 B | |
| | LPS | 8 | 4.0 B | 0.6 B | |
| 2 | PRRSV–5d–LPS–3 h–LPS | 8 | 8.8 A | 15.5 A | |
| | LPS–3 h–LPS | 4 | 4.3 B | 7.0 B | |
| 3 | PRRSV–5d–LPS–24 h–LPS | 6 | 9.8 A | 11.0 A | |
| | PRRSV | 4 | 8.5 A | 2.5 AB | |
| | LPS–24 h–LPS | 4 | 0.5 B | 0.0 B | |

A,B Within each experiment, values with different letters in the same column are significantly different by standard two-sample Mann–Whitney test ($P < 0.05$).

a Body temperature (0: $\leq 39.9$ °C; 1: $\geq 40.0$ °C–$\leq 40.9$ °C; 2: $\geq 41.0$ °C) and depression (0, absent; 1, present).

b Respiration rate/min (0: $\leq 45$; 1: $\geq 46$–$\leq 59$; 2: $\geq 60$), abdominal breathing and dyspnoea (0, absent; 1, present).
in all the pigs and respiratory signs in 87% of the pigs (Fig. 1). Respiratory signs were characterized by tachypnoea (peak 154 breaths/min), abdominal breathing and dyspnoea and lasted until the end of the monitoring period. Two out of the 15 pigs did not show respiratory signs after LPS administration. Mean general and respiratory scores were
significantly higher in PRRSV–LPS exposed pigs than in singly LPS exposed pigs (Table 1).

3.3. Clinical signs after two LPS administrations with a 3 h interval

In non-infected pigs, which received two LPS administrations with a 3 h interval, both general and respiratory signs were observed (Fig. 2). Clinical signs were significantly higher in PRRSV-infected pigs not only with regard to the number of affected pigs but also with regard to the clinical scores. All pigs reacted severely. The mean clinical scores after the second LPS administration are presented in Table 1.
3.4. Clinical signs after two LPS administrations with a 24 h interval

Non-infected pigs had recovered at the time of the second LPS administration, 24 h after the first one. This second LPS administration again induced general signs within 2 h, but no respiratory signs (Fig. 3). PRRSV-infected pigs had not yet recovered 24 h after the first LPS administration (Fig. 3). The second LPS administration however increased the number of pigs with general and respiratory signs and mean clinical scores (Fig. 3). Here again, mean general and respiratory scores were significantly higher in PRRSV–LPS exposed pigs than in singly LPS exposed pigs.

Fig. 3. Evolution of clinical signs after two LPS administrations with a 24 h interval in PRRSV- and non-infected pigs.
3.5. Lung inflammatory findings

Total BAL cell numbers and differentials are shown in Table 2. BAL cell numbers and differentials in PRRSV–LPS exposed pigs were essentially similar to those of pigs exposed to PRRSV or LPS only. However, there was great individual variation within all three groups.

Mean PRRSV titres in lungs and BAL fluids are shown in Table 3. Virus titres were similar in lungs and BAL fluids of singly PRRSV-inoculated pigs and PRRSV–LPS exposed pigs. The lungs and BAL fluids of LPS controls and non-inoculated controls were negative for PRRSV.

Bacterial culture of lung tissue yielded negative results for all pigs.

4. Discussion

It has become generally accepted that PRRSV plays an important role in respiratory disease problems in the field, particularly in multi-factorial respiratory disease. However, it has been most difficult to reproduce respiratory signs in experimental infection studies with PRRSV and a second infectious agent. The present PRRSV–LPS combination induces clear respiratory signs in 87% of the pigs. Unlike in our previous studies with PRRSV–SIV
and PRRSV–PRCV combinations (Van Reeth et al., 1996), mean clinical scores were higher than those of control pigs in every experiment. Only two out of the 15 PRRSV-infected pigs did not develop respiratory signs upon LPS exposure. It is important to mention, however, that individual variation in disease severity is unavoidable with respiratory pathogens. Such an individual variation has even been reported in experimental infection studies with primary respiratory pathogens such as Actinobacillus pleuropneumoniae (Baarsch et al., 2000) or SIV (Van Reeth et al., 1998).

We used two LPS administrations with the purpose to extend the duration of clinical signs. The clinical effect of a second LPS administration was dependent on the time interval between the two LPS administrations. In non-infected pigs, a second LPS administration at a 24 h interval caused milder clinical signs than the first one. On the other hand, a second LPS administration within a 3 h interval seriously aggravated and prolonged general and respiratory signs. These observations suggest that two LPS administrations within a short time interval lead to an accumulation of LPS in the lungs. Indeed, it has been demonstrated that the clinicopathological manifestations of LPS are strictly dose-dependent. For example, if sufficient amounts of LPS are given to animals and man, cytokine induction, lung inflammation and decreased lung function are observed. Slightly smaller LPS doses, on the other hand, will cause only a mild lung inflammation (Michel et al., 1997). In PRRSV-infected pigs, the clinical effect of a second LPS administration was difficult to assess since pigs had not yet recovered at the moment of the second LPS administration, 3 or 24 h after the first one. There is little information on the effect of repeated LPS administrations to farm animals in the literature. It appears logical, however, that the mediators or mechanisms responsible for the clinical effects of LPS may become exhausted if high LPS doses are administered frequently.

Respiratory signs following PRRSV–LPS exposure could not be explained by the extent of virus replication or inflammatory changes in the lungs. Indeed, virus titres were similar in PRRSV–LPS or singly PRRSV-inoculated pigs. Total BAL cell numbers and neutrophil infiltration were similar in PRRSV–LPS or singly LPS exposed pigs. Also, the two PRRSV–LPS exposed pigs, which remained healthy, had similar BAL cell profiles as their clinically affected group mates. This suggests that inflammatory changes in the lungs have little or no effect on the synergism between PRRSV and LPS. We hypothesize therefore that functional lung changes, such as bronchial hyper-responsiveness, are more important in the pathogenesis of PRRSV–LPS induced disease than structural changes. Similar findings were made in a previous experimental infection study with PRCV followed by LPS (Van Reeth et al., 2000). In this study, disease development was tightly correlated with lung production of proinflammatory cytokines, among which tumor necrosis factor-alpha (TNF-α). Interestingly, TNF-α has been shown to cause bronchial hyper-responsiveness in laboratory animal models (Kips et al., 1992; Thomas et al., 1995).

Under field circumstances, most pigs become infected with PRRSV at growing age and they are continuously exposed to airborne endotoxins. Also, during gram-negative infections of the lungs, excessive amounts of endotoxins are released locally. The present PRRSV–LPS infection model therefore is relevant for the study of PRRSV-induced respiratory problems in the field. The synergism was observed in 87% of the pigs. So, it can be considered as reproducible and may be used to test the efficacy of preventive and therapeutic measures.
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