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# Cytokines and acute phase proteins associated with acute swine influenza infection in pigs

Filip Barbé, Kalina Atanasova, Kristien Van Reeth *

*Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium*

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ABSTRACT

This study set out to investigate the cytokines and acute phase proteins (APPs) associated with the acute stages of experimentally-induced swine influenza virus (SIV) infection in 3-week-old, colostrum-deprived, caesarean-derived piglets. The piglets were inoculated intratracheally with $10^{7.5}$ 50% egg infective dose [$EID_{50}$] Swine/Belgium/1/98 (H1N1) SIV and were euthanased at time-points between 0 and 120 h post-inoculation (PI). Broncho-alveolar lavage fluid (BALF), lung homogenates and sera were examined for inflammatory mediators by bioassay or ELISA. Interferon (IFN)-α, interleukin (IL)-6, IL-1 and tumour necrosis factor (TNF)-α peaked in BALF 24–30 h PI, when virus titres and the severity of clinical signs were maximal.

Whereas IFN-γ and IL-12, but not IL-18, increased in tandem in BALF, serum cytokine concentrations were either undetectable or were up to 100-fold lower. The APP C-reactive protein (CRP) and haptoglobin peaked 24 h later than the cytokines and reached higher levels in serum than in BALF. In contrast, lipopolysaccharide (LPS)-binding protein (LBP) only increased in BALF. Lung virus titres tightly correlated with BALF IFN-α, IL-6, IL-1, TNF-α, IFN-γ and IL-12, as well as with serum IL-6, IFN-α and IFN-γ. Signs of disease correlated with the same cytokines in BALF and serum, as well as with BALF LBP and serum CRP. The findings suggest that IFN-γ and IL-12 play a role in the pathogenesis of SIV and that APPs are induced by cytokines. This influenza infection model may have value in assessing the therapeutic potential of cytokine antagonists.

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## Introduction

Swine influenza virus (SIV) is a major cause of acute respiratory disease in pigs (Loeffen et al., 1999). Clinical signs include high fever, depression, anorexia and laboured abdominal breathing (Shope, 1931; Olsen et al., 2006), and the virus replicates in epithelial cells of the nasal mucosa, tonsils, trachea and the lungs, the major target organ. Infected susceptible pigs exhibit extensive gross lung lesions with microscopic evidence of necrosis, desquamation of bronchiolar epithelium and neutrophil infiltration (Olsen et al., 2006). Infection is generally limited to the respiratory tract and attempts to demonstrate viraemia or virus replication outside the tract have been largely unsuccessful.

Swine influenza can be reproduced experimentally by intratracheal (IT) inoculation of naïve pigs with a high dose of virus ($10^{7.5}$ 50% egg infective dose [$EID_{50}$]). This results in respiratory and more systemic clinical signs, high lung titres of virus ($\geqslant 10^{8.0}$ 50% tissue culture infective dose [$TCID_{50}$]/g), the activation of several pro-inflammatory cytokines and in neutrophils making up >50% of the broncho-alveolar lavage fluid (BALF) cells 1 day post-inoculation (PI). Experimental infection with representative H1N1, H3N2 and H1N2 SIV strains demonstrated highly significant correlations between clinical signs and the levels of interferon (IFN)-α, interleukin (IL)-6 and tumour necrosis factor (TNF)-α in BALF (Van Reeth et al., 2002). In contrast, correlation between signs of disease and IL-8 or IL-1 was much weaker.

The clinical signs and pathology of influenza in pigs are similar to those in humans and the cytokine profile in the BALF of affected pigs is similar to that found in nasal lavage fluids of experimentally-infected human volunteers (Hayden et al., 1998). Given that pigs are naturally susceptible to the same influenza A virus subtypes (H1N1, H3N2, H1N2) as humans, and that they have similar physiological features, this species may form a valuable experimental model to study the pathogenesis of influenza (Kuiken and Taubenberger, 2008).

IL-12, IL-18 and IFN-γ may also play a role in the pathogenesis of influenza; IL-12 is a potent regulator of cell-mediated immune responses such as proliferation of and IFN-γ production by T- and NK-cells (Gately et al., 1991; Monteiro et al., 1998). IFN-γ has numerous immunological functions such as enhancement of

* Corresponding author. Tel.: +32 9 264 73 69; fax: +32 9 264 74 95.
*E-mail address:* kristien.vanreeth@UGent.be (K. Van Reeth).

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MHC expression and anti-viral activity and IL-18 is an IFN-γ-inducing factor (Konishi et al., 1997).

Intranasal inoculation of human volunteers with A/Texas/36/91 (H1N1) resulted in an increase in IFN-γ in nasal lavage fluid 2–5 days PI (Fritz et al., 1999). In mice inoculated with A/PuertoRico/8/34 (H1N1), both IFN-γ and IL-12 were found in BALF 3–7 days PI (Monteiro et al., 1998) and a study in IL-18 'knock-out' mice suggested a role for IL-18 in viral clearance from the lungs and in the induction of IFN-γ (Denton et al., 2007). However, the activities of these cytokines in a swine influenza infection model have not been studied (Jung et al., 2004).

Acute phase proteins (APPs) such as haptoglobin (HG), lipopolysaccharide (LPS)-binding protein (LBP) and C-reactive protein (CRP) are produced by the liver in response to cytokine activity and play a role in the pathogenesis of respiratory disease (Moshage et al., 1988). Haptoglobin binds free haemoglobin thus removing it from the circulation, LBP binds and neutralises LPS and CRP has roles in macrophage activation and opsonisation. The cytokines IL-1, IL-6 and TNF-α appear to be the main inducers of APPs (Petersen et al., 2004). Following experimental influenza virus infection of human volunteers, CRP in sera peaked 3 days PI (Whicher et al., 1985), and in natural infection CRP is significantly higher in acute relative to convalescent sera (Falsey et al., 2001; Melbye et al., 2004). Haptoglobin concentrations peaked 7–10 days PI in horses following experimental infection with influenza virus (Kent and Goodall, 1991) and in horses naturally infected with equine influenza virus, serum amyloid A was elevated during the acute stage of the disease (Hulten et al., 1999). To our knowledge the activities of APPs have not been studied in pigs with influenza.

The objectives of this study were to investigate the cytokine and APP responses in the lungs and circulation of SIV-infected pigs and to correlate these responses with lung virus titres and inflammation. The findings will enhance our understanding of the pathogenesis of influenza virus infection in pigs and other species.

## Materials and methods

### *Preparation of virus inoculum*

A SIV Swine/Belgium/1/98 (H1N1) strain had been isolated from the lungs of fattening pigs during an outbreak of acute respiratory disease. The stock used for inoculation represented the third passage in eggs. Inoculations of $10^{7.5}$ $EID_{50}$ of virus in 3 mL of phosphate-buffered saline (PBS) were given IT, using a 20-gauge needle attached to a syringe inserted through the skin cranial to the thoracic inlet.

### *Experimental design and BALF cell analysis*

All experimental procedures were approved by The Local Ethical Committee of The Faculty of Veterinary Medicine, Ghent University (authorisation reference number EC 2005/88).

Sixteen, 3-week-old caesarean-derived, colostrum-deprived pigs were used. The pigs were from two sows and were housed in Horsefall-type isolation units with positive-pressure ventilation and were fed ultra-high-temperature-treated cow's milk supplemented with antibiotics. Two pigs were sham-inoculated with PBS and euthanased the next day. The 14 remaining animals were inoculated IT with SIV and euthanased at 24 h ($n = 3$), 30 h ($n = 2$), 48 h ($n = 3$), 72 h ($n = 3$) and 120 h ($n = 3$) PI. Euthanasia was by intravenous injection of sodium pentobarbital (Natrium Pentobarbital 20%, Kela). A 'score' of clinical disease severity was attributed to each animal just prior to euthanasia based on each of the following clinical signs: anorexia, depression and coughing whereby one point was assigned for the presence of each of these signs. Where the respiration rate was 60–90/min and >90/min, one and two additional points were added to the clinical score, respectively.

At euthanasia, blood samples were collected, the whole lung was excised and the right lung lavaged with cold PBS to obtain BALF (Van Reeth et al., 1999). Samples of the left lung lobes were pooled and tissue homogenates prepared for virus titration and cytokine quantification. The BALF was separated into cells and cell-free fluids by centrifugation. Cell-free BALF was concentrated 20-fold by dialysis against polyethylene glycol, was cleared of residual virus by centrifugation and was used to measure cytokine and APP concentrations (see below). Total numbers of BALF cells were counted using a Türk chamber. Cytocentrifuge preparations were stained using Diff-Quik (Medion Diagnostics) to determine neutrophil numbers.

**Table 1**
Details of clinical scores at euthanasia, lung virus titres and inflammatory changes in broncho-alveolar lavage fluid (BALF) following intratracheal inoculation of pigs with swine influenza virus. PI, post-inoculation.

| PI (h) | *n* | Mean clinical score ± SD | Mean virus titre ± SD (log10 $TCID_{50}$/g) | Mean cell numbers in BALF (x$10^6$) ± SD | | |
|---|---|---|---|---|---|---|
| | | | | Total | Neutrophils | Mononuclear cells |
| 0 | 2 | 0.0 ± 0.0 | <1.7 ± 0 | 131 ± 18 | 1 ± 0 | 130 ± 18 |
| 24 | 3 | 1.7 ± 1.5 | 9.6 ± 0.3 | 458 ± 310 | 332 ± 336 | 126 ± 50 |
| 30 | 2 | 3.5 ± 0.7 | 9.2 ± 0.2 | 614 ± 303 | 481 ± 211 | 133 ± 92 |
| 48 | 3 | 1.7 ± 1.2 | 8.7 ± 0.3 | 331 ± 131 | 114 ± 113 | 217 ± 122 |
| 72 | 3 | 0.0 ± 0.0 | 7.5 ± 0.7 | 118 ± 24 | 11 ± 3 | 107 ± 26 |
| 120 | 3 | 0.0 ± 0.0 | 5.8 ± 0.6 | 228 ± 18 | 15 ± 5 | 213 ± 20 |

### *Virological examination and quantification of cytokines*

Virus titration of lung homogenates was carried out in Madin–Darby canine kidney cells (Van Reeth et al., 2002) and the virus titres calculated as previously described (Reed and Muench, 1938). IFN-α, TNF-α, IL-1 and IL-6 were quantified in sera, BALF and lung homogenates by bioassay as previously described (Van Reeth et al., 1999, 2002). In summary, IFN-α was quantified using a cytopathic effect reduction test with Madin-Darby bovine kidney cells and vesicular stomatitis virus. TNF-α concentrations were determined using a cytotoxicity test in porcine kidney sub-clone 15 (PK 15) cells and IL-1 and IL-6 activity was measured using proliferation assays in D10(N4)M and B9 cells, respectively. All bioassays were repeated twice or thrice and geometric means of the concentrations were calculated.

Assay specificity was demonstrated by neutralising the samples with specific antibodies for IFN-α, TNF-α and IL-6 or by pre-incubation of the D10(N4)M cells with IL-1 receptor antagonist. Various ELISAs were used to determine the concentrations of IFN-γ (Swine IFN-γ ELISA, Biosource), IL-18 (Pig IL-18 ELISA, BenderMed Systems) and IL-12 (Porcine IL-12/IL-23 p40 ELISA, R&D Systems). The detection limits of these assays were 2, 23 and 18 pg/mL, respectively. All samples were tested in duplicate according to manufacturers' instructions.

### *Quantification of acute phase proteins*

C-reactive protein, HG and LBP were measured in serum and BALF and the concentrations of CRP (Phase Range Porcine C-reactive Protein Assay, Tridelta Development Ltd.) and LBP (LBP ELISA, Hycult Biotechnology) were determined by ELISA. A colorimetric assay was used to measure HG (Phase Range Porcine Haptoglobin Assay, Tridelta Development Ltd.). The detection limits for CRP, LBP and HG were 47 ng/mL, 1.6 ng/mL and 50 μg/mL, respectively. All samples were tested in duplicate according to manufacturers' instructions.

### *Statistical analysis*

Spearman rank correlation coefficients ($\rho$) were calculated to compare individual cytokine and APP levels in the lungs and circulation, lung virus titres, neutrophil numbers in BALF and clinical scores. A $P$-value <0.01 was taken as significant. Standard Mann–Whitney tests were used to compare cytokine or APP concentrations between SIV-inoculated and control pigs.

## Results

### *Clinical signs, virus titres and lung inflammation*

The PBS-inoculated control pigs did not exhibit clinical signs, did not have virus in their lungs and had negligible numbers of neutrophils ($1 \times 10^6$) in BALF. In SIV-inoculated animals, clinical signs consisted of depression and tachypnoea with abdominal breathing. These signs peaked 24–30 h PI and had completely resolved 72 h PI (Table 1). Lung virus titres were maximal 24–30 h PI ($10^{9.6}$ $TCID_{50}$/g) and had decreased substantially by 5 days PI ($10^{5.8}$ $TCID_{50}$/g). Increased numbers of neutrophils were noted in BALF 24–30 h PI whereas numbers of mononuclear cells remained constant (Table 1). Neutrophil numbers had returned to baseline values by 5 days PI (8–20 $\times 10^6$).

### *Cytokine profile in BALF, serum and lung tissue*

The concentrations of bioactive IFN-α, IL-6, TNF-α and IL-1 in BALF, serum and lung tissue are illustrated in Fig. 1. IFN-α, TNF-α,

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