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Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity

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SUMMARY

The acute stages of infection with swine influenza virus (SIV), porcine respiratory coronavirus (PRCV) and porcine reproductive-respiratory syndrome virus (PRRSV) were shown to differ in terms of clinical and lung inflammatory effects and proinflammatory cytokine profiles in bronchoalveolar lavage (BAL) fluids. Caesarian-derived colostrum-deprived pigs were inoculated intratracheally with one of the three viruses. SIV infection was followed within 1 day post inoculation (d PI) by characteristic respiratory and general signs, and excessive lung epithelial desquamation and neutrophil infiltration (38 to 56 per cent of BAL cells at 1 d PI vs 0 to 1 per cent in controls). High concentrations of bioactive interferon-α (IFN-α), tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) coincided with peak symptoms and neutrophil infiltration. PRCV infection was asymptomatic and produced a mild bronchointerstitial pneumonitis and neutrophil infiltration (13 to 22 per cent of BAL cells at 4 d PI). IFN-α titres parallelled those found during SIV infection, TNF-α was negligible and IL-1 undetectable. PRRSV infection induced anorexia and lethargy between 3 and 5 d PI. There was marked infiltration with mononuclear cells in alveolar septa and BAL fluids between 7 and 10 d PI, while neutrophils remained at less than 11 per cent of BAL cells at any time. IL-1 was produced from three throughout 10 d PI, while IFN-α production was minimal and TNF-α undetectable. These data strongly suggest that proinflammatory cytokines can be important mediators of viral respiratory disease.

SWINE influenza virus (SIV), porcine respiratory coronavirus (PRCV) and porcine reproductive-respiratory syndrome virus (PRRSV) are ubiquitous in swine producing areas of Europe. All three viruses are transmitted via the respiratory route and infect the pig lung, but they differ in terms of lung target cells and replication kinetics. SIV and PRCV replicate principally in lung epithelial cells and cause acute, self-limiting infections (Haesebrouck and Pensaert 1986, O'Toole et al 1989, Cox et al 1990, Brown et al 1993). PRRSV, on the other hand, has a predilection for lung alveolar macrophages, and persists in these cells during at least 3 to 4 weeks (Duan et al 1997). The clinical importance of these infections is frequently a matter of debate. Still, critical analysis of field and experimental data indicates differences in pathogenicity between these viruses. SIV has been associated with outbreaks of fever, anorexia, tachypnoea, dyspnoea and coughing; and the typical clinical picture has been reproduced experimentally (Haesebrouck and Pensaert 1986). PRCV or PRRSV infections on swine farms frequently pass without being noticed. Most experimental PRCV infections have remained asymptomatic (O'Toole et al 1989, Cox et al 1990). Similarly, after experimental inoculation of European PRRSV isolates, respiratory disease has been difficult to reproduce and a transient fever was the most consistent clinical manifestation (Paton et al 1992, Nielsen and Botner 1997).

Cytokines produced locally at the site of infection regulate many of the host responses to respiratory pathogens. Interferon-α (IFN-α), tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are the first cytokines to be produced following pulmonary virus infection (reviewed by Bielefeldt-Ohmann 1995). Both TNF-α and IL-1 induce a striking increase in intra-alveolar neutrophils when injected intratracheally into rats, and they exert profound stimulating effects on neutrophil and macrophage functions. IFN-α also stimulates a variety of phagocyte functions (reviewed by Tizard 1995). In addition, all three cytokines share systemic effects known as the 'acute phase response', such as fever, lethargy and anorexia. Severe lung inflammation and disease result in particular when cytokines occur at high levels or in combination with each other (Murtaugh et al 1996). In experimental infections of pigs with *Mycoplasma hyopneumoniae* (Asai et al 1993) or with *Actinobacillus pleuropneumoniae* (Baarsch et al 1995), lung neutrophil infiltration and pathology have been associated with TNF-α and IL-1. As for virus infections of the pig lung, the possible involvement of cytokines is largely unexplored.

In this study, we compared SIV, PRCV and PRRSV for clinical manifestations and for lung proinflammatory cytokine profiles in a gnotobiotic pig model. In addition, we tried to identify correlations between these parameters.

MATERIALS AND METHODS

Virus strains

The A/Sw/Belgium/1/83 (H1N1) strain of influenza virus was used at the 3rd passage in embryonated eggs, the 91V44

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TABLE 1: Results of virus titration of lung tissue at various times after inoculation with SIV, PRCV or PRRSV

Inoculation	Time post inoculation	Number of pigs	Range of virus titres ($log_{10}ID_{50}g^{-1}$ lung)
PBS	1d	3	–
SIV	18h	4	8.2–9.7
SIV	1d	3	8.4–9.0
SIV	2d	3	7.7–8.2
SIV	3d	2	6.5–7.0
PBS	1d	4	–
PRCV	1d	2	5.6–6.0
PRCV	2d	2	6.6–6.6
PRCV	3d	4	7.3–8.3
PRCV	4d	4	7.5–8.5
PBS	1d	3	–
PRRSV	3d	2	3.4–4.2
PRRSV	5d	3	4.2–6.7
PRRSV	7d	3	5.5–6.7
PRRSV	9d	2	5.0–7.0
PRRSV	10d	2	5.0–5.8

PBS, phosphate-buffered saline; h, hours; d, days(s); -,no virus isolated

strain of PRCV was at the 2nd passage in swine testis cells (Van Reeth and Pensaert 1994), and the Lelystad virus strain of PRRSV (kindly provided by Dr. Wensvoort, Institute for Animal Science and Health, The Netherlands) was at the 5th passage in primary alveolar macrophages (AMS) from gnotobiotic pigs. Pig inoculation doses were $10^{7.5}$ EID_{50} of SIV, $10^{7.0}$ $TCID_{50}$ of PRCV, and $10^{6.0}$ $TCID_{50}$ of PRRSV.

Pigs and experimental design

Forty-six 3- to 4-week-old caesarian-derived colostrum-deprived (CDCD) pigs were used (Table 1). Three groups of 12 pigs each were inoculated intratracheally with SIV, PRCV or PRRSV and killed between 18 hours (h) and three days post inoculation (d PI), between 1 and 4 d PI, and between 3 and 10 d PI, respectively. These times were selected to represent the acute stages of the respective virus infections. Ten littermates of virus-inoculated pigs were mock-inoculated with phosphate-buffered saline (PBS) and killed after 24 h.

All pigs were monitored for respiratory and general signs. At necropsy, gross lung lesions were recorded. Samples from the left diaphragmatic lung lobe were collected for standard histopathology, virus titrations, fluorescent antibody stainings (Van Reeth and Pensaert 1994, Duan et al 1997), and standard bacteriologic examination. The right lung was used for bronchoalveolar lavage.

Bronchoalveolar lavage (BAL) and BAL cell analysis

The right lung was lavaged with 60 ml cold Dulbecco's PBS without Ca^{2+} and Mg^{2+} (Gibco) via an 18-gauge blunt needle inserted through the trachea. Recovered BAL fluids (45–47 ml) were separated into cells and supernatants by centrifugation (400 **g**, 10 min, 4°C). Cells were counted using a Türk chamber and cytocentrifuge preparations were stained with DiffQuik (Baxter, Düdingen, Switzerland) to determine percentage of neutrophils. Cell-free BAL fluids were concentrated 20 times by dialysis against a 20 per cent w/v solution of polyethylene glycol (MW 20,000), cleared of residual virus by ultracentrifugation at 100,000 *g*, aliquoted and stored at –70°C, until analysis in cytokine bioassays.

Cytokine bioassays

IFN-α, TNF-α and IL-1 concentrations in BAL fluids were determined by bioassay and expressed in biological units ml^{-1} BAL fluid. Bioassay data were corrected using laboratory standards with assigned potencies. Samples were tested in three (IFN-α, TNF-α) or 2 (IL-1; duplicates of samples) independent assays, and geometric mean titres of the corrected values were calculated.

IFN titres were determined in a cytopathic effect (CPE) reduction assay using Madin-Darby bovine kidney (MDBK) cells and vesicular stomatitis virus (VSV) (La Bonnardière and Laude 1981). MDBK cells were seeded at 2.5×10^5 cells per well in 96-well microtitre plates in 100 µl Dulbecco's modified Eagle medium with 10 per cent fetal calf serum (FCS; Gibco), 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 100 µg ml^{-1} kanamycin. Following overnight incubation (37°C, 5 per cent CO_2), the medium was removed, and 100 µl serial two-fold dilutions of samples were added. After a further 18 hours incubation, samples and virus control wells were challenged with 50 µl VSV and cell control wells received 50 µl of medium. Two days later, media were decanted, 50 µl of a 0.1 per cent neutral red solution was added and plates were further incubated for 1 h. Stained cells were rinsed, air dried and 150 µl dissolving solution (50 µl sodium dodecyl sulphate (SDS), 100 µl 0.2 M HCl in H_2O) was added. Optical densities (ODS) were read at 498 nm in a Titertek Multiskan. One unit of IFN activity was defined as the reciprocal of the dilution producing 50 per cent inhibition of CPE. To assign the antiviral effect to IFN-α, samples were incubated with a rabbit antiserum against recombinant porcine IFN-α (gift from C. La Bonnardière, INRA, Jouy en Josas, France).

TNF-α was determined in a cytotoxicity assay in PK(15) subclone 15 cells (Bertoni et al 1993). PK(15) cells were seeded at 2.5×10^4 cells per well in microtitre plates in 100 µl Eagle's minimum essential medium with 7 per cent FCS, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. Following overnight incubation (37°C, 5 per cent CO_2), the medium was removed and 100 µl basal Iscove's medium containing bactopeptone and actinomycin D (final culture concentrations of 0.25 per cent (w/v) and 1.5 µg ml^{-1} respectively) was added and incubated for 2 hours. Next, 50 µl of serial two-fold dilutions of samples or 50 µl medium (cell controls) were added and the plates were incubated for a further 18–20 hours at 39.5°C, 5 per cent CO_2. To determine percentage cytotoxicity, the cells were washed and stained with 0.5 per cent crystal violet in 22 per cent ethanol-8 per cent formaldehyde for 4 minutes. Stained cells were rinsed, air dried and solubilized in 100 µl of 33 per cent acetic acid. ODS were read in a Titertek Multiskan at 550 nm. One unit of TNF activity was defined as the reciprocal of the dilution producing 50 per cent cytotoxicity. Cytotoxic activity was confirmed to be TNF-α by neutralization of samples with rabbit anti-human TNF-α (Innogenetics, Zwijndrecht, Belgium).

IL-1 activity was determined in a proliferation assay using D10(N4)M cells (Hopkins and Humphreys 1989). Before assay, all samples were treated with a 24 per cent w/v polyethylene glycol (MW 8,000) (Sigma, St Louis, MO) solution in RPMI to remove non-specific inhibitors (Hopkins and Humphreys 1990). Serial two-fold dilutions of samples were

made in RPMI 1640 containing 10 per cent FCS, 0.5×10^{-4} M 2-mercaptoethanol and 50 µg ml^{-1} gentamicin and 150 µl volumes were added to microtitre plates. Cell controls received 150 µl of medium. D10(N4)M cells at a concentration of 1×10^4 cells per well in 100 µl RPMI with recombinant human IL-2 (Genzyme, Cambridge, MA) and concanavalin A (Sigma) (final culture concentrations of 10 U ml^{-1} and 2 µg ml^{-1} respectively) were incubated with the cells during 72 hours (37°C, 5 per cent CO_2). Cell proliferation was measured by the thiazolyl blue (MTT) conversion procedure: 50 µl of a 3 mg ml^{-1} solution of MTT (Sigma) was added to the wells and further incubated during 18 h. Thereafter, 200 µl of medium was removed and 150 µl dissolving solution (0.5 per cent SDS, 36 mM HCl in isopropanol) added to each well. The resulting formazan crystals were dissolved by 15 minutes sonication in an ultrasonic water bath and ODS were read at 550 nm in a Titertek Multiskan. One unit of IL-1 was defined as the reciprocal of the dilution producing 50 per cent proliferation of D10(N4)M cells. To assign bioactivity to IL-1, monoclonal rat anti-mouse IL-1 receptor type 1 antibodies (Genzyme) were included in the assay.

Statistics

Standard two-sample *t*-tests were used to compare virus titres or total and mononuclear BAL cell numbers at various times during each virus infection, and Kruskal-Wallis rank sum tests were used for comparison of cytokine titres. Spearman rank correlation tests were used to compare cytokine titres and other parameters. $P<0.05$ was considered significant.

RESULTS

Clinical signs

Uninoculated control and PRCV-inoculated pigs remained healthy. SIV-inoculated pigs developed typical respiratory and general signs. By 24 h PI, seven out of eight pigs showed moderate to severe tachypnoea, dyspnoea, anorexia, lethargy and shivering. Two and 3 d PI these symptoms started to resolve, but coughing was noticed. PRRSV-inoculated pigs developed anorexia and lethargy between 3 and 5 d PI, with pigs of one of the three groups being more severely affected. None of the PRRSV-infected pigs showed respiratory signs.

Virus replication

The lungs of control pigs were negative for viruses. SIV-, PRCV- and PRRSV-inoculated pigs showed distinct kinetics of virus production (Table 1) and patterns of viral antigen expression. The localization of virus-positive cells was in accordance with that in previously published reports (Haesebrouck and Pensaert 1986, Cox et al 1990, Duan et al 1997).

SIV titres were highest 18 h (8.2 to 9.7 $\log_{10}$ $TCID_{50}$ g^{-1} lung) and 1 d PI (7.1 to 8.7 $\log_{10}$ $TCID_{50}$), and dropped significantly 2 and 3 d PI ($P<0.049$). By immunofluorescence (IF), SIV antigen was visualised in all sections examined. An approximate 90 per cent of bronchial/bronchiolar and 30 per cent of alveolar epithelial cells were positive at 18 h and 1 d PI. By 2 and 3 d PI, large areas of bronchiolar epithelia were denuded, but fluorescence in the alveoli still increased.

PRCV titres were higher at 3 (7.3 to 8.3 $\log_{10}$ $TCID_{50}$) and 4 (7.5 to 8.5 $\log_{10}$ $TCID_{50}$) d PI than at 1 and 2 d PI ($P<0.001$). The extent of IF was limited 1 and 2 d PI, and gradually increased at 3 and 4 d PI. At that time, between 20 and 30 per cent of the alveolar tissue and a few epithelial cells of smaller bronchioli were found PRCV-positive.

PRRSV titres tended to increase more slowly and remained at a plateau between 5 (4.2 to 6.7 $\log_{10}$ $TCID_{50}$) and 10 (5.0 to 5.8 $\log_{10}$ $TCID_{50}$) d PI. Fluorescence remained focal and did not encompass more than 15 to 20 per cent of the lung at any time PI. PRRSV antigen was found exclusively in AM-type cells in the alveolar interstitia.

The lungs of all pigs were culture-free of bacteria.

Lung pathological changes

Mean total BAL cell, mononuclear and neutrophil cell numbers in pigs killed at various times during SIV, PRCV and PRRSV infection and in mock-inoculated controls are shown in Fig. 1.

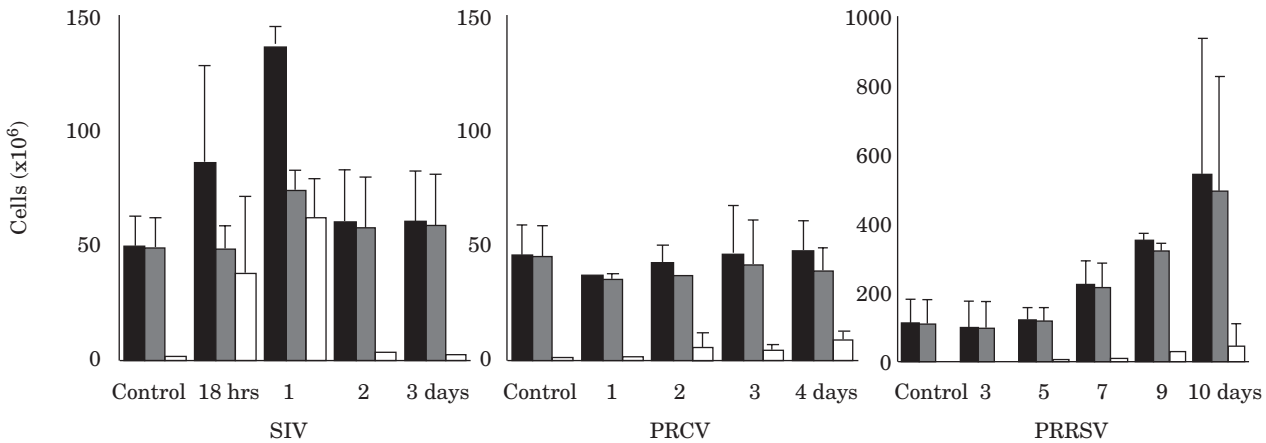

FIG 1: Numbers of total bronchoalveolar lavage cells (■), mononuclear cells (▩), and neutrophils (□) at various times post infection with SIV, PRCV or PRRSV. All values are mean values with standard deviation, where applicable.

Controls of SIV-, PRCV- and PRRSV-infection groups had mean total BAL cell counts of 49.5, 46 and 110×10^6 respectively. In all control pigs, ≥ 98 per cent of BAL cells had macrophage morphology, and ≤ 2 per cent of cells were neutrophils. Microscopic or macroscopic lung lesions were absent.

SIV infection produced a 2- to 3-fold increase in total BAL cells within 1 d PI ($P < 0.001$ at 1 d PI), which was largely due to neutrophil infiltration (18 to 60 per cent and 38 to 56 per cent of BAL cells at 18 h and 1 d PI respectively). Two and 3 d PI, total cell and neutrophil numbers had mostly returned to normal. Histopathologic examination of lung tissue sections revealed degeneration of large areas of bronchiolar epithelia within 1 d PI, and a massive neutrophil infiltration, as previously observed by Haesebrouck and Pensaert (1986). Two and 3 d PI, neutrophils had largely disappeared and deposits of proteinaceous debris with necrotic cells filled bronchioli and alveoli. Gross lung lesions, involving 4 to 18 per cent of the total lung, were seen 2 and 3 d PI.

After PRCV infection, total and mononuclear cell numbers remained unchanged ($P > 0.839$ and 0.474 respectively). A slight increase in neutrophils occurred 3 and 4 d PI (6 to 18 per cent and 13 to 22 per cent of BAL cells respectively). At that time, histopathological lung changes involved a mild to moderate, multifocal airway epithelial necrosis, and infiltration with macrophages and neutrophils as described by Cox et al (1990). Eosinophilic staining material with necrotic cells was occasionally observed in alveolar ducts – respiratory bronchioli. Gross lung lesions increased progressively between 2 and 4 d PI, when they ranged from 5 to 34 per cent of the lungs.

Following PRRSV infection, total BAL and mononuclear cell numbers were significantly higher 7–10 d PI than at 3–5 d PI ($P < 0.040$ and 0.034 respectively). Mononuclear cells could not be differentiated confidently from lymphoblasts, but ≥ 85 per cent of cells morphologically resembled monocytes-macrophages. Neutrophils remained at less than 11 per cent of BAL cells at any time PI. On histopathology, the lesions were typical of experimental PRRSV infection (Paton et al 1992, Collins et al 1992). There was marked thickening of alveolar septa with mononuclear cells from 5 d PI on, but there was little evidence of lung epithelial damage. Macroscopic lung changes were characterised by a failure to collapse, and a mild interlobular oedema. Lung consolidation was rare and involved less than 3 per cent of the lungs.

Cytokine profiles

IFN-α, TNF-α and IL-1 were undetectable in BAL fluids of control pigs. Figure 2 displays cytokine levels in the individual SIV-, PRCV-, or PRRSV-infected pigs.

SIV infection was followed by the concurrent production of all three cytokines within 18 h to 1 d PI in all seven pigs examined. In these pigs, IFN-α titres were between 27800 and 337400 U, and TNF-α and IL-1 titres were between 30–225 U and 108–520 U respectively. By 2 and 3 d PI, IFN-α titres had decreased significantly ($P < 0.019$), and TNF-α and IL-1 were undetectable. Production of all three cytokines was correlated with neutrophils in BAL fluids ($P < 0.001$ for each cytokine) and coincided with the onset of typical flu symptoms and lung pathological changes.

PRCV infection induced IFN-α from 1 throughout 4 d PI, in all 12 pigs examined. Peak IFN-α titres were similar to those seen after SIV infection, but there was considerable individual variation in IFN-α levels at set times PI. TNF-α appeared 3 and 4 d PI, in three pigs out of eight. Two of these three pigs had TNF-α titres near to the detection limit of the bioassay (40 and 57 U respectively). IL-1 was undetectable.

PRRSV infection induced IFN-α from three throughout 10 d PI in all 12 pigs. Peak IFN-α titres were at least 1000-fold lower than those found during SIV or PRCV infection. TNF-α was undetectable. Substantial amounts of IL-1 were found from three throughout 10 d PI, in nine pigs out of 12. IL-1 titres were between 41 and 335 U.

DISCUSSION

Several considerations are important with regard to the experimental design and interpretation of this study. Firstly, we selected different times of euthanasia for each virus infection, because of the known differences in replication kinetics. The results of virus titrations of lung tissue later justified this approach. Secondly, we used the highest possible pig inoculation dose of each virus. These infectious doses differed between the three viruses. Thirdly, we included pigs from one single litter in the three virus infection groups where possible, to minimise the effects of litter variations.

The cytokine patterns detected here are compatible with those described in previous reports. We have recently been able to demonstrate for the first time that SIV infection induces high bronchoalveolar levels of INF-α, TNF-α and IL-1 (Van Reeth et al 1998). An early rise of these three cytokines also occurred in the respiratory tract of influenza virus-infected mice or human volunteers (Wyde et al 1982, Vacheron et al 1990, Hayden et al 1998). PRCV infection of 10-week-old conventional pigs, as in this study, induced high BAL IFN-α and minimal TNF-α levels (Van Reeth and Pensaert 1995). Experiments in PRRSV-infected pigs also revealed weak IFN-α titres in BAL fluids (Albina et al 1998) and increased expression of IL-1β in BAL cells (Zhou et al 1992).

Most important, our study documents intrinsic differences in respiratory pathogenicity of SIV, PRCV and PRRSV. SIV, but not PRCV or PRRSV, produced acute respiratory disease, and severe lung necrosis and inflammation. Moreover, SIV symptoms and pathology could be linked with an excessive and concurrent production of IFN-α, TNF-α and IL-1 in the lungs. There was a strong temporal association between peak levels of these cytokines and the onset of typical flu symptoms, lung neutrophil infiltration and pathology. Individual cytokine levels as well correlated largely with clinical severities and lung pathology, as shown in our previous study (Van Reeth et al 1998). Finally, potent synergistic interactions between IFN-α, TNF-α and IL-1 are known, and the 'cytokine triad' will probably exert much greater effects than the individual cytokines. Conversely, the lack of such a 'cytokine combination' during PRCV or PRRSV infection may in part explain the mild respiratory pathology and the absence of respiratory disease.

We found a perfect coincidence of peak SIV replication and cytokine secretion, and cytokines appeared during the very early phase of virus replication. Others have shown

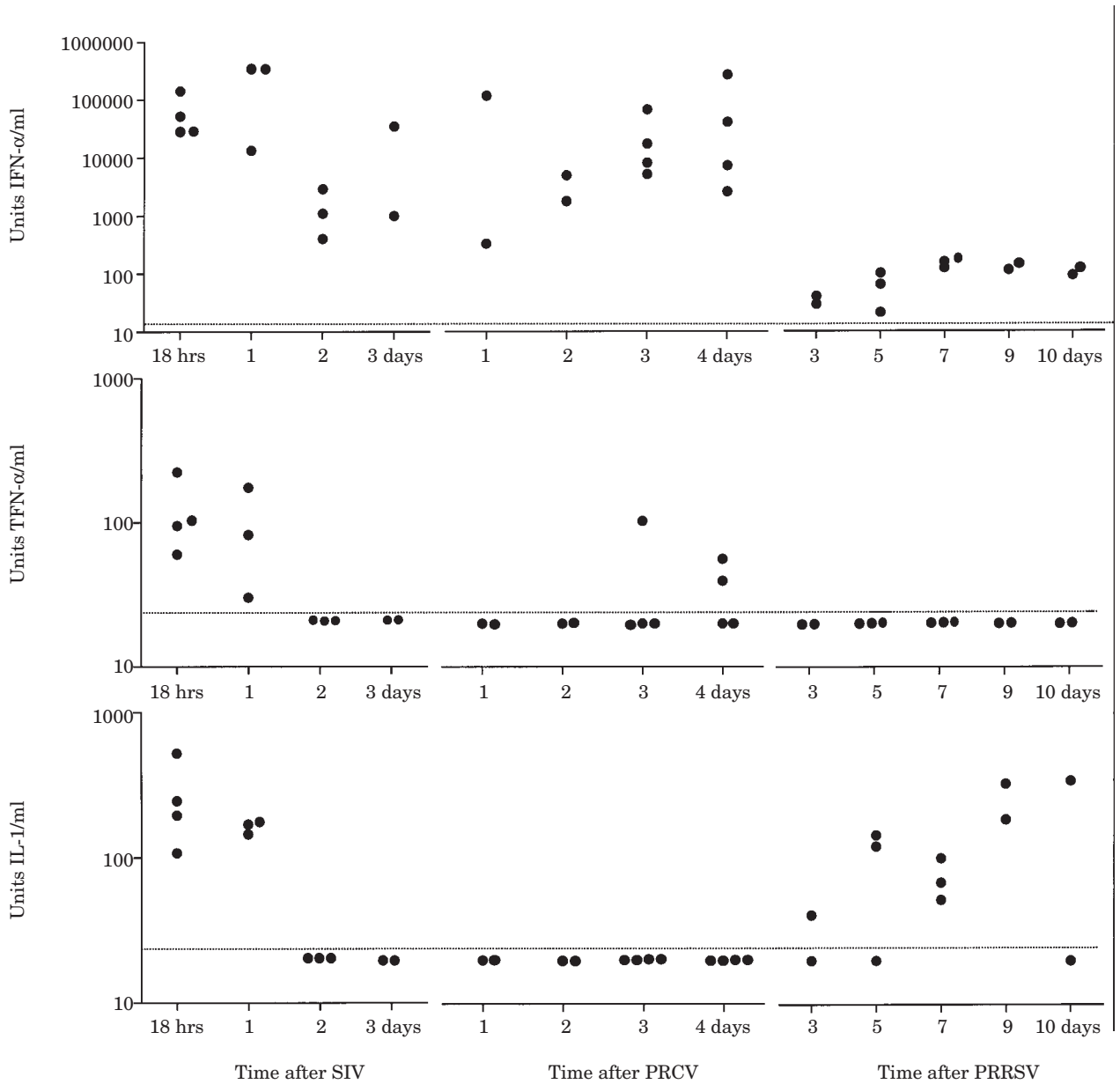

FIG 2: Bioactive levels of interferon-α (IFN-α), tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) in bronchoalveolar lavage (BAL) fluids at various times post infection with SIV, PRCV or PRRSV. Titres are expressed in biological units ml^{-1} BAL fluid. Each dot represents the value for an individual pig. Dotted lines indicate detection limits of the assays (10 U for IFN α; 20 U for TNF-α and IL-1). Cytokines were undetectable in all control pigs (not shown).

that influenza virus induces cultures of mononuclear cells to synthesize IFN-α, TNF-α and IL-1 (Nain et al 1990). In addition, the influenza virus haemagglutinin was the first viral envelope protein that was shown to activate the transcription factor NF-kappa B, which controls gene expression of a series of (pro)inflammatory cytokines (Pahl and Baeuerle 1995). All these data support that SIV directly stimulates lung cells to release cytokines, and that cytokines are not merely a side-effect of cellular destruction or immune activation. The fact that SIV replicates vigorously in the lung may further contribute to a strong cytokine response, for cytokine production is virus dose-dependent.

PRRSV causes minimal airway epithelial damage or gross lung pathology, and as such does not behave like a 'classic' respiratory virus. On the other hand, systemic symptoms and a massive mononuclear cell infiltration of the lung have been found in our and other studies (Paton et al 1992, Collins et al 1992, Nielsen and Botner 1997, Shibata et al 1997). Both features possibly relate to the production of IL-1 during PRRSV infection. Localised production of IL-1 has shown to induce fever and anorexia (Hill et al 1997), and IL-1 is one of the main stimuli for the production of monocyte- attracting chemokines (Zoja et al 1991). Two 'monocyte chemotactic proteins' (MCPs), MCP-1 and MCP-2, have been cloned from porcine cDNA libraries (Hosang et al 1994 a, b), but their in-vivo production or significance has not yet been demonstrated, nor has their significance. It is our working hypothesis that chemokines produced by PRRSV-infected AMS mediate the influx of new monocytes-macrophages, which in turn serve as targets for virus infection and sources of chemokines.

In conclusion, our findings indicate that there is differential cytokine production during different respiratory virus infections. Furthermore, they strongly suggest that proinflammatory cytokines can be important mediators during viral respiratory disease. Further studies are needed to define the roles of specific cytokines in symptom formation and lung pathology. Anti-cytokine strategies in pigs are still dreams of the future, we thus have to search for alternative and creative experimental approaches.

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