Changes of leukocyte phenotype and function in the broncho-alveolar lavage fluid of pigs infected with porcine reproductive and respiratory syndrome virus: a role for CD8+ cells

Janneke N. Samsom, Tiny G. M. de Bruin, John J. M. Voermans, Janneke J. M. Meulenberg, Jan M. A. Pol and Andre T. J. Bianchi

Department of Mammalian Virology, Institute for Animal Science and Health, PO Box 65, NL-8200 AB Lelystad, The Netherlands

Porcine reproductive and respiratory virus (PRRSV) primarily infects and destroys alveolar macrophages of the pig. The aim of the present study was to characterize the changes of leukocyte populations in the broncho-alveolar lavage fluid (BALF) of PRRSV-infected pigs. Piglets were inoculated intranasally with PRRSV strain LV ter Huurne. On various days post-infection the piglets were sacrificed and the lungs removed, washed semi-quantitatively and analysed by flow cytometry. The total number of recovered BALF cells increased approximately 10 times between day 10 and day 21 of infection and decreased thereafter. The number of small low-autofluorescent cells (SLAC), i.e. lymphocytic and monocytic cells, increased very strongly from day 2 until day 21 of infection; in contrast, the number of large highly autofluorescent cells (LHAC), i.e. mostly macrophages, remained constant until day 14 of infection, increased slightly on day 21 and then decreased. On day 21 of infection in specific-pathogen-free piglets approximately 60% of the SLAC consisted of CD2+CD8+CD4−γδTCR− cells, which were partly CD8+CD6+ and partly CD8+CD6−. These phenotypes correspond to that of cytotoxic T-cells and natural killer cells respectively. From these results we can conclude that during a PRRSV infection the total number of BALF cells increases mainly due to an influx of lymphocytic cells with a cytolytic phenotype.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-strand RNA virus that belongs to the arterivirus family (Meulenberg et al., 1993; Conzelmann et al., 1993). An important characteristic of the arterviruses is their strong tropism for cells that belong to the monocyte–macrophage lineage (Tong et al., 1977; Plagemann & Moening, 1992; Pol & Wagenaar, 1992; Voicu et al., 1994). PRRSV primarily infects alveolar macrophages of the pig which, in young piglets, may result in severe respiratory distress, whereas in sows infection often leads to reproductive failure (Hill, 1990; Lindhaus & Lindhaus, 1991; Cromwijk, 1991; Paton et al., 1991).

The effect of PRRSV infection on the lungs has been studied extensively. Reports on the pathogenesis of PRRSV in the lungs have revealed the presence of necrotic cell debris in the alveolar lumina and a thickening of the alveolar septa due to infiltration of mononuclear cells during the first week of infection (Pol et al., 1993; Collins et al., 1992; Halbur et al., 1993). The virus can be detected in macrophages in the alveolar septa and in the alveolar spaces (Pol et al., 1993; Halbur et al., 1994). However, even during the acute stage of an experimental infection no more than 2% of the alveolar macrophages stain positive for PRRSV antigen (Duan et al., 1997; Mengeling et al., 1995).

Analysis of the composition of the alveolar cell-population in the lungs of infected pigs yields variable results. Based on histological examination of haematoxylin–eosin-stained lung sections it has been reported that the alveolar lumina are filled with neutrophils and macrophages during the first days of infection (Rossow et al., 1994; Halbur et al., 1996). Similarly, microscopical examination of broncho-alveolar lavage fluid...
(BALF) on day 7 of infection revealed that 35% of the BALF cells were neutrophils, 50% were macrophages and 15% were lymphocytes (Zhou et al., 1992). However, others have reported that the percentage of neutrophils in the BALF-cell population remained low during a 56 day observation period after intranasal (i.n.) infection, whereas the percentage of macrophages decreased and the percentage of lymphocytes increased between days 0 and 28 of infection (Shibata et al., 1997). Furthermore, others report an increase in the number of macrophages in the broncho-alveolar lavage fluid of piglets that were intracheally infected with PRRSV and sacrificed during the first days of infection (Van Reeth & Pensaert, 1997). These contradictory data may be due to a number of factors. Firstly, the porcine alveolar macrophage population is a very heterogeneous population and therefore relatively difficult to study microscopically (Choi et al., 1994). This problem may be avoided by using flow cytometry. With this technique it is possible to identify and quantify subsets of cells within a heterogeneous population by measuring size, granularity and presence of surface markers on the cells (Berndt & Müller, 1997). Secondly, a difference in microbiological status of the pigs may determine their susceptibility to PRRSV infection and may lead to variability in results. For example, it has been suggested that gnotobiotic piglets are more sensitive to PRRSV infection than specific-pathogen-free (SPF) piglets. Also, variation in the composition of the alveolar cell population in pigs with a different microbiological status may affect their response to PRRSV infection. Thirdly, the changes in the alveolar cell population may vary at different times of PRRSV infection. Lastly, most results have focused on relative changes in leukocyte populations, thus neglecting possible changes in absolute numbers.

The aim of our study was to characterize the phenotypic changes of leukocyte populations in BALF of PRRSV-infected piglets using flow cytometry.

Methods

First experiment. Gnotobiotic piglets were born by closed hysterectomy from F1 (Dutch Landrace × Large White Yorkshire) sows. The piglets were housed in stainless steel isolator units at a temperature of approximately 29.5 °C and were fed a commercial sterile milk substitute (Nutricia, Zoetermeer, the Netherlands). The animals were randomly allocated to an infected (n = 9) or a control (n = 9) group. At 7 days of age one group of piglets was infected with 0·5 ml per nostril of PRRSV strain ter Huurne at a TCID<sub>50</sub> of 10<sup>7</sup>/ml. Control SPF piglets received uninfected culture supernatant in a similar manner. On days 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 post-infection the piglets were sacrificed, and the lungs removed and washed semi-quantitatively.

The experiments described in this study were performed according to regulations of the Animal Care Committee of the Institute.

Semi-quantitative broncho-alveolar lavage. Prior to the lavage the weight of each lung was determined. Thereafter, the lung was washed on ice with a fixed volume of ice-cold PBS. After gently squeezing the lung several times to spread the PBS over the alveoli, the BALF was recovered and its volume was determined. The BALF cells were collected by centrifugation at 300 g for 10 min at 4 °C. The cells were washed twice with ice-cold PBS and resuspended in a fixed volume of RPMI 1640 Dutch modification medium (ICN Biomedicals) containing 10% heat-inactivated foetal bovine serum (Integro), 200 U/ml sodium penicillin-G (Yamanouchi), 0·2 mg/ml streptomycin (Biochemie), 0·3 mg/ml l-glutamine (Flow Laboratories) and 5×10<sup>–6</sup> M β-mercaptoethanol (Sigma), hereafter referred to as medium. An aliquot of the cell-suspension was used for virus isolation and the total number of recovered cells per lung was determined. Thereafter, the cells were adjusted to a final concentration of 1×10<sup>6</sup> cells/ml in medium. A portion of the BALF cells was dispensed in aliquots of 50 µl per well in a 96-well V-bottomed microtitre plate (Nunc) for flow cytometric analysis. In addition, cytospin preparations were made and a portion of the remaining cells was used for the induction of cytokine release.

Flow cytometric analysis of BALF cells. BALF cells and PBMC were spun down in the V-bottomed microwell plates by centrifugation (230 g at 4 °C). The supernatant was discarded and the cells were incubated for 20 min on ice with various combinations of murine monoclonal antibodies (MAbs) directed against, or cross-reactive with, porcine leukocyte differentiation antigens (Fig. 1). The MAbs used were: SWC1 (clone 76-6-7, IgM), SWC3 (clone 74-22-15, IgG), MHC II (clone MSA3, IgG2a), CD14 (clone MY4, Ig2b) (Coulter), CD11b (clone C25, IgG2a), CD5 (clone b53b7, IgG1), CD6 (clone a38b2, IgG1), CD3 (clone CVI 517.2, IgG2b), CD13 (clone pp3, IgG1), CD2 (clone MSA4, IgG2a), CD4 (clone 74-12-4, IgG2b), CD8 (clone 295/33 IgG2a), CD5 (clone b53b7, IgG1), CD6 (clone a38b2, IgG1) and γδT-cell receptor (clone pp16, IgG2b); all were diluted to optimal concentrations in PBS containing 2 μg/ml sodium azide (FACS buffer). After incubation the cells were washed three times with PBS buffer. The cells were incubated a second time for 20 min on ice with the appropriate fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated goat anti-murine IgG isotype specific antibodies diluted in FACS buffer. After the second incubation the cells were washed three times and resuspended in FACS buffer. The cells were transferred to tubes and fluorescence was measured using a FACScan. For BALF cells a population of large highly autofluorescent cells and a population of small low-autofluorescent cells were distinguished and analysed separately.

Second experiment. Dutch Landrace piglets aged 8 to 10 weeks were obtained from the SPF herd of the ID-DLO. After 1 week of acclimatization 20 piglets were infected with 0·5 ml per nostril of PRRSV stain ter Huurne at a TCID<sub>50</sub> of 10<sup>7</sup>/ml. Control SPF piglets received uninfected culture supernatant in a similar manner. On days 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 post-infection the piglets were sacrificed, and the lungs removed and washed semi-quantitatively.

The experiments described in this study were performed according to regulations of the Animal Care Committee of the Institute.

First experiment. Dutch Landrace piglets aged 8 to 10 weeks were housed in stainless steel isolator units at a temperature of 29.5 °C and were fed a commercial sterile milk substitute (Nutricia, Zoetermeer, the Netherlands). The animals were randomly allocated to an infected (n = 9) or a control (n = 9) group. At 7 days of age one group of piglets was infected with 0·5 ml per nostril of PRRSV strain ter Huurne at a TCID<sub>50</sub> of 10<sup>7</sup>/ml. Control SPF piglets received uninfected culture supernatant in a similar manner. On days 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 post-infection the piglets were sacrificed, and the lungs removed and washed semi-quantitatively.

The experiments described in this study were performed according to regulations of the Animal Care Committee of the Institute.

Second experiment. Dutch Landrace piglets aged 8 to 10 weeks were obtained from the SPF herd of the ID-DLO. After 1 week of acclimatization 20 piglets were infected with 0·5 ml per nostril of PRRSV stain ter Huurne at a TCID<sub>50</sub> of 10<sup>7</sup>/ml. Control SPF piglets received uninfected culture supernatant in a similar manner. On days 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 post-infection the piglets were sacrificed, and the lungs removed and washed semi-quantitatively.

The experiments described in this study were performed according to regulations of the Animal Care Committee of the Institute.
Results

Experimental design

Results of two different experiments will be described separately. In the first experiment the changes of leukocyte phenotype and function during PRRSV infection in one-week-old gnotobiotic piglets were assessed at one time-point during infection. To assess whether the observations were not exclusive for a PRRSV infection in young gnotobiotic piglets and to study the kinetics of these changes a second experiment was performed in 8 to 10-week-old SPF piglets.

Characterization of BALF cells recovered from the lungs during a PRRSV infection in gnotobiotic or SPF piglets: number, phenotype and cell-associated virus

Gnotobiotic piglets. In one-week-old gnotobiotic piglets the total number of BALF cells isolated from the lung-halves of PRRSV-infected piglets (2.92 ± 1.88 × 10^7) on day 5 of a PRRSV infection was not significantly different from that of control piglets (4.3 ± 2.09 × 10^7).

Tris buffer supplemented with 2% H_2O_2 to eliminate endogenous peroxidase activity prior to immunoperoxidase staining. For the immunoperoxidase staining the sections were incubated for 20 min at room temperature with either anti-PRRSV-nucleoprotein antibody (SDOW 17) or anti-CD8 (clone 295 33, IgG2a), diluted to optimal concentrations in PBS containing 0–2% (v/v) BSA (PBS–BSA). Control sections were stained with an irrelevant MAb of the same isotype. After incubation the sections were washed thoroughly with PBS–BSA and incubated for 20 min at room temperature with peroxidase-labelled rabbit anti-mouse Ig diluted 1/200 in PBS–BSA. The sections were washed and peroxidase activity was visualized by incubation with 0–17 mg/ml 3'3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris–HCl buffer (pH 7.6) supplemented with 0.02% H_2O_2. The sections were counterstained with haematoxylin.

Statistical analysis. For experiment 1 results are expressed as dots for individual animals or as mean ± SD for nine pigs per group. Analysis was performed with two-sample Student’s t-tests.

In experiment 2 statistical analysis was performed using analysis of variance followed by a Fisher’s least significant difference test. It should be noted that the results are expressed as means of two pigs per time-point. With larger groups even more significant differences might have been obtained. Values for uninfected animals are not shown but were comparable to the values on day 0.
During PRRSV infection in SPF piglets the percentage of SLAC steadily increased to a maximum of 50% on day 14 of infection whereas the percentage of LHAC decreased from 90% to 39% on day 14 of infection (Fig. 2 B). When expressed in total numbers, the number of SLAC increased dramatically from day 2 until day 21 of infection whereas the total number of LHAC increased from day 14 until day 21 of infection.

The amount of PRRSV that was isolated from the BALF cells reached a peak on day 7 of infection, decreased to undetectable levels on day 21 of infection and remained undetectable until day 42 of infection (Fig. 2 C).

**Flow cytometric analysis of SLAC in the BALF of gnotobiotic and SPF piglets**

**Gnotobiotic piglets.** On day 5 of infection the percentages of SWC1+ SLAC and SWC3+ SLAC in PRRSV-infected piglets were significantly increased in comparison with the percentages of SWC1+ and SWC3+ in control piglets (Fig. 3). Since SWC1 is a common marker for monocytes, granulocytes and resting T-lymphocytes and SWC3 is a common marker for myeloid cells, staining of more specific myeloid and lymphoid markers was used to characterize the differences observed (Fig. 1).

Analysis of macrophage and monocytic markers revealed that the percentage of both 517-2L+ and MHC II+ SLAC were significantly increased in PRRSV-infected piglets in comparison with controls. On the other hand, the number of CD14+ SLAC was only slightly but not significantly increased in comparison with controls (Fig. 3).

Analysis of lymphoid markers revealed that the percentage of CD2+CD3+ SLAC in PRRSV-infected piglets was higher than in control piglets (Fig. 3). Furthermore, the percentage of CD2+CD3+ SLAC in PRRSV-infected piglets was also slightly but significantly higher than in control piglets (Fig. 3). The
percentage of CD2$^-$CD3$^+$ cells was similar in both groups (Fig. 3). Double staining of SLAC for CD8 and CD4 revealed that the percentage of SLAC with the cytolytic phenotype CD8$^+$CD4$^-$ was higher in PRRSV-infected piglets in comparison with controls but that the percentages of CD8$^-$CD4$^+$ and CD8$^+$CD4$^+$ SLAC were similar in both groups (Fig. 3).

Cytolytic cells can be separated into natural killer cells with a CD8$^+$CD6$^-$ phenotype and γδT-cells with a CD8$^+$γδTCR$^+$ phenotype (Fig. 1). Further analysis of the increased CD8$^+$CD4$^-$ SLAC showed that significant percentages of CD8$^+$CD6$^-$ and CD8$^+$γδTCR$^-$ SLAC were present in the BALF of PRRSV-infected piglets whereas these were virtually absent in control piglets (Fig. 3). In addition, no CD8$^-$CD6$^+$, CD8$^+$CD6$^+$, CD8$^+$γδTCR$^+$ or CD8$^+$γδTCR$^-$ SLAC could be detected in the lungs of piglets in either of the groups (Fig. 3).
SPF piglets. During PRRSV infection in SPF piglets changes in populations of SLAC in the BALF were comparable to those seen for gnotobiotic piglets. The percentages of SWC1+517-2L- and SWC1+CD14- SLAC slightly increased during the early stages of infection and remained constant thereafter. In contrast, the percentages of SWC1+517-2L+, SWC1+CD14+ and 517-2L+CD11b+ SLAC decreased slightly during the first 14 days of infection and remained almost constant during the following weeks (Fig. 4). The percentage of 517-2L-CD11b+ SLAC increased during the first 10 days of infection but returned to basal levels thereafter (Fig. 4).

Analysis of lymphocytic markers revealed that CD2+CD3+ SLAC dramatically increased starting on day 10 of infection, peaked on day 21 and decreased thereafter (Fig. 4). In addition, the percentage of CD2+CD3- SLAC increased during the first week of infection and remained almost constant during the days following (Fig. 4). Most prominent was the change in the percentage of CD8+CD4- SLAC, which peaked on day 21 of infection at 40% above the level of day 0 (Fig. 4). Further analysis of the CD8+ SLAC revealed that the percentage of CD8+CD6+ increased during the first 7 days of infection and reached a maximum of 20% above base level. After day 7 of infection the percentage of CD8+CD6- SLAC also increased to a maximum of 20% above base level (Fig. 4). The percentages of CD8+CD5low SLAC, CD8+CD6TCR-SLAC and CD3+γδTCR-SLAC followed a pattern similar to that of the CD8+CD4-SLAC (Fig. 4).

Cytospin staining and histology

Cytospin preparations of BALF cells from gnotobiotic piglets isolated on day 5 of infection were stained with a MAb against the nucleocapsid protein of the European PRRSV strain ter Huurne. The mean percentage of LV-nucleocapsid-positive cells was 1.91 ± 3.13 (n = 9), whereas no positive cells could be detected in control piglets.
piglets were sacrificed and the in both gnotobiotic and SPF piglets with a PRRSV infection, approximately 30% of the total number of BALF cells consisted of CD8+ lymphocytes occurs in the lungs. This is demonstrated by the presence of CD8+ cells which stained positive with anti-CD6 (Pauly et al., 1996) and by the absence of CD8+CD4+, CD8+γδ T-cells. The increase in the percentage of MHC-restricted cytotoxic T-cells occurs in the lungs starting on day 7 of infection correlated precisely with a rapid decrease of the amount of BALF cell-associated PRRSV. This finding strongly suggests that the presence of cytolytic cells in the lungs during a primary infection is protective. Cytotoxic T-cells and natural killer cells are potent at lysis of infected cells and may thus prevent spread of the virus (Kimman et al., 1996). Furthermore, both types of cell have been shown to regulate cellular immunity via the production of interferon-γ (Trinchieri, 1995). Future research will focus on directly demonstrating the protective role of cytolytic cells during the host defence against PRRSV.

It should be noted that there was no increase in the percentage of other types of lymphocyte, such as CD4+CD8−, CD4−CD8+ or other myeloid cells such as polymorphonuclear cells (data not shown), in the lungs of PRRSV-infected pigs. This finding seems to indicate a response to very selective chemotactic signal.

Based on the findings that PRRSV causes apoptosis of infected macrophages (Suárez et al., 1996) and that PRRSV-infected pigs from the field often carry secondary infections, it has been suggested that infection with the virus may cause immunosuppression in its host due to a decrease in the number of macrophages in the lungs (Molitor et al., 1992). Our study clearly demonstrates that overall the number of macrophages in BALF does not decrease during a PRRSV infection in gnotobiotic or SPF piglets. However, the percentage of macrophages does decrease during PRRSV infection. Support for these conclusions is provided by the finding that semi-quantitative broncho-alveolar lavage yielded either constant (gnotobiotic piglets) or increasing (SPF piglets) numbers of LHAC, i.e. macrophages, during infection. The percentage of alveolar macrophages decreased steadily during infection due to a large influx of SLAC, i.e. lymphocytes and monocytes. The latter finding agrees with Shibata et al. (1997) who performed a microscopical evaluation of BALF cells from PRRSV-infected pigs and demonstrated that the ratio of macrophages decreased during infection whereas the ratio of lymphocytes gradually increased and the ratio of neutrophils remained unchanged. It

Discussion

Our study demonstrates that during a PRRSV infection in pigs a strong influx of natural killer cells and cytotoxic T-lymphocytes occurs in the lungs.

Support for this conclusion is provided by the following observations. Semi-quantitative lavage of the lungs of PRRSV-infected piglets revealed an increase in the total number of BALF cells between days 10 and 21 of infection. Subsequent flow cytometric analysis of BALF cells showed that the percentage of CD8+ cells increased dramatically during infection, with a peak between days 14 and 21 when approximately 30% of the total number of BALF cells consisted of CD8+ cells. This increase in CD8+ BALF cells was observed in both gnotobiotic and SPF piglets with a PRRSV infection, whereas it was not seen prior to infection or in control piglets. Further analysis of the CD8+ cells revealed that during the first days of infection mainly CD8+CD6+ cells were detected in the lungs of gnotobiotic and SPF piglets. As described by others this phenotype corresponds to that of MHC-non-restricted cytolytic cells (Pauly et al., 1996). Double staining against CD8 in combination with other markers revealed that these cells were mostly CD2+, CD4−, CD5− or CD5+γδ but γδTCR−, indicating that the phenotype of the CD8+CD6+ cells corresponds to a natural killer cell and not to the other cytolytic CD8+γδ T-cells. After day 7 of infection an additional increase in the percentage of MHC-restricted cytotoxic T-cells in the lungs occurs. This is demonstrated by the presence of CD8+ cells which stained positive with anti-CD6 (Pauly et al., 1996) and by the absence of CD8+CD4+, CD8+γδ T-cells.

To substantiate our flow-cytometric findings, which suggest that an influx of CD8+ cells occurs in the alveolar lumen, histology was used to determine whether increased numbers of these cells could also be found in the lung tissue. Tissue sections from three different areas in the right lung-half of control and PRRSV-infected piglets were stained with an anti-CD8 MAb on day 5 of infection. A segment of the frontal lobe (A), cardiac lobe (B) and dorsal lobe (C) of the right lung-half was cryosectioned and stained with a MAb against CD8. The number of CD8+ cells was assessed microscopically. Data are means (SD; n = 9).

![Image](image_url)

**Fig. 5.** Number of CD8+ cells per mm² tissue section in the lungs of PRRSV-infected gnotobiotic piglets. One-week-old gnotobiotic piglets were inoculated i.n. with 0.5 ml 10⁶ TCID₅₀ PRRSV strain ter Huurne. At day 5, of infection control (■) and infected (□) piglets were sacrificed and the lungs were removed. A segment of the frontal lobe (A), cardiac lobe (B) and dorsal lobe (C) of the right lung-half was cryosectioned and stained with a MAb against CD8. The number of CD8+ cells was assessed microscopically. Data are means (SD; n = 9).
should be noted that on day 2 of infection in SPF piglets the total number of BALF cells does decrease slightly. However, it is unlikely that this small change could cause continuous immunosuppression.

In this study two infection models were used to assess whether differences in the microbiological status of piglets affects the changes in BALF cell population during PRRSV infection. In general, we did not identify different responses in the two models. The influx of CD8⁺ CD6− natural killer cells during the first days of a PRRSV infection was seen in both models. Unfortunately, we cannot conclude that responses in the lungs in these two models do not differ because we have no data on PRRSV infection in gnotobiotic piglets during later times of infection.

In summary, our findings demonstrate that during a PRRSV infection the total number of BALF cells increases mainly due to an influx of natural killer cells and cytotoxic T-lymphocytes. The number of macrophages in the lung does not decrease during infection.

The authors wish to express their gratitude to Dr A. Saalmüller for his comments and suggestions for Fig. 1. We also thank Dr M. Denyer for providing the C25 MAb and E. P. de Kluijver, G. Kok and R. Autar for technical assistance.

References


J. N. Samsom and others
CD8+ cells in the lungs of pigs with PRRS


Received 1 June 1999; Accepted 5 October 1999