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Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Kinetics of Infection in Lymphatic Organs and Lung

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With 2 tables and 7 figures

(Received for publication July 20, 1998)

Summary

Pigs were infected by the oronasal route with European isolates of the porcine reproductive and respiratory syndrome virus (PRRSV; I10 and Cobbelsdorf). The kinetics of infection in lymphatic organs and the lung were analysed by immunofluorescence detection of virus antigen, re-isolation of the virus and reverse transcription–polymerase chain reaction (RT–PCR) for PRRSV-specific RNA. The kinetics of PRRSV infection proceeded in three phases, irrespective of the varying infestation of lymphatic organs within the first days post-infection (p.i.). First, an early acute infection of lymphatic organs developed within the first week and was characterized by a high number of antigen-positive macrophages. Second, a delayed acute infection of the lung was observed, which was most pronounced during the second and third week p.i. when a high number of infected alveolar macrophages was observed. The acute infection of lymphatic organs had resolved at this time. Infected cells in the lung were predominantly located in pneumonic lesions. Third, a persistent infection was demonstrated by RT–PCR and immunohistology when the experiments were terminated at day 49 p.i. The virus persisted in lymphatic organs, especially in the tonsils, and in the lung. At this stage, indications for a re-occurrence of acute infection were observed in restricted areas of the lung.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was observed for the first time during 1987 in the USA (Keffaber, 1989) and occurred in 1990 in Europe, both in Germany and The Netherlands (Nienhoff, 1991; Lindhaus and Lindhaus, 1991; Wensvoort et al., 1991). PRRS is now distributed worldwide (reviewed by Meredith, 1995). Characteristic signs are an infertility of sows, in particular abortions around the 110th day of pregnancy, stillbirth or weak piglets and a febrile respiratory disease affecting piglets and older swine.

PRRS virus (PRRSV) was first isolated in 1991 in The Netherlands (Lelystad virus; Wensvoort et al., 1991) and shortly thereafter in the USA (Benfield et al., 1992; Yoon et al., 1992; Collins et al., 1992). The genome of PRRSV consists of a single-stranded RNA of positive polarity and is closely related to equine arteritis virus, lactate dehydrogenase-elevating virus of mice and simian haemorrhagic fever virus (Conzelmann et al., 1993; Meulenbergh et al., 1993, 1997). These four species form the genus arterivirus of the monogeneric family Arteriviridae (Cavanagh, 1997).

PRRSV isolates from the USA show a large variation in virulence and genetics (Halbur et al., 1995, 1996). The differences within European strains are less pronounced (Wensvoort et al., 1992; Nelson et al., 1993; Katz et al., 1995; Meng et al., 1995). But between European and US

isolates remarkable genetic and antigenic differences have been defined. Concerning virulence, Lelystad virus, the prototype of European PRRSV, corresponds to the low-virulent US isolates.

The respiratory form, morphologically characterized by interstitial pneumonia, can be induced by intranasal infection of piglets and weaner pigs (Ohlinger et al., 1991; Pol et al., 1991; Collins et al., 1992; Fichtner et al., 1993; Halbur et al., 1993; Rossow et al., 1994). Therefore, together with influenza virus and the porcine respiratory coronavirus (Andries et al., 1981; Pensaert et al., 1986; Van Reeth and Pensaert, 1994a,b) PRRSV represents a major viral factor causing respiratory diseases in pigs.

The first isolation of PRRSV demonstrated the predominant tropism for alveolar macrophages (Wensvoort et al., 1991). Results obtained by further experiments showed that this virus also infects macrophages in lymphatic tissues and other organs (Rossow et al., 1994, 1996; Halbur et al., 1995, 1996). Most studies on the course of infection and disease were performed with US isolates, so that knowledge of the pathogenesis of European virus isolates is to some extent insufficient. Recently, Duan et al. (1997b) performed quantitative immunohistological and virological studies with Lelystad virus. They demonstrated PRRSV during the acute phase of infection predominantly in macrophages of lymphatic tissue and lungs. Later the virus persisted in macrophages of the lung.

As a first step to extend our knowledge of the respiratory form of PRRS, we initiated studies with two European PRRSV isolates. Here we describe the kinetics of infection in lymphatic organs and the lung in view of the pneumonic lesions which develop during the disease process.

Materials and Methods

Viruses

The PRRSV isolate 10 (I10) was isolated in The Netherlands (Conzelmann et al., 1993; received from V. Ohlinger) and the isolate Cobbelsdorf was obtained in the then GDR (D 2/91; Fichtner et al., 1993). These viruses were originally isolated in porcine alveolar macrophages and were propagated in MARC-145 cells or in subclones of this line (Kim et al., 1993; catalogue no. 277, collection of cell lines, Insel Riems). In total the virus isolates were passaged four to six times in alveolar macrophages and then six to 10 times in MARC-145 cultures. The final virus concentration was 10^4 TCID₅₀/ml.

Animals

Conventionally raised suckling piglets (24 days old) and weaner pigs (8 weeks old) were used. They represented crosses between German thoroughbred pigs and German landrace pigs. Four highly pregnant sows free of antibodies against PRRSV were housed in separate stables. The piglets were left together with their mothers until the experiments were terminated. Weaner pigs had been separated from their mothers 2 weeks before the experiment was started.

Experimental design

The experiments were performed with three groups of piglets, derived from three litters. PRRSV-inoculated pigs were euthanized and necropsied at days 1, 2, 3, 4, 7, 14, 21, 28, 35 and 49 post-infection (p.i.) as described below for the analysis of the kinetics of infection. PRRSV antigen-positive cells were identified and counted by immunofluorescence, infectious virus was re-isolated in tissue culture, and viral RNA monitored by reverse transcription-polymerase chain reaction (RT-PCR). Histopathology was performed to characterize pneumonic lesions and a bacteriological examination was performed to exclude changes caused by pathogenic bacteria. The pneumonic lesions are described here only to the extent necessary to explain the kinetics of infection.

Group 1. Eight suckling piglets (24 days old) were inoculated with PRRSV I10. Two uninoculated piglets from this litter were euthanized at the beginning of the experiment and necropsied for controls.

Groups 2 and 3. Weaner pigs were inoculated with PRRSV isolate Cobbelsdorf at 8 weeks of age. Group 2 consisted of a litter of six piglets and group 3 comprised 11 piglets of another litter. Six pigs of the same age and breed were used as controls.

The animals were infected by the oronasal route using a dose of 10^5 TCID₅₀ PRRSV in 10 ml of cell culture medium per pig as a spray. All control animals received culture medium without virus. The body

temperature and clinical signs were monitored daily. Necropsy was performed as follows: group 1, from day 1 to day 28 p.i.; group 2, from day 2 to day 21 p.i.; group 3, from day 1 to day 49 p.i. On each day one pig was euthanized, except for group 3. In this group, two pigs were necropsied on each of days 28 and 35. For groups 2 and 3, the six non-infected control animals were euthanized on days 2, 3, 4, 7, 14, 21 p.i.

Gross pathology was investigated and the organ samples processed for immunofluorescence, histology, virus isolation, bacteriology and RNA extraction. Blood and tissue samples of all lung lobes, tonsils, spleen, thymus, five lymph nodes (Lymphonodus = Ln. bifurcationis, Ln. mandibularis, Ln. retropharyngeus medialis, Ln. iliacus, Ln. inguinalis superficialis), liver, kidney and brain were examined. For lung tissue, regions with pneumonic lesions were preferred. For groups 2 and 3, the nasal turbinates were also examined.

Histological techniques

Samples of lung tissue (2–3 mm thick) were fixed for 20 h in 4% neutral-buffered formalin and subsequently embedded in paraffin using an automatic tissue processor. Paraffin sections were stained with haematoxylin–eosin and sealed with balsam. For histological evaluation and microphotography a Jenaval microscope (Carl Zeiss Jena) was used.

Immunofluorescence in cryostat sections

Preparation. PRRSV antigen-positive cells were identified with the indirect immunofluorescence test (IFT) in cryostat sections. Tissue samples from all lobes of the lung and eight lymphatic organs were examined. For groups 2 and 3 the nasal turbinates were also included. The tissue samples were snap-frozen in *n*-heptane with CO₂-ice and stored at –70°C. Cryostat sections were fixed with acetone (–20°C) for 10–15 min and stored at –20°C. The sections were equilibrated to room temperature and pre-incubated with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 20 min. Then the sections were incubated with monoclonal antibody (mAb) P3/27 (isotype IgG1, diluted in PBS containing 2% BSA). The mAb is specific for the PRRSV 15 kDa nucleocapsid protein ORF7 (Wieczorek-Krohmer et al., 1996). As the second antibody, a fluorescein isothiocyanate (FITC)-labelled goat F(ab)₂ anti-mouse IgG + IgM (H + I) antibody was applied (Caltag Laboratories, MEDAC, Hamburg, Germany). The antibody was diluted in 2% BSA–PBS and mixed at a ratio of 3:1 with 0.005% Evans blue solution. In parallel, sections were incubated with an irrelevant mAb (anti-Newcastle disease virus, isotype IgG1, obtained from B. Köllner, Insel Riems). Sections from tissues of non-infected pigs were processed in parallel as further negative controls. All sections were sealed with DABCO fluorescence maintenance buffer. For evaluation and photography an Optiphot 2 fluorescence microscope (Nikon) was used.

Quantification of PRRSV antigen-positive cells. All antigen-positive cells within a section were counted (magnification 400 ×). The area of the section varied between 10 mm² and 60 mm², with an average of 20–30 mm². The size of the sections was determined by the number and size of the visual fields at 100 × magnification. Finally, the number of antigen-positive cells was calculated as an average value per 10 mm², independent of their localization and distribution within the section. One section was analysed per lymphatic organ or lung lobe. With these data the average number of antigen-positive cells per 10 mm² lymph node or lung tissue was calculated.

Virus isolation

The tissue samples were homogenized in tissue culture medium (1:10 w/v). After 2 h at 4°C the homogenate was clarified by centrifugation. A fresh suspension of MARC-145 cells was distributed into a 24-well tissue culture plate (Greiner, Frickenhausen, Germany) and each well was inoculated with 50 µl of the homogenate. These cultures were kept for 3 days at 37°C and were then heat-fixed by incubation at 80°C for 2 h. The virus was detected by indirect immunofluorescence (Fichtner et al., 1994).

Viral nucleic acid extraction, cDNA synthesis and PCR

RNA extraction. RNA was obtained from 20–50 mg of homogenized tissue samples with the PURE-script RNA isolation kit (Gentra Systems, Biozym, Hess. Oldendorf, Germany). Purified RNA was ethanol precipitated and dissolved in distilled RNase-free water.

Table 1. Primer design for reverse transcription–polymerase chain reaction (RT–PCR) amplification

ORF2/ORF3 site	Pos. 1769–1792 (upper strand) 5' TGC TCC GCG CTT CTC CGT TCG CGC 3' Pos. 2434–2411 (lower strand, reverse) 5' ACG ACC GGG CTC GAG CCT TTG GCG
ORF2 site	Pos. 1926–1949 (upper strand) 5' GAT GAG ATG GTC TCT CGT CGC ATT 3' Pos. 2150–2127 (lower strand, reverse) 5' GTT GTA CTG TAG GCT CAC ATT GCC 3'

Primers. Two oligonucleotide primer pairs, as listed in Table 1, were delineated from the published PRRSV sequence (Conzelmann et al., 1993; Accession no. L04493, L04927). One primer pair corresponded to an overlapping ORF2/ORF3 site and resulted in a product of 666 bp. A second pair, also used for nested PCR and Southern blot hybridization to confirm the PCR results, yielded a product of 225 bp and corresponded to part of the ORF2 site.

RT. Priming by oligo(dT) was used to enable additional studies on the transcriptional expression of immunomodulatory molecules in parallel to viral RNA. Five micrograms of RNA in 10 μ l of distilled water was incubated at 70°C for 10 min in the presence of 1 μ g oligo(dT)_{12–18} as a template primer. Then, the following components were added to a final volume of 25 μ l: 5 \times first strand buffer, 0.1 mM dithiothreitol (DTT), 2.5 mM each of dNTPs, 40 U RNAsin and 200 U of Superscript II RNase H⁻ reverse transcriptase (Gibco, Karlsruhe, Germany). The reaction was incubated for 75 min at 37°C and finally heated at 90°C for 4 min. After ethanol precipitation, the cDNA was ready for PCR.

PCR. The cDNA (270 ng) was amplified in a 50 μ l reaction containing 1.5 mM MgCl₂, 0.3 mM of each dNTP, 25 pmol of each primer and 2.5 U of *Taq* polymerase. Cycle conditions were 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. After 35 cycles, a final extension step was performed for 10 min at 72°C. The PCR amplicon was analysed by electrophoresis in a 1% agarose gel and assessed semi-quantitatively (scoring + to + + + +).

Bacteriological studies

Samples (1 g each) of five lung lobes, tonsils, spleen, liver and kidney were investigated. Tissue suspensions in caseine-soya-peptone-bouillon (SIFIN, Berlin, Germany) were diluted from 10⁻¹ to 10⁻⁴ and incubated under aerobic and anaerobic conditions on Columbia agar (OXOID, Wesel, Germany) with 5% bovine blood. All isolates were differentiated employing the API system (bioMerieux, Nürtingen, Germany). Selected isolates were tested for toxicity with embryonal porcine lung cells (cell line 113, collection of cell lines, Insel Riems) according to Schimmel et al. (1994). Furthermore, suspensions of lung tissue were incubated on mycoplasma agar (DIFCO, Nordwald KG, Hamburg, Germany) with 0.1% nicotinamide adenine dinucleotide under microaerophilic conditions.

Results

Clinical observations

Moderate clinical signs were observed throughout the study. Two of the three animal groups developed a biphasic course of fever. In group 1, the first peak was observed at days 2 and 3 p.i. with temperatures between 40.0°C and 40.9°C. The two pigs left alive developed a second peak (40.0–40.2°C) at 7 and 9 days p.i. Three animals of group 3 showed fever between 3 and 5 days p.i. and 7–13 days p.i. (40.0–40.6°C), respectively. Only one animal of group 2 showed a slight increase of temperature (40.0°C) at day 7 p.i. Moderate dyspnoea and tachypnoea corresponding to the onset of pyrexia were recognized in infected animals of groups 1 and 3, in particular when they were stressed during handling procedures. In addition, the pigs appeared slightly apathetic and were occasionally coughing. The food uptake was reduced during phases with fever. No clinical signs were observed in non-infected control pigs.

Pathomorphological changes in the lung

In all groups, small macroscopically visible lesions were first noted at day 4 p.i., but only a few lobuli were affected. These slight to moderate changes were comparatively more pronounced between 7 and 21 days p.i. Disseminated focal subpleural changes affected not more than two to eight lobuli located in apical, middle and accessory lobes, and five to 20 lobuli in the diaphragmatic lobe. The affected lobuli were greyish-red in colour with consolidated parenchyma. Animals of groups 1 and 3, necropsied 28 and 35 days p.i., revealed no macroscopical lung lesions. However, in the pig necropsied at day 49 p.i., some consolidated lobuli were encountered in the lobus medius.

The histopathological examination revealed slight changes, such as submiliar foci in up to three lobes on day 3 p.i. An acute alveolitis was found both in these early stages and later in the periphery of more advanced lesions. An increasing number of type II pneumocytes was observed. Accumulated cells, predominantly alveolar macrophages and desquamated degenerating type II pneumocytes were found in the alveoli. In addition, polymorphonuclear neutrophilic granulocytes and lymphocytes were involved. In some regions, a high amount of cell detritus was visible. The advanced stages of pneumonia (7–21 days p.i.) were characterized by thickened alveolar septa due to hyperplasia and hypertrophy of type II pneumocytes. These changes were most pronounced in the animals of group 1 (Fig. 1a). In a few areas, a slight infiltration of alveolar septa by histiocytes was detected. However, interlobular septae were more distinctly infiltrated with mononuclear cells. Alveolar macrophages, desquamated and decaying type II pneumocytes, single neutrophils and lymphocytes accumulated within the alveolar spaces. In some spots, the alveolar spaces were filled with massive cell accumulations consisting of alveolar macrophages and type II pneumocytes. A large number of these cells

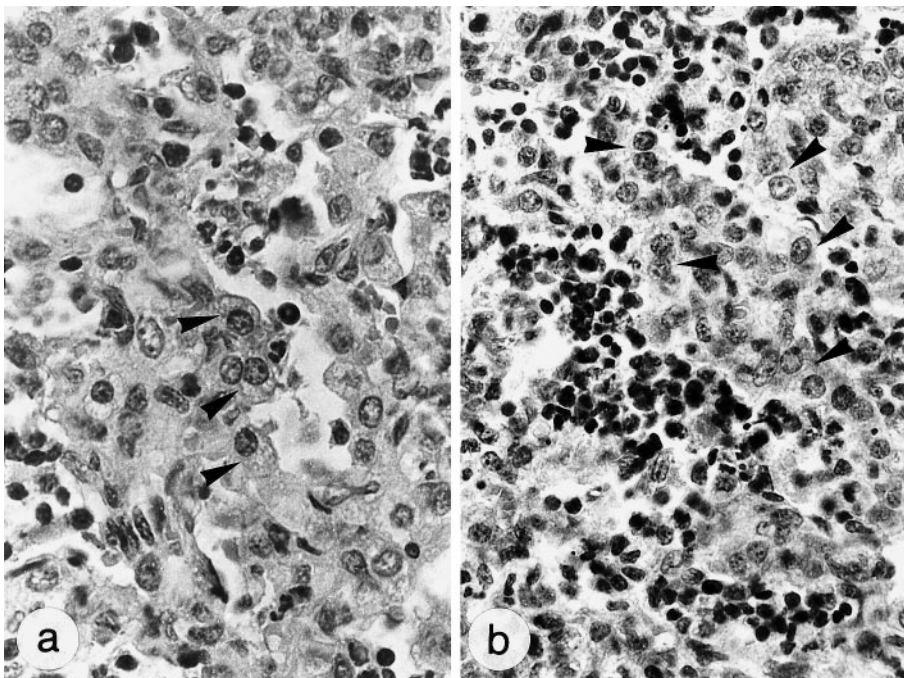


Fig. 1. Porcine reproductive and respiratory syndrome virus (PRRSV)-induced interstitial pneumonia (group 1, 14 days post-infection). Hyperplasia and hypertrophy of type II pneumocytes (arrows). (a) Decaying cells in alveolar clefts and cell detritus; haematoxylin-eosin (HE); $\times 600$. (b) Massive accumulation of pyknotic cells in alveolar clefts. HE; $\times 480$.

showed pyknotic nuclei and karyorrhexis, respectively (Fig. 1b). These histological lesions are typical for a moderate multifocal interstitial pneumonia.

Quantitative evaluation of PRRSV antigen-positive cells by immunofluorescence

The number of PRRSV antigen-positive cells detected in lymphatic organs and lung from days 1 to 49 during the course of infection was determined. The results are shown in Figs 2 and 3.

Group 1. No antigen-positive cells were detected on day 1 p.i. in the lymphatic organs of PRRSV-infected piglets. However, on days 2 and 4 p.i., numerous antigen-positive cells reached their peak compared with later points in time. No or a few antigen-positive cells were found in lymphatic organs between 7 and 21 days p.i. In lung tissue, only a few antigen-positive cells were detected on days 2 and 4 p.i., but accumulations of infected cells were noted in pneumonic regions 3 and 4 days p.i. A peak was reached in the samples from pigs necropsied on day 7 p.i., but the level of infection decreased to some extent on days 14 and 21 p.i.

Group 2. As described above in group 1, predominantly lymph nodes and other lymphatic organs contained PRRSV antigen on days 2 and 4 p.i. However, the total number of antigen-positive cells was smaller than counted in group 1. An exception was the relatively high number of PRRSV antigen-positive cells in the spleen on day 2 p.i. No antigen-positive cells were detected in lymphatic organs obtained on day 7 p.i. and later, with the exception of the tonsils, where positive cells on day 21 p.i. were encountered. In lung tissue, the highest number of antigen-positive cells was observed on day 7 p.i.

Group 3. Again in this group, lymph nodes were predominantly infected at days 2 and 4 p.i., and yielded a similar number of infected cells as group 2. The number of antigen-positive cells in the other lymphatic organs was rather low, except in the tonsils, where a relatively high number of positive cells was observed on day 4 p.i., and a smaller number 28 days p.i. Between 7 and 21 days p.i., the lungs were predominantly affected. Numerous positive cells were already detected in a few spots on day 7 p.i., and more extended on days 14 and 21 p.i. On day 49 p.i., only in one animal, many positive cells could be detected in an area of the lobus medius which contained several pneumonic lobuli. Taken together, in all groups the lymphatic organs contained the highest number of antigen-positive cells from day 2 to 4 p.i., but the lungs were predominantly affected 7–21 days p.i.

Type and localization of PRRSV antigen-positive cells

Lymphatic organs. The specific cytoplasmic fluorescence appeared grainy, dusty or diffuse and had a brilliant appearance. In the lymph nodes, positive cells were scattered or localized in groups in sinus regions or were disseminated in the lymphatic parenchyma (Fig. 4a,b). These cells had morphological features of histiocytic reticulum cells (macrophages), characterized by their large size, irregular shape and abundant cytoplasm. Occasionally, large positive cells were also found, which were distributed in loosely attached groups. The cytoplasm of these cells was brilliantly fluorescing and some contained roundish clods of cell detritus with non-specific yellow fluorescence. These cells were large macrophages of the germinal centres (starry sky cells, tingible body macrophages; Fig. 4c). A number of brilliantly fluorescing cells were thin and elongated, and partially branched. These cells may have been dendritic reticulum cells, but an unequivocal differentiation from histiocytic reticulum cells was not possible in our studies.

Within the lymphatic parenchyma of the tonsils and spleen, in general, antigen-positive cells were only scattered or in small groups. Occasionally, such cells were also found in subepithelial regions of the crypts. In the spleen, those cells were located in the periarterial lymphatic tissue. In the thymus, infected cells were predominantly detected in the medulla (Fig. 4d). They were concentrated in foci, leaving most areas of the sections free of viral antigen. In the cortical region, antigen-positive cells were only rarely encountered. By morphology, the antigen-positive cells in the tonsils, spleen and thymus were similar to those identified in the lymph nodes, with the exception of starry sky cells, which were only found in lymph nodes.

Lung. In non-pneumonic regions, antigen-positive cells were scattered loosely. Near to

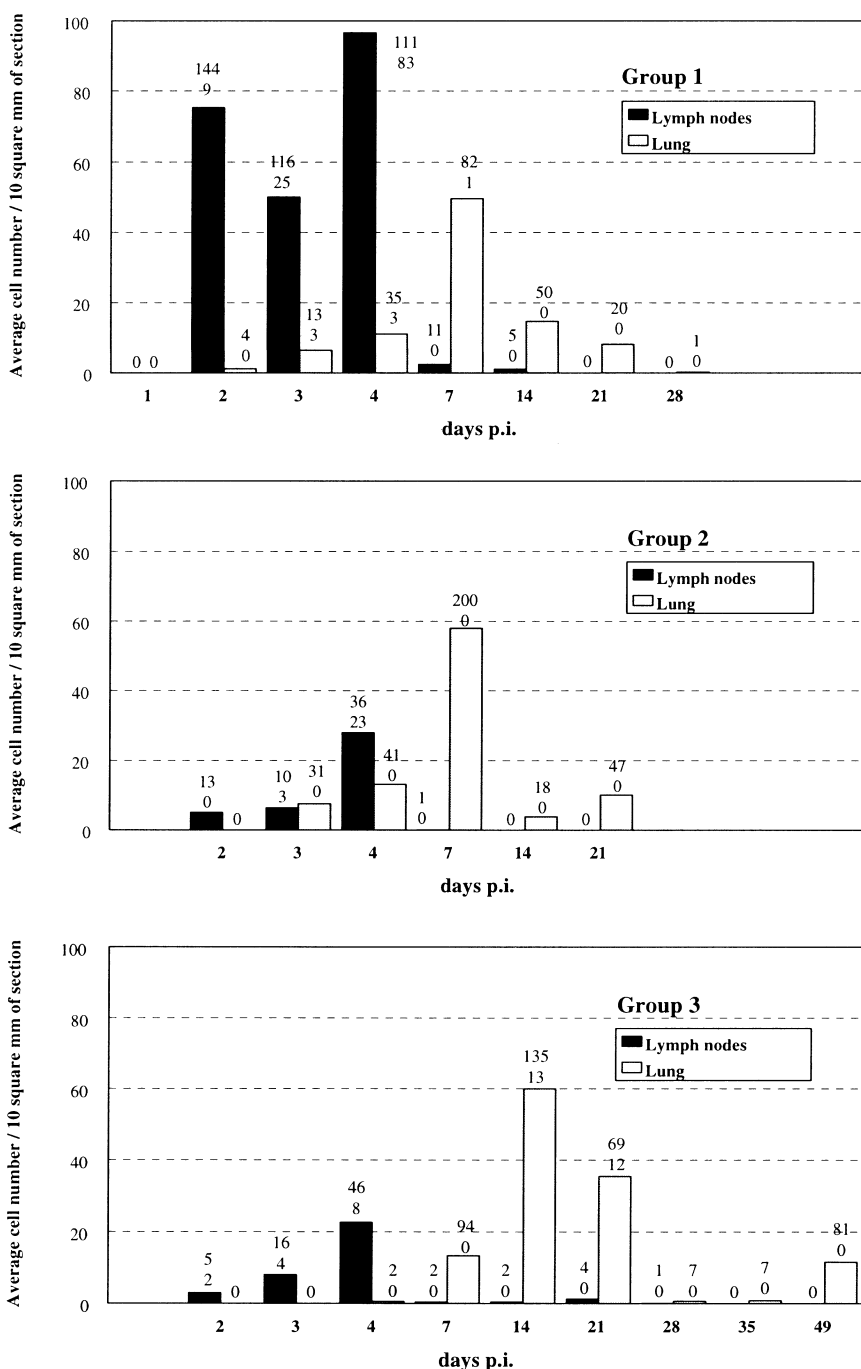


Fig. 2. Kinetics of porcine reproductive and respiratory syndrome virus (PRRSV) antigen-positive cells in lymph nodes and lung (groups 1, 2, 3). Results of the indirect immunofluorescence test on cryostat sections. The average number of antigen-positive cells per 10 mm² section is shown. Each day post-infection (p.i.) one section from each of the five lymph nodes and seven lung lobes per animal was evaluated; additionally, in group 3 a second animal was investigated on days 28 and 35 p.i. To indicate the variability, the figures above each column denote the minimal and maximal number of antigen-positive cells per section.

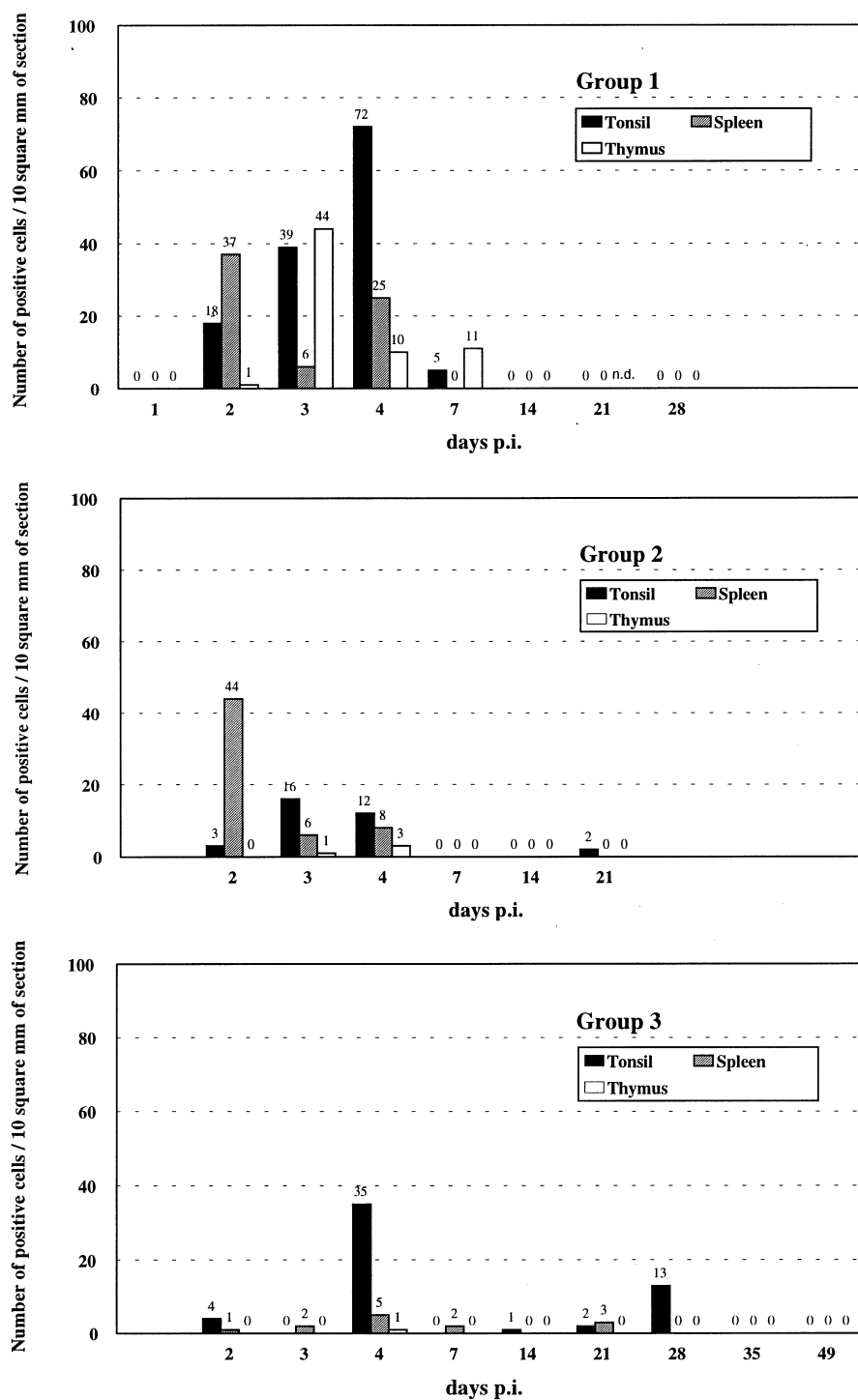


Fig. 3. Kinetics of porcine reproductive and respiratory syndrome virus (PRRSV) antigen-positive cells in tonsils, spleen and thymus (groups 1, 2, 3). Results of the indirect immunofluorescence test on cryostat sections. For each day post-infection (p.i.) one section from each organ of one animal was evaluated, and for group 3 a second animal was investigated on days 28 and 35 p.i.

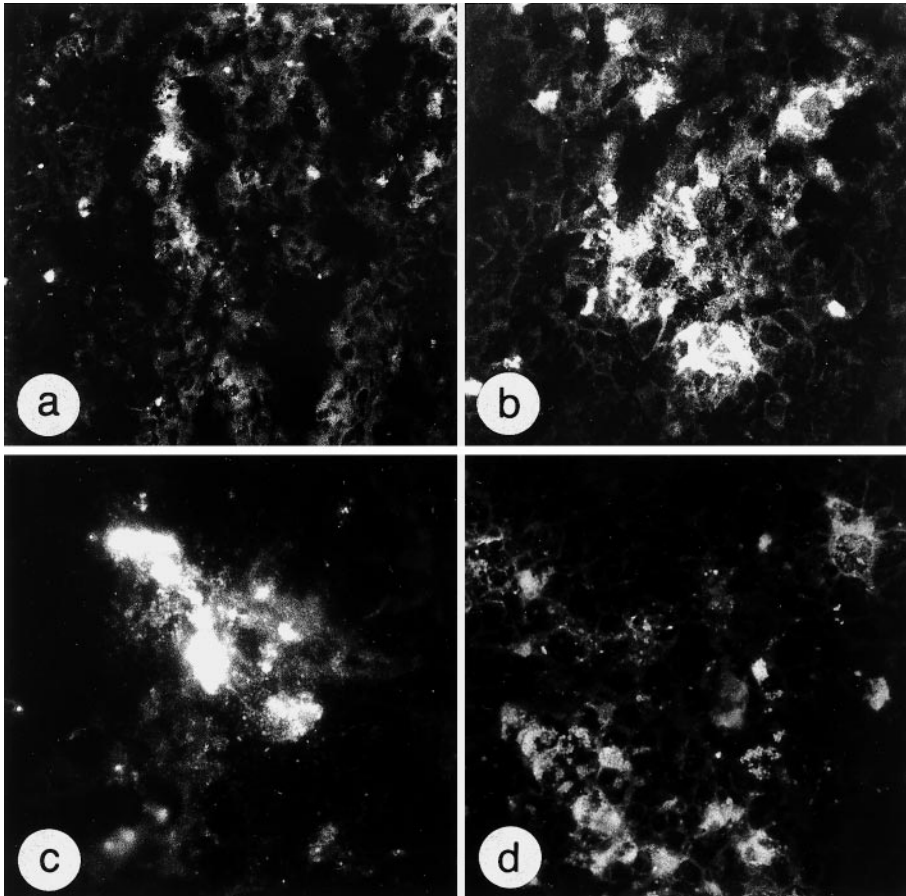


Fig. 4. Porcine reproductive and respiratory syndrome virus (PRRSV) antigen-positive histiocytic reticulum cells (macrophages) in lymph nodes and thymus (group 1). Indirect immunofluorescence test on cryostat sections with anti-PRRSV monoclonal antibody P3/27. (a) Positive cells in the sinus region, Ln. iliacus, 2 days post-infection (p.i.), $\times 230$. (b) Positive cells in lymphatic parenchyma, Ln. iliacus, 2 days p.i., $\times 460$. (c) Large tingible body macrophage in a germinal centre, Ln. inguinalis superficialis, 4 days p.i., $\times 1150$. (d) Positive cells within the thymus medulla, 3 days p.i., $\times 460$.

pneumonic lesions their numbers increased significantly. They showed brilliant cytoplasmic fluorescence and were located in alveoli, near to the alveolar wall (Fig. 5a,b). Positive cells were also located in thickened alveolar septae. The majority of these cells had a large area of cytoplasm (Fig. 5b,c). These cells were alveolar macrophages by morphology, localization and distribution. Within pneumonic regions, massive accumulations of clustered and partially pyknotic alveolar cells occurred (Fig. 5d). Occasionally alveoli contained fluorescing and non-fluorescing cell detritus. Within advanced pneumonic regions, which showed intensive hyperplasia of type II pneumocytes, the proportion of antigen-positive cells was low. The bronchial epithelial cells did not contain virus antigen. Furthermore, no antigen-positive cells were found in the nasal turbinates. All control sections were clearly negative for fluorescing cells as described above.

Virus isolation

Infectious virus was recovered first at day 2 p.i. from blood, tonsils, spleen, kidney and brain. The thymus and lymph nodes contained infectious virus on day 3, and the lungs were

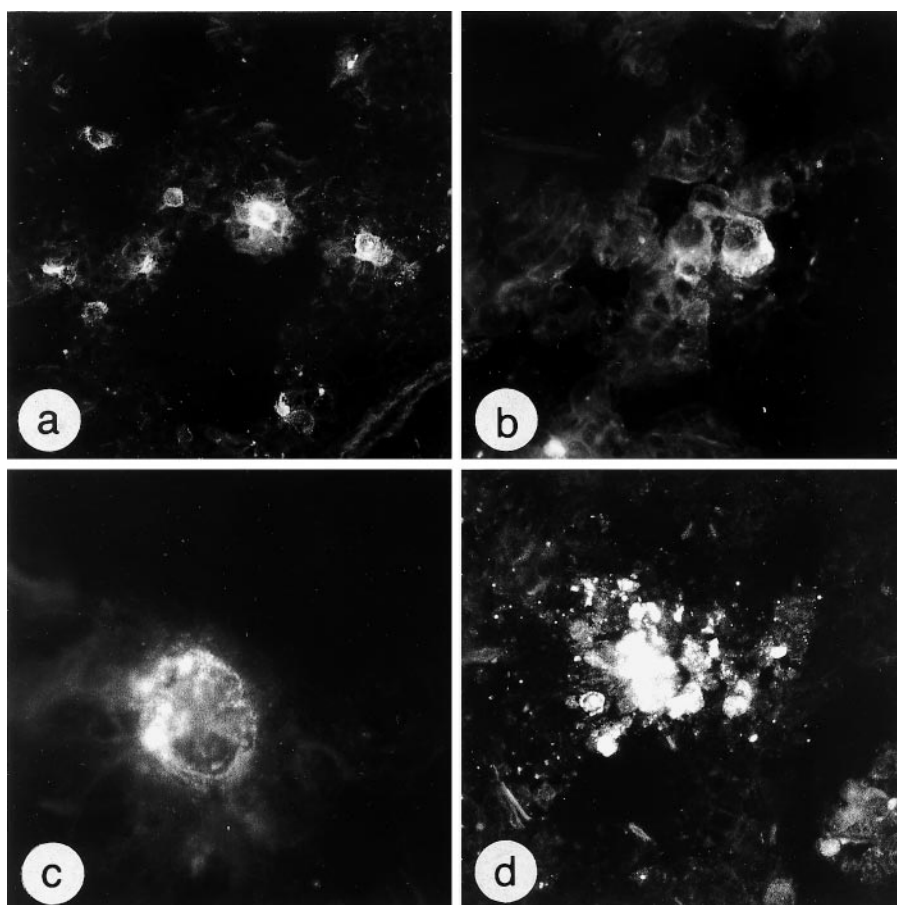


Fig. 5. Porcine reproductive and respiratory syndrome virus (PRRSV) antigen-positive macrophages in the lung. Indirect immunofluorescence test on cryostat sections with anti-PRRSV monoclonal antibody P3/27. (a)–(c) Positive macrophages in alveolar clefts or in septa near to a pneumonic lesion; (b) and (c) cells with pronounced, extended cytoplasm. (d) Accumulation of positive alveolar macrophages and cell detritus in a pneumonic lesion with some pyknotic cells. (a) Group 3, 14 days post-infection (p.i.), $\times 230$. (b) Group 1, 7 days p.i., $\times 460$. (c) Group 3, 14 days p.i., $\times 1150$. (d) Group 1, 7 days p.i., $\times 460$.

positive for the first time 4 days after infection. From blood, tonsils, thymus and lymph nodes, virus isolation was possible up to 21 days p.i., from lung tissue infectious virus was recovered 35 days p.i. In group 1, virus was isolated from 33 of 126 samples (26%), in group 2 from 16 of 96 samples (17%) and in group 3 from 19 of 160 samples (12%). All samples from non-infected control animals were negative for infectious virus.

Kinetics of PRRSV-specific RNA

From group 1, all samples were investigated. From the other two groups, we analysed samples from the seven lung lobes, tonsils, spleen and three lymph nodes (Ln. bifurcationis, Ln. mandibularis, Ln. inguinalis superficialis). By RT-PCR, viral RNA was detected beginning day 1 p.i. in the lung and thymus (group 1), 4 days p.i. in the lymph nodes and 7 days p.i. in

blood. Two pairs of primers were compared, which amplified a 225 bp and a 666 bp sized fragment, respectively. The amplification of the shorter fragment yielded clearer results.

The PCR results for groups 2 and 3 are shown in Figs 6 and 7. In both groups, the positive signal for viral RNA obtained from the lymph nodes necropsied 2–4 days p.i. was stronger than for lung tissue. For the lymph nodes, the intensity of the signal increased in most samples from day 2 to day 4. By contrast, the lung tissue samples yielded stronger signals between day 7 and day 21 or 28. The peak of intensity was observed around 14–21 days after infection. In most cases, the tonsils, spleen and thymus were positive for viral RNA. The latest samples examined were from day 49 p.i.; the tonsils, thymus, lymph nodes and lung were positive. The tonsils and pneumonic lung regions displayed the strongest signals for PRRSV RNA. All organ samples derived from non-infected control animals were negative.

Bacteriological studies

The dominant species identified in lung tissue were *Streptomyces* and *Nocardia*, accompanied by a few Gram-positive cocci and bacilli. In addition, coagulase-negative *Staphylococci* and

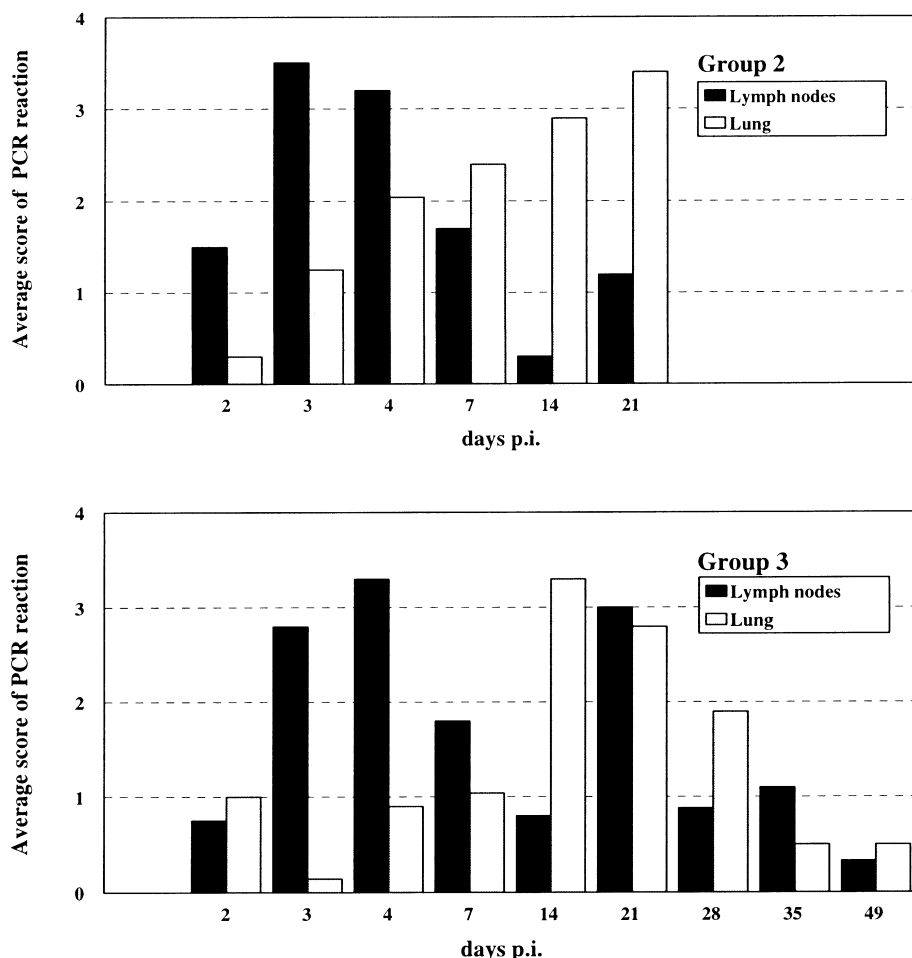


Fig. 6. Chronological demonstration of porcine reproductive and respiratory syndrome virus (PRRSV)-specific RNA (ORF2) by reverse transcription–polymerase chain reaction in three lymph nodes and seven lung lobes (groups 2 and 3). Average values derived from semi-quantitative evaluation of the reaction with a four-cross (++++) score.

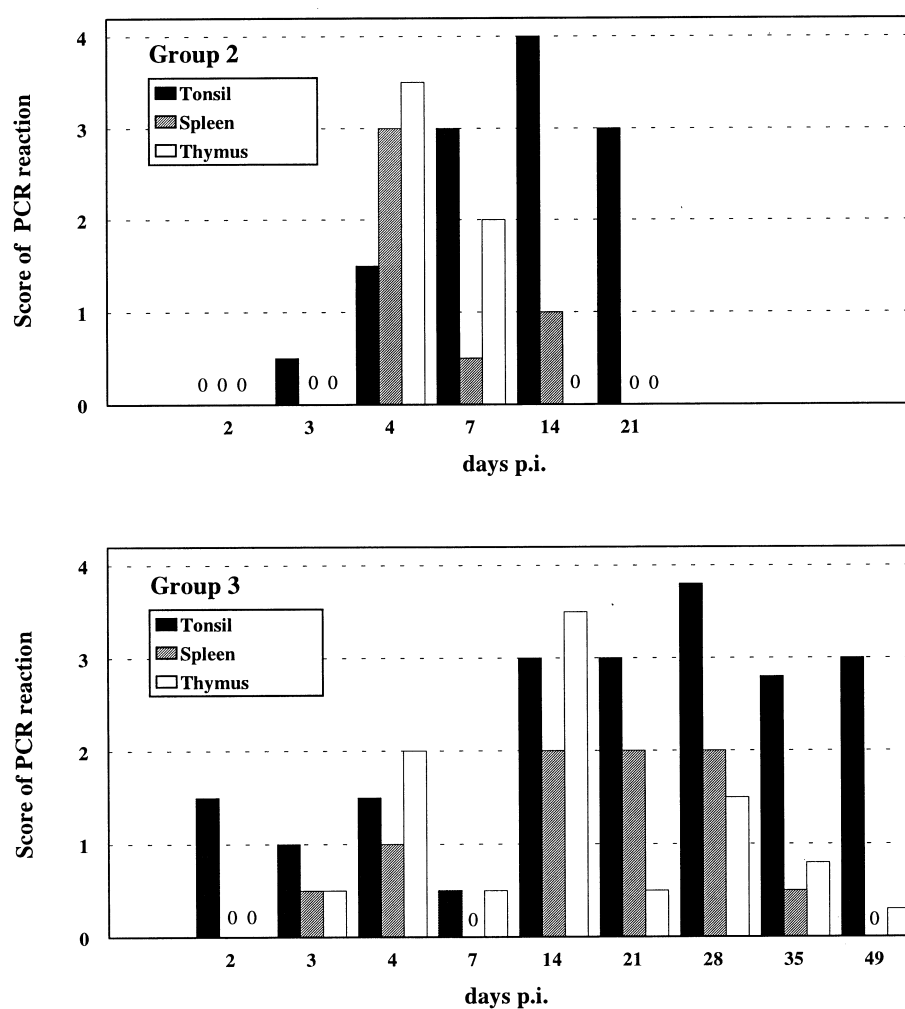


Fig.7. Chronological demonstration of porcine reproductive and respiratory syndrome virus (PRRSV)-specific RNA (ORF2) by reverse transcription–polymerase chain reaction in tonsils, spleen and thymus (groups 2 and 3). Semi-quantitative evaluation of the reaction using a four-cross (++++) score.

haemolytic *Streptococci* were isolated. The total number of bacteria ranged from 9×10^1 to 3×10^5 CFU/g lung tissue. Pathogenic bacteria (including mycoplasmas) were not detected. The growth of porcine lung cells in culture was not impaired by cocultivation with culture supernatants of selected isolated bacteria.

Discussion

In order to characterize essential and common features of PRRSV infections, we used two European virus isolates for the inoculation of pigs. The results show that the kinetics of infection appeared similar for all three groups analysed so far, irrespective of quantitative differences in the infestation of lymphatic organs within the first days p.i. Furthermore, the acute phase of the PRRSV infection showed a biphasic course. At first, an early acute infection

of macrophages in lymphatic organs developed which was then followed by a delayed acute infection of lung macrophages.

In lymph nodes and other lymphoid organs we detected PRRSV antigen in histiocytic reticulum cells (macrophages) including starry sky cells of germinal centres. In addition, some antigen-positive cells had the appearance of dendritic reticulum cells. In the lung, PRRSV antigen was found in alveolar macrophages. These findings correspond with other studies in which virus antigen was identified by immunohistochemistry in macrophages of the lung and other organs, tingible body macrophages and dendritic cells of lymphatic organs (Magar et al., 1993; Halbur et al., 1994, 1996; Larochelle and Magar, 1995; Rossow et al., 1996; Duan et al., 1997b). Evidence for virus replication within those cell types was also obtained by *in situ* hybridization (Haynes et al., 1997; Lawson et al., 1997). Both antigen and RNA of PRRSV was also observed in macrophages of other organs, such as nasal turbinates, heart, liver, kidney, adrenal gland and brain. Macrophages are widely distributed in all organs (mononuclear phagocyte system; Van Furth, 1980), which makes the systemic character of the PRRSV infection comprehensible. The infection affects especially lymphatic organs and the lung because of the high number of target cells. However, the susceptibility for PRRSV varies among types of macrophages. PRRSV has a strongly restricted tropism for only some subpopulations of porcine monocytes/macrophages (Duan et al., 1997a).

A number of publications described PRRSV antigen in the cells of bronchial epithelia (Pol et al., 1991), epithelia of the nasal mucosa (Rossow et al., 1996), in type II pneumocytes (Halbur et al., 1994) and endothelial cells (Halbur et al., 1995, 1996). In contrast to these findings, we did not observe PRRSV antigen in these cell types. Further studies are indicated to clarify whether these cells are susceptible or not.

In our study, inoculation with PRRSV rapidly resulted in viraemia and the infection of several organs. From 2 days p.i. onwards it was possible to isolate virus from blood, tonsils, spleen, kidney and brain, at 3 days p.i. the lymph nodes and thymus were positive and lung tissue yielded infectious virus 4 days p.i. By RT-PCR, evidence for viral RNA was obtained in the lung, thymus and kidneys on day 1 p.i. Other groups have reported that virus was isolated from 1 or 2 days p.i. onwards (Rossow et al., 1995; Halbur et al., 1996) from sera and several organs. With the highly virulent US isolate VR-2332 viraemia was already demonstrated 12 h p.i. (Rossow et al., 1995, 1996). In addition, PRRSV antigen was detected at this early time p.i. in epithelial cells, in macrophages of the nasal mucosa and in tonsils, including interstitial or alveolar lung macrophages.

We identified PRRSV antigen-positive cells 2–4 days p.i. predominantly in lymphatic organs, whereas lung tissue was much less involved. These results indicate that the first phase of infection is characterized by an acute infection of the lymphatic organs, in which the virus replicates in many cells. This early acute infection is accompanied by fever, as shown previously (Fichtner et al., 1993). We detected infected cells in all the lymphatic organs examined, although not at all sites and to somewhat variable levels. This acute lymphatic organ phase diminished by the onset of the second week p.i. There were fewer infected cells in groups 2 and 3, inoculated with PRRSV isolate Cobbelsdorf, than in group 1, for which I10 was used. Additionally, the animals in this group were younger at the time of infection than those in groups 2 and 3. This may be one of the important factors for the spread of the virus. The pigs used for groups 2 and 3 were infected at the age of 8 weeks. Possibly the resistance to infection increases with the age of the animals. Further experiments are necessary to clarify whether these two European PRRSV isolates differ in their tropism for target cells in the lymphatic tissue of pigs.

In the lung tissue of all groups, PRRSV antigen-positive cells were detected between 7 and 21 days p.i. to a similar extent. During this phase, the animals suffered from a second period of fever and the pathomorphological changes in the lung tissue were most pronounced. These results indicate that lung macrophages are infected very early, but their rate of infection is much lower than in lymphatic organs. In lung tissue, the infection appears to spread slowly and reaches its highest level after 2–4 weeks p.i. The immunofluorescence study correlates to some extent with the chronological detection of viral RNA by RT-PCR.

Similar observations on the course of infection were reported by Duan et al. (1997b) using the less virulent Lelystad virus. They found a high number of antigen-positive cells in all sections

from lymph nodes on day 3 p.i., but only in a few sections from lung tissue. However, on day 14 p.i. most regions of the lung were strongly infected and only a few infected cells were found in the lymph nodes and tonsils. In contrast, using the highly virulent American virus isolate VR-2385, a high number of infected alveolar macrophages was already detected on day 3 p.i. (Halbur et al., 1995, 1996). Obviously the virulence of the strain used for infection correlates with the manifestation of clinical disease and the level of infection.

The acute infection was then followed by a period of virus persistence in some, but not in all, animals. In our experiments, viraemia was detected until 21 days p.i. Others detected virus in blood until day 23 p.i. (Wills et al., 1997), day 28 p.i. (Halbur et al., 1996; Duan et al., 1997b) and day 35 p.i. (Yoon et al., 1993). In our studies, the isolation of infectious virus from the lung and tonsils was successful up to day 28 p.i. Virus-specific RNA was demonstrated on day 49 p.i. especially in the tonsils and the lung. PRRSV antigen-positive cells were found on day 28 p.i. (tonsils, lymph nodes) and on days 35 or 49 p.i. (lung). In the animal necropsied on day 49 p.i. the number of antigen-positive cells in a defined pneumonic region was surprisingly large. In other studies, infectious virus or viral antigen was detected up to 28 days p.i. in lymphatic organs and lung (Halbur et al., 1996). Duan et al. (1997b) isolated infectious virus 35 days p.i. from lung tissue. Alveolar macrophages obtained by bronchoalveolar lavage were virus positive up to days 49 or 70 p.i. (Shibata et al., 1997; Mengeling et al., 1995). Wills et al. (1997) reported that infectious virus was isolated up to 157 days p.i. from oropharyngeal tissue, especially from tonsils. These data indicate that an early acute infection of lymphatic organs and lung is followed by a stage of persistent infection lasting for several months. The virus persists in lymphatic organs, obviously with a predilection for the tonsils, and in alveolar macrophages. Our observations emphasize that an acute inflammatory process can reappear in restricted regions of the lung based on a persistent infection.

In spite of the observed variations within affected organs and between individual animals, a three-phase pathogenesis can be defined for European PRRSV infections (Table 2):

- 1 An early acute infection of the lymphatic organs develops quickly during the initial viraemia within 1 week p.i. This phase is characterized by a rapid increase in infected macrophages.
- 2 A delayed acute infection of lung macrophages follows. A small number of macrophages becomes infected at an early point in time. The infection spreads slowly to a peak, which is reached 2–3 weeks p.i. and involves numerous cells. At that time, the acute infection of lymphatic tissues has diminished to only a few cells containing virus antigen. Highly virulent virus isolates reach a peak of infection more rapidly than isolates with moderate or low virulence.
- 3 A late persistent infection with restricted virus replication lasts several months. In this phase, attempts to isolate the virus are in most cases unsuccessful.

Table 2. Phase of infection and relative number of porcine reproductive and respiratory syndrome virus (PRRSV)-infected cells counted in lymphatic organs and the lung

Organ (cell type involved)	Phase 1 Acute infection	Phase 2 Delayed acute infection	Phase 3 Persistent infection
Lymphatic organs (macrophages)	+++	+	(+)
Lung (alveolar macrophages)	+	+++	(+) or +++ ¹

+++ , many PRRSV antigen-positive cells, isolation of infectious virus positive; + , small amount of PRRSV antigen-positive cells, isolation of infectious virus positive; (+) , few or no PRRSV antigen-positive cells, isolation of infectious virus inconsistent, PRRSV RNA present; +++¹ , relapse of inflammation involving many PRRSV antigen-positive cells.

The exposure of mucosal surfaces to PRRSV results in an infection of residential macrophages, where the initial replication of PRRSV takes place (Rossow, 1998). Following oronasal inoculation or natural infection, an initial viraemia develops quickly. This viraemia is probably induced by virus replication in subepithelial macrophages of nasal and pharyngeal mucosa. In addition, macrophages located directly beyond the crypt epithelia of the tonsils could be a major target for infection. The pathogenesis of pneumonic lesions may be initiated by a small number of alveolar macrophages which are infected early during viraemia. The multifocal spread of the virus within the lung supports this conclusion. In addition, the infection may spread directly by the respiratory route. In the following days, more alveolar macrophages are probably infected via viraemia or locally by virus released from already infected macrophages. It is unlikely that blood monocytes are involved in the shift of infection to the lung because they are hardly susceptible (Duan et al., 1997a).

The lesions in the lung are characterized by an accumulation of macrophages in alveoli. Type II pneumocytes show hyperplasia, hypertrophy and desquamation. The septa are only infiltrated to a small extent by mononuclear cells. Taken together, these criteria are typical for an acute interstitial pneumonia as described for the respiratory form of PRRS (Ohlinger et al., 1991; Pol et al., 1991; Collins et al., 1992; Yoon et al., 1992; Halbur et al., 1993, 1995; Rossow et al., 1994, 1995). Numerous antigen-positive alveolar macrophages form clusters and their nuclei show pyknosis and karyorrhexis. A part of these cells might be apoptotic, like in tissue culture after PRRSV infection (Suarez et al., 1996). We have no evidence that PRRSV can replicate in type II pneumocytes. Therefore, an immediate damage of alveolar cells by the virus infection is unlikely. It is conceivable that the infection and necrosis of alveolar macrophages lead to desquamation of alveolar epithelia and, thus, the production of surfactant could be impaired at an early stage. The repair of alveolar epithelial cells involves proliferation of type II pneumocytes. We suggest that hyperplasia of these cells is caused by an overshooting reaction and a dysregulation associated with the inflammatory process. Further studies will be of interest to elucidate the pathogenetic mechanisms which regulate the cellular responses to pneumonic lesions and local immune reactions.

Acknowledgements

We thank Brigitte Nolting, Gabriele Czerwinski, Hanna Wege, Birgit Goerl, Margitta Sachse and Sylvia Großklaus for excellent technical assistance. We thank also Klaus Depner and Jens Teifke for helpful discussions and critical reading of the manuscript.

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