Circulating cortisol, tumor necrosis factor-alpha interleukin-1beta, and interferon-gamma in pigs infected with Actinobacillus pleuropneumoniae


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ABSTRACT: This study evaluated the time course of systemic cytokine concentrations in an acute model of pneumonia in pigs challenged intranasally with *Actinobacillus pleuropneumoniae*. Feed intake and serum cortisol were measured as overt clinical and systemic markers of disease onset, respectively, and serum tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) as representative systemic inflammatory markers. Crossbred barrows (*n* = 15), approximately 5 wk of age, were used in the study. Pigs were housed in an environmentally controlled facility at 25°C and under continuous illumination in pens measuring approximately 1.5 m². Pigs had free access to water and an unmedicated diet. Approximately 1 wk prior to disease challenge, pigs were fitted nonsurgically with venous catheters. At challenge, pigs were given 5 × 10⁸ CFU *Actinobacillus pleuropneumoniae* intranasally (*n* = 8) or a similar volume of sterile growth media intranasally (Control; *n* = 7). Feed intake was estimated by the change in feeder weight at 12-h intervals from −12 to 72 h relative to the time of disease challenge. Blood sampling began 12 h prior to challenge and continued until 72 h after challenge. Pigs were sampled at −12, −6, and 0 h, then at 90-min intervals until 12-h post-challenge, continuing at 3-h intervals until 24-h post-challenge, then again at 6-h intervals until 72 h after challenge. Serum was harvested and frozen until assayed for cortisol, tumor necrosis factor-α, interleukin-1β, and interferon-γ. Feed intake was reduced in *Actinobacillus pleuropneumoniae*-challenged pigs during the intervals 0 to 12 h (*P* < 0.001), 24 to 36 h (*P* < 0.001), 48 to 60 h (*P* < 0.05), and 60 to 72 h (*P* < 0.05). The *Actinobacillus pleuropneumoniae*-challenged pigs had elevated serum cortisol from 180-min to 18-h post-challenge (*P* < 0.001) and also at 36 (*P* < 0.05), 42 (*P* < 0.001), and 60 h (*P* < 0.05) following infection. Circulating cytokines were not affected by disease challenge. Thus, in this experimental model of pneumonia, weaned pigs demonstrated expected behavioral and endocrine characteristics of disease in the absence of significant changes in circulating inflammatory cytokines.

Key Words: Diseases, Hydrocortisone, Interferon, Interleukin, Pigs, Tumor Necrosis Factor

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Introduction

Bacterial lipopolysaccharide (LPS) is a potent inflammatory mediator and has been used to model bacterial infection experimentally in animals. Treatment of pigs with LPS evokes a multitude of symptoms of sickness (Johnson and von Borell, 1994); activation of the pituitary-adrenal axis (Warren et al., 1997; Wright et al., 2000); and elaboration of systemic inflammatory cytokines (Stabel et al., 1995; Warren et al., 1997; Webel et al., 1997). The profound inflammatory cytokine response to LPS has led to the view that systemic cytokines may be obligatory upstream regulators of other pathophysiologic events. However, swine models of sep-
sis utilizing live bacterial challenge have not been consistent with this notion. Infection of pigs with *Salmonella typhimurium* produced a marked febrile response and a prolonged surge in plasma cortisol in the absence of disease-induced changes in circulating tumor necrosis factor alpha (TNFα) (Balaji et al., 2000). Similarly, no significant changes were observed in lung TNFα mRNA (Baarsch et al., 1995; Baarsch et al., 2000) or TNFα in serum (Fossum et al., 1998) of pigs infected with *Actinobacillus pleuropneumoniae*. Also, oral administration of *S. typhimurium* to weaned pigs failed to alter serum TNFα, although intranasal administration resulted in a delayed elevation (weeks post-challenge) (Stabel et al., 1995).

Thus, elaboration of systemic inflammatory cytokines, at least TNFα, may not be requisite for development of other early sequelae of disease onset, such as fever, anorexia, somnolence, and a surge in serum corticosteroids. However, there are few studies that have examined systemic cytokine profiles in detail in the early hours of infection with pathogenic bacteria. Therefore, the current study was designed to test the hypothesis that challenge of pigs with pathogenic bacteria could evoke inappetence and a surge in plasma cortisol in the absence of significant changes in circulating inflammatory cytokines.

### Materials and Methods

The physical facilities and management of pigs were described in detail in previous reports of disease challenge experiments published from our laboratory (Balaji et al., 2000). For the current study, 15 crossbred barrows, approximately 5 wk of age, were used. Pigs were randomly assigned to one of two identical environmentally controlled rooms and housed at 25°C under continuous illumination in 1.5 m²-pens. Pigs had free access to water and an unmedicated diet that was cornsoybean meal-based containing 10% spray-dried whey, 4.5% select menhaden fish meal, and 3% choice white grease, and was formulated to contain 1.40% lysine, 0.90% Ca, and 0.79% P. All animals were free of clinical signs of disease prior to housing.

Approximately 1 wk prior to disease challenge, pigs were fitted nonsurgically with venous catheters equipped with remote sampling devices that allowed for frequent blood sampling without disturbing the experimental animals (Carroll et al., 1999). Catheter patency was maintained by flushing twice daily with heparinized saline. At challenge, pigs were given *A. pleuropneumoniae* (serotype 1, biotype 1, strain 4074) intranasally (*A. pleuropneumoniae*; n = 8) or a similar volume of sterile growth media intranasally (Control; n = 7). Feed intake was estimated by the change in feeder weight at 12-h intervals from −12 to 72 h, relative to the time of disease challenge. Blood sampling also began 12 h prior to challenge and continued until 72 h after challenge. Pigs were sampled at −12, −6, and 0 h, then at 90-min intervals until 12-h post-challenge. Blood sampling continued at 3-h intervals until 24-h post-challenge, then at 6-h intervals until 72 h after challenge. Serum was harvested and frozen at −20°C until assay for cortisol by RIA (Griffith and Minton, 1992); and for TNFα (catalog EPTNFA), interleukin (IL)-1β, and interferon (IFN)γ (catalog EPIINF) utilizing swine specific ELISA (Pierce Endogen, Rockford, IL; validation data for the TNFα, and IFNγ assays were provided by the manufacturer). The same TNFα and IFNγ assays utilized in the current study were reported to detect elevations of TNFα (Warren et al., 1997; Webel et al., 1997; Wright et al., 2000) and IFNγ (Daniel et al., 1999; Zannelli et al., 2000) in serum of LPS-treated pigs. Serum cortisol concentrations were estimated in a single assay that had a CV of 4.0%. The intra- and interassay CV averaged 2.35 and 3.56%, respectively, for TNFα, and 5.07% and 9.30%, respectively, for IFNγ.

The IL-1β sandwich ELISA was developed from commercially available reagents. Both capture and detection antibodies were from polyclonal sera of rabbits immunized against recombinant swine IL-1β (Pierce Endogen catalog, pp 425). Recombinant pig IL-1β (Pierce Endogen catalog, RPI1B5) was used as the standard in the assay, and the range of the curve was 31.25 to 2000 pg/mL. The ELISA did not detect other swine cytokines tested (pTNFα, pIFNγ, pIL-2, pIL-6, pIL-8, pIL12p40, pIL12p70, and pGMCSF). Recovery of pIL-1β added to six separate porcine serum samples averaged 93.4%. These same six samples of porcine serum were diluted 1:2, 1:4, 1:8, and 1:16 and the concentration of IL-1β estimated in the assay. The regression of the volume-corrected concentrations measured in the assay on the expected concentrations of IL-1β had a slope of 0.99 (R² = 0.9896). The assay was sensitive to 15.6 pg/mL. Tissue culture supernatants from porcine alveolar macrophages that had been treated with LPS, and serum from pigs treated with LPS were evaluated in the assay. The ELISA could detect a robust and unmistakable LPS-induced increase in IL-1β in both culture media and serum (data not shown). All samples from the current study were evaluated in two assays. Unfortunately, the control pool chosen in the first assay contained a concentration of IL-1β that was below the sensitivity of the assay. Thus, no intrassay CV could be calculated. A different internal pool was used in the second assay and it had an intrassay CV of 11.7%.

All data were analyzed as a split-plot with repeated measures (Gill and Hafs, 1971) using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included effects of treatment in the main-plot (tested by the pig-within-treatment variance), and effects of time and treatment × time interaction in the subplot. Feed intake data were obtained from all animals in the study. Catheters remained patent in six Control pigs and six *A. pleuropneumoniae* pigs, and cortisol was evaluated in samples from these animals. Because of a limitation on available reagents, serum cytokines were estimated in a subset of three animals from each treatment and
Figure 1. Feed intake of pigs infected with *Actinobacillus pleuropneumoniae* (n = 8) or given sterile growth media (n = 7) intranasally. Data are least squares means ± SEM. Probability levels denote intervals when feed intake differed significantly between infected and control pigs.

only in samples from −12 to 24 h. The animals to be evaluated for circulating cytokines were chosen solely on the basis of having complete or nearly complete sets of sequential serum samples from −12 to 24 h relative to challenge. The rationale for limiting the cytokine analyses to the first 24 h following treatment was based upon the course of inflammatory cytokine mRNA expression in lung lavage cells and the presence of cytokine bioactivity in lung lavage fluid from pigs infected with *A. pleuropneumoniae* (Baarsch et al., 1995). Significant treatment × time interactions were only observed for feed intake and cortisol (*P* < 0.001). Treatment × time means were compared using least significant difference tests.

Results and Discussion

We estimated feed intake (from feed disappearance) in the current study as an indicator of the clinical progress of the disease. Feed intake was chosen over other potential clinical signs of disease (e.g., fever) because it could be estimated without substantial disruption of the experimental animals and potentially biasing both the cortisol and systemic cytokine data. Feed intake in *A. pleuropneumoniae* and Control pigs was similar in the 12 h prior to treatment (Figure 1). As expected, intake was reduced in *A. pleuropneumoniae* pigs and the reduction was significant during the intervals 0 to 12 h (*P* < 0.001), 24 to 36 h (*P* < 0.001), 48 to 60 h (*P* < 0.05), and 60 to 72 h (*P* < 0.05). Intake was similar between pigs in the two treatments at 12 to 24 h and 36 to 48 h post-treatment. However, the lack of treatment differences at those times appeared to be related more to periodic nadirs in intake in Control pigs since the intake in *A. pleuropneumoniae* pigs remained reduced, but rather constant from the 12- to 24-h interval through the remainder of the study.

Virtually all of the feeding bouts of pigs occur during the light hours of the light/dark cycle, with the greatest feeding activity occurring at the beginning and conclusion of the photophase (Wangsness et al., 1980). Whereas the reductions in intake in sick pigs in the current study were entirely expected as a consequence of the development of the disease (Johnson and von Borell, 1994), it is interesting to note the apparent diurnal variation in intake in Control pigs as all pigs were housed in constant environmental lighting. On the other hand, we have shown that certain endocrine rhythms free run in pigs under constant lighting conditions (Griffith and Minton, 1991). Even though the current study was not designed to evaluate critically the effects of environmental lighting on behavioral rhythms, the feed intake data from Control pigs suggest that rhythms of feed consumption also are maintained in pigs under constant environmental illumination. It is acknowledged that our study was of short duration, and rhythms may have been lost with continued housing under constant illumination.

Cortisol is known to surge following injection of bacterial LPS in pigs (Parrott et al., 1997; Matteri et al., 1998; Wright et al., 2000). Recently, we demonstrated that infection with *S. typhimurium* produced a surge in plasma cortisol that remained elevated in challenged pigs for approximately 48 h (Balaji et al., 2000). In the current study, pigs treated with *A. pleuropneumoniae* had elevated serum cortisol from 180-min to 18-h post-challenge (*P* < 0.001; Figure 2). Cortisol also was increased at 36 (*P* < 0.05), 42 (*P* < 0.001), and 60 (*P* < 0.05) h following challenge with *A. pleuropneumoniae*. Thus, both bacterial models of acute enteric disease (Balaji et al., 2000) and pneumonia (current study) pro-
voke activation of the hypothalamic-pituitary-adrenal (HPA) axis that is consistent with the effect seen in LPS models of disease. However, the total duration of the cortisol surge in both models outlasts those achieved by LPS, even with relatively high doses (Wright et al., 2000). From these results, it can generally be concluded that the neuroendocrine drive for activation of the HPA axis provided by a replicating pathogen appears to be more potent than that achieved with the bacterial cell wall product. The mechanism(s) by which bacterial pathogens and LPS activate the neuroendocrine stress axis are not precisely known, and may be different, but a pathway including peripheral neural signaling and central hypothalamic activation appear to be most likely (Elmquist et al., 1997). However, as discussed below, activation of the HPA axis subsequent to significant changes in peripheral inflammatory cytokines would not appear to be a major mechanism in the swine bacterial disease models evaluated in our laboratory to date (Balaji et al., 2000 and current study).

Data from our laboratory do not suggest a role for circulating cortisol in restraining systemic inflammatory cytokines in diseased pigs, but cortisol may play a role locally in regulating cytokine production within the lungs of infected pigs (Sapolsky et al., 2000). The surge in cortisol in diseased pigs may, however, have other immune modulating roles beyond their well-known anti-inflammatory role. Specifically, glucocorticoids recently have been shown to positively regulate proliferation in activated lymphocytes (Wiegers et al., 1995; Wiegers and Reul, 1998), and glucocorticoids may assist in regulation of the cytokine milieu associated with type 1 vs type 2 immune responses. Generally it is reported that glucocorticoids favor immune cytokine bias from a so-called type 1 to a type 2 pattern (reviewed in Sapolsky et al., 2000). However, such a role for glucocorticoids has not been established unequivocally and would seem to be an unlikely regulatory role for the surge in cortisol observed in the early hours in bacterially infected pigs (Balaji et al., 2000 and the current study).

An additional minor point should be made regarding the cortisol concentrations observed in control pigs in the current study. As noted above, this study was not designed to track circadian variations, but clear circadian rhythmicity in cortisol can be observed in the Control animals (Figure 2). This pattern of secretion is noteworthy in that it is consistent with the persistence of cortisol rhythms in pigs maintained in constant light (Griffith and Minton, 1991). Furthermore, the presence of circadian profiles, especially within the first 24 h of sampling, confirms that the experimental procedures, including frequent blood sampling and handling associated with sham infection, were not themselves overly stressful to the animals. Cortisol circadian rhythmicity in pigs is known to be acutely sensitive to experimental manipulations (Becker et al., 1985).

Neither serum TNFα (Figure 3), IL-1β (Figure 4), nor IFNγ (Figure 5) was affected by infectious challenge. Again, we measured TNFα, IL-1β, and IFNγ as representative inflammatory cytokines. Our failure to observe systemic disease-induced changes in these mediators in this model of bacterial pneumonia is completely consistent with another report of A. pleuropneumoniae infection in pigs (Fossum et al., 1998), although in this study and one other (Johansson et al., 2001), A. pleuropneumoniae stimulated a measurable increase in systemic IL-6 in pigs in response to infection. Our findings in the current study also are consistent with the lack of effect of S. typhimurium on plasma TNFα in pigs (Balaji et al., 2000).

Although we did not observe systemic changes in inflammatory cytokines, there is considerable evidence of enhanced production of mRNA for these inflammatory...
mediators in lung tissue of pigs infected with *A. pleuropneumoniae* (Baarsch et al., 1995; Choi et al., 1999; Huang et al., 1999; Baarsch et al., 2000). In fact, in contrast to our findings, another study reported increased TNFα and IL-1β in serum 3 to 4 d after infection of pigs with *A. pleuropneumoniae* (Huang et al., 1999). Several differences may account for the apparent contrast in findings between that study and ours. First, in that study, pigs were infected with aerosolized bacteria, so the total dosage of bacteria actually reaching the lungs may have been considerably greater than in our experiment. Second, the strain of *A. pleuropneumoniae*, and therefore associated virulence factors, differed between the two studies. Third, in the study by Huang et al. (1999), cytokines were estimated using single blood samples taken at sacrifice. Finally, and perhaps most importantly, systemic cytokines were measured 3 to 4 d post-infection in that study, and we limited sequential measurement of cytokines to the 24 h following infection. We do not know whether the cytokines measured in the current study would have increased later post-challenge. However, our data demonstrate unequivocally the ability of pigs to mount other behavioral (feed intake) and systemic (cortisol) mechanisms in response to disease challenge in the absence of measurable changes in systemic inflammatory cytokines. Similarly, other pathophysiological correlates of disease, in particular fever genesis, originally thought to be conveyed to the brain via blood-borne cytokines, now appear to be triggered by factors other than systemic cytokines (Blatteis et al., 2000).

Thus, in contrast to conclusions drawn largely from LPS models of disease, disease induced by actual bacterial pathogens do not necessarily evoke significant systemic TNFα, IL-1β, or IFNγ secretion, at least within the first 24 h following infection. Elaboration of these inflammatory cytokines undoubtedly occurs, even in the absence of significant changes in peripheral concentrations, but they seem to be confined locally within affected tissues (Baarsch et al., 1995; Baarsch et al., 2000). The localization of cytokine production very likely is important in limiting the immune response only to infected tissues, and in preventing widespread engagement of cytokine receptors and activation of inflammatory cells in otherwise unaffected tissues (unless, perhaps, the animal becomes septicemic).

### Implications

The results of the current study clearly suggest that infection of young pigs with *Actinobacillus pleuropneumoniae* is associated with pathophysiologic symptoms of disease, in particular inappetence and an unmistakable and prolonged surge in serum cortisol, and that both of these symptoms occurred in the absence of changes in selected systemic cytokines in the first 24 h following infection.

### Literature Cited


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Figure 5. Serum interferon gamma (IFNγ) in pigs infected with *Actinobacillus pleuropneumoniae* (n = 3) or given sterile growth media (n = 3) intranasally. Data are least squares means ± SEM.
Circulating cytokines in pigs with pneumonia


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