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ARTERIVIRUS PRRSV

Experimental Studies on the Pathogenesis of Respiratory Disease

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1. ABSTRACT

Pigs were infected with the porcine respiratory and reproductive syndrome virus (PRRSV) by the oronasal route. We studied the development of histological lesions, sites of virus infection and of inflammatory infiltrates by quantitative evaluation of reactive cells. The animals developed a multifocal interstitial pneumonia. Clinical signs of pneumonia were observed from day 7 to 21. In the first stage, an acute alveolitis was found, which was characterised by a hyperplasia of type II pneumocytes within the septa and an accumulation of macrophages in the alveolar spaces. Within 2–4 days p.i., virus infected cells were prominent in lymphatic organs, but their number declined rapidly during the following days. In the following period, the number of virus antigen positive cells increased in the lung. An interesting discrepancy existed between the relatively small number of virus specific cells and the degree of intensive pneumonia. As a first step to analyse mechanisms leading to the induction of pneumonia, we studied transcriptional expression of cytokines and other immunomodulatory molecules by semiquantitative RT-PCR.

2. INTRODUCTION

The porcine reproductive and respiratory syndrome (PRRS) is a viral disease of the swine of increasing economical importance (reviewed by Meredith 1995). Retrospective

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studies indicate, that this syndrome can be followed back to the early 1980s in the USA and spread rapidly until 1990. The syndrome comprises a spectrum of symptoms ranging from early farrowings, late abortions and a highly prevalent respiratory disease. In particular the respiratory syndrome is often accompanied by a number of other pathogenic bacterial or viral agents. In the field PRRS is of increasing concern. PRRS was described under different nomenclature and the incertainity concerning its true etiology complicated the diagnosis. In Europe the first PRRS cases were noticed 1990 and the disease spread rapidly from Germany to the Netherlands, Belgium and England. An important progress was the first isolation and definition of this virus ("Lelystad virus", Wensvoort et al. 1991, Terpstra et al., 1991). The virus is highly variable in terms of virulence. In addition, between the strains from USA and Europe remarkable serological and genetic differences were found (Kapur et al., 1996; Suárez et al., 1996; Wieczorek-Krohmer, 1996; Halbur et al., 1995 Rossow et al., 1995). Nowadays, live vaccines are available but are debated because of their potential to induce persistent infections and reproductive failures. It prooved to be difficult to reproduce the disease experimentally. The knowledge on the immune response and the pathogensis of PRRS is limited. Only contradictory data are available on the suspected synergism with other potential pathogens (Molitor et al., 1997; Van Reeth, 1997). Furthermore, PRRSV infections offer an opportunity to study inflammatory mechanisms and immunological defense in the lung as a model for human respiratory diseases. In order to investigate the pathogenesis of pneumonia in conventionally raised pigs, we initiated experimental studies with PRRSV (Fichtner et al., 1993). Here we describe the pathological changes, virus replication and transcriptional expression of proinflammatory cytokines during a kinetic study.

3. MATERIALS AND METHODS

Conventionally raised piglets (6–7 weeks old) were infected by the oronasal route with an European strain (Cobbelsdorf) of PRRSV. At day 2, 3, 4, 7, 14, 21, 28 and 35 p. i. each time one uninfected control animal and two infected piglets were euthanized and slaughtered for dissection. Organ samples were shock frozen and stored at -70°C before processing for immunofluorescence on cryostate sections or RT-PCR. Immunostaining was performed with mAbs specific for PRRSV ORF 7 protein (Wieczorek-Krohmer, 1996) and alveolar macrophages. Organ samples for histology and immunohistology were fixed in 4% neutral formalin and embedded in paraffin. For RT-PCR, RNA extracted from organ samples were reversely transcribed to cDNA (oligo dT primed). The optimal conditions for PCR were established for each primer set. The primers were delineated from published sequence informations and data banks (Murtaugh et al., 1994). The correct identity of the PCR-product was monitored by Southern-blot hybridisation. Isolation of infectious virus was performed by inoculation of MARC 145 cell monolayers with homogenised tissue samples.

4. RESULTS

4.1. Pathomorpholcgical Findings

Histological changes typical for a mild, multifocal interstitial lobular pneumonia were noticed first at the third day post infection. Macroscopic lesions were first observed on day 4 p.i. and developed further from day 7 to 21 p.i., but in general they remained mild to moderate (Figure 1). By contrast, the affected lobuli displayed pronounced histo-

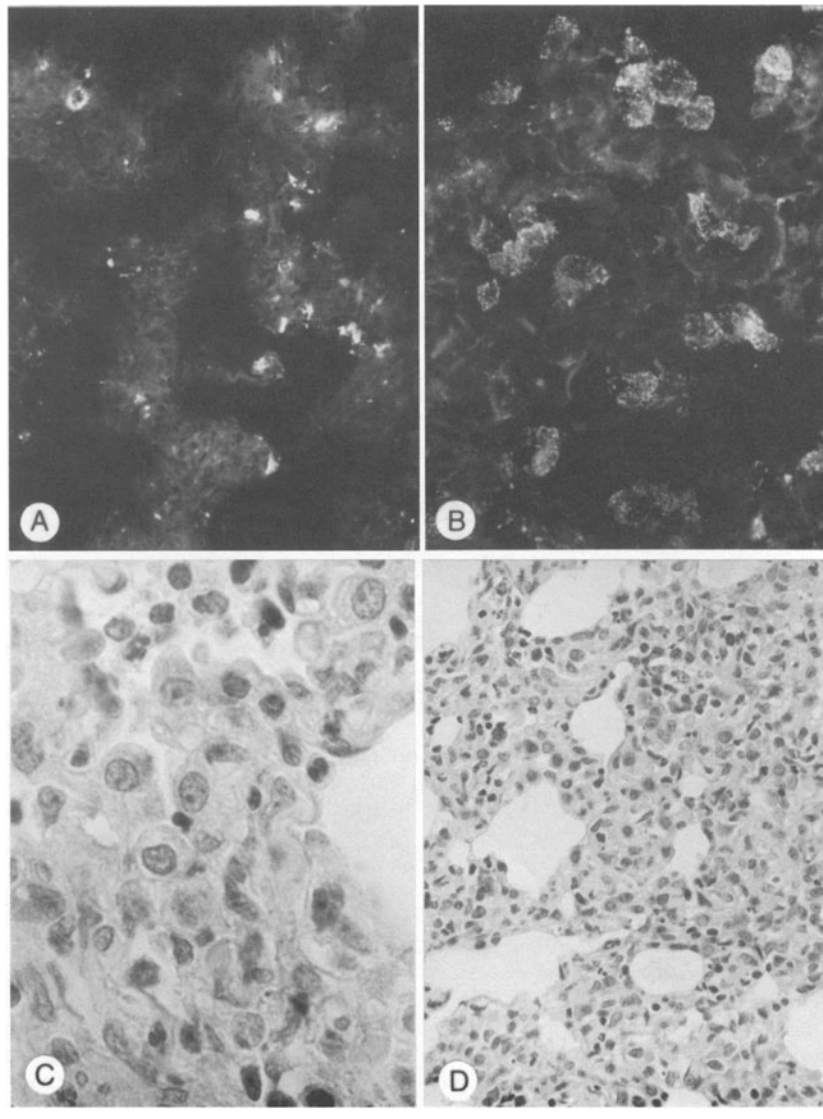

Figure 1. Typical features of PRRSV induced pneumonia. A) PRRSV - antigen in alveolar macrophages, 7 days p. i. Indirect immunofluorescence on cryostate section, anti-PRRSV mAb P3/27. 235:1. B) Accumulation of alveolar macrophages in an early stage of pneumonia, 21 days p. i. Indirect immunofluorescence on cryostate section, anti-macrophage mAb 2G6. 470:1. C) Interstitial pneumonia.Type II pneumocytes displaying hyperplasia and hypertrophy, alveolar macrophages are scattered in between. 14 days p. i., HE staining, 960:1. D) Pronounced interstitial pneumonia, 21 days p.i., thickening of septal walls. HE staining, 300:1.

logical lesions. Early stages are histologically characterised by an acute alveolitis. Within these lesions, the number of alveolar macrophages increased and a hyperplasia of type II pneumocytes was visible. In some areas, the alveolar macrophages appeared destroyed. Advanced stages of pneumonia displayed a thickening of the alveolar septa and alveolar spaces were often filled with cell detritus. As a consequence of the strong hyperplasia and hypertrophy of type II pneumocytes, the alveolar spaces were increasingly restricted. Desquamated, decaying type II pneumocytes and alveolar macrophages accumulated in the remaining alveoli. Solid pneumonic lesions were first seen on day 7 p. i. and consisted of proliferating type II pneumocytes including a quite variable proportion of macrophages scattered in between. In localised areas, necrotic type II pneumocytes and aggregations of decaying alveolar macrophages were encountered.

4.2. Kinetics of Virus Spread and Cell Tropism

The time course of virus spread and the infiltration process was analysed employing virus specific monoclonal antibodies, immunofluorescence and RT-PCR technology. In lymphatic organs, a high number of virus infected cells was found within 2–4 days p.i., which declined rapidly within the following days. At the same time, only a few virus positive cells were noted in lung tissue. From day 7–21 p.i., the number of virus antigen positive cells increased in the lung tissue (Figure 1 A). It is noteworthy, that the degree of intensive pneumonia did not correlate with the relatively small number of virus specific cells. The virus antigen was located predominantly in alveolar macrophages. In addition, cells which were not yet unequivocally identified displayed virus antigen.

4.3. Transcriptional Expression of Virus RNA and Mediators of Inflammation

As a first step towards an analysis of local immunity and inflammatory mechanisms we monitored the organ specific transcriptional expression of viral RNA in parallel to proinflammatory cytokines IL-1a, IL1–1b, IL-6, IL-8, AMCFII (alveolar macrophage chemotactic factor), TNFa and TNFb. In correlation to the results of virus detection by immunofluorescence and reisolation, transcriptional expression of viral RNA was already detectable within 2 days p. i. in tonsils and lymph nodes. Starting at 4 days p. i. also lung tissue, spleen and thymus were highly positive. Strong involvement was typical for tonsils and at variable degrees for thymus and spleen until the experiment was terminated 35 days p. i. However, in lung tissue of both healthy control animals and infected pigs, a strong transcriptional expression of the cytokines IL-1a, IL1–1b, IL-8, TNFa and TNFb was found, with not much variation throughout the time kinetics. Since conventional raised pigs were employed, this result may reflect the reactivity of the mucosal defence system to bacteria. Attempts of semiquantitation by taking a series of samples at different PCR-cycle numbers and comparing to standardised probes did not reveal significant differences between infected and uninfected controls.

Remarkable correlations between PRRSV induced pneumonia and the transcriptional expression of the cytokines IL-6 and AMCFII were noticed. Table 1 illustrates the kinetics of transcriptional expression throughout the experiment concerning virus, IL-6, IL-8 and AMCFII. From day 4 p. i. onwards IL-6 expression in lung tissue was significantly enhanced compared to uninfected controls. The transcriptional expression of IL-6 was also increased in organ samples from lymphnodes, spleen, thymus and tonsils, indicating a systemic upregulation. Furthermore, the transcriptional expression of AMCFII

Table 1. Transcriptional expression of PRRSV and cytokines: kinetics in lung tissue

Days p.i.	Status	Organ	PRRSV	AMCF II	IL-6	IL-8
2	control	lung	–	±	±	++++
2	infected	lung	±	±	±	++
2	infected	lung	–	+	±	+++
3	control	lung	–	±	+	+++
3	infected	lung	++	±	±	++++
3	infected	lung	–	+	+	+++
4	control	lung	–	±	+	++++
4	infected	lung	++++	±	+	+++
4	infected	lung	++	±	+	+++
7	control	lung	–	–	+	+++
7	infected	lung	++++	+++	+++	+++
7	infected	lung	+	±	+	+++
14	control	lung	–	+	+	++++
14	infected	lung	+++	++	+++	++++
14	infected	lung	+++	++	+	+++
21	control	lung	–	±	±	+++
21	infected	lung	++++	++	+	+++
21	infected	lung	++++	++	++	+++

was selectively upregulated in PRRSV-infected lung tissue, beginning from day 7 throughout the following time points studied. Rather independent of the stage of inflammation this chemokine was strongly expressed in tonsils and at a variable degreee in thymus and spleen.

5. DISCUSSION

Field observations and experimental data indicate, that the pathogenicity and virulence of PRRSV strains differ widely and are strongly influenced by host factors (Kapur et al., 1996; Suárez et al., 1996; Halbur et al., 1995; Rossow et al., 1995). The European strains in general appear to induce a milder disease than strains from the USA. Unrecognised subacute and persistent infections with PRRSV may lead to long lasting health problems in pig herds. Therefore we started to analyse the pathogenesis of pneumonia employing deliberately conventionally raised pigs and an European strain of PRRSV to obtain informations reflecting the situation in the field (Fichtner et al., 1993).

It is not yet clear, whether the virus infection spreads to the septal walls of the lung strictly via the respiratory route or if a hematogeneous transmission is involved. Alveolar macrophages are obviously the major target, but by morphological criteria type II pneumocytes are probably involved. We observed widespread inflammation in advanced stages of pneumonia, although the number of infected cells remained rather small. A number of results indicate, that the interactions between PRRSV and macrophage populations play a pivotal role determining the outcome of infection (Molitor et al., 1997). The balance between tissue injury and protection strongly depends on the pattern of proinflammatory cy-

tokines produced by alveolar macrophages (Murtaugh et al., 1994 and 1996). Furthermore, chemokines which attract mononuclear phagocytes and neutrophils may have a great impact on the disease process.

We employed the RT-PCR to obtain preliminary information on the expression of mRNA specific for cytokines and immunomodulatory substances. The protein levels of many cytokine genes are regulated by transcription. Therefore, RT-PCR technology can complement bioassays or immunoassays both in terms of sensitivity and specificity, especially when no other options are available. However, RT-PCR results should be interpreted with caution. First, for some cytokines transcriptional expression does not correlate with biological activity. Secondly, only limited conclusions can be drawn because of the lack of localisation and insufficient information on the morphological context. This ambiguity is illustrated by the observation, that by RT-PCR potentially harmful cytokines appeared to be transcriptionally expressed in healthy control pigs. Further studies are in progress to clarify these points.

PRRSV replicated in early stages in lymph organs, later lung tissue was involved. It is an open question, whether the infection results directly in immunosuppression because of a functional and quantitative impairment of alveolar macrophages. In other studies, at least a temporal disturbance of lung defence mechanisms against secondary infections were observed. However, the diminished number of lymphocytes and macrophages recovered within a few weeks p. i. (Molitor et al., 1997; Shimizu et al., 1996). An infection with PRRSV may possibly booster the immune response induced by vaccinations possibly through activation of macrophages. Furthermore, it was observed that certain antibodies to PRRSV can even enhance the uptake of virus by alveolar macrophages (Yoon et al., 1997).

IL-6 was transcriptionally upregulated in infected lung tissue as well as in other organs. This cytokine is produced by several cell types. It mediates an early protection by acute phase proteins and enhances the antibody response. A most interesting observation was the selective induction of the alveolar macrophage chemotactic factor AMCF II, a chemokine important for attraction of neutrophils. The peptide is related to the chemokine family GRO and CINC (Goodman et al., 1991 and 1992). It is structurally different from the chemokine IL-8, which belongs to the integrine family but has similar biological properties. In our studies, IL-8 was upregulated independently of AMCFII. Further studies are of interest to analyse the relations of PRRSV infection to signal mediated induction of immunomodulatory substances.

The clinical course of PRRSV induced interstitial pneumonia was relatively mild. The pathology differs clearly from the pleuropneumonia induced by some gram-negative bacteria, which may be triggered by the immunological effects of lipopolysaccharides. One striking difference concerns the kinetics of disease. Only a few hours following infection of swine by Pasteurella multocida pronounced infiltrations were observed (Berndt and Müller, 1995). Furthermore, following endotracheal infection with Actinobacillus pleuropneumoniae lavage cells expressed already within 2 hours p. i. proinflammatory cytokines IL-1 and IL-8 followed later by IL-6 (Baarsch et al., 1995). It is conceivable, that mutual interactions between viral and bacterial agents occur, which finally precipitate or exacerbate a clinical disease. PRRSV infections may thus complicate the enzootic pneumonia of pigs, which represents a multifactorial disease associated with a number of bacterial infections (Molitor et al., 1997; Van Reeth, 1997). This health problem is of increasing importance for animal welfare. We hope, that further studies of PRRS will help to understand more precisely the relations between inflammation, virus infection and the immune response.

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