

Immune responses of pigs after experimental infection with a European strain of *Porcine reproductive and respiratory syndrome virus*

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The purpose of this experiment was to study the immune response of pigs during an experimental infection with a European strain of *Porcine reproductive and respiratory syndrome virus* (PRRSV). Five pigs were challenged intranasally with PRRSV strain VP21 and another five were kept as controls. Clinical course and humoral and cell-mediated responses were monitored for 70 days post-infection (p.i.). Infected pigs developed mild signs at 24 h p.i. Viraemia was detectable by nested RT-PCR until day 14 p.i. Earliest seroconversions (ELISA) were seen by day 7 p.i. (three of five animals) and, by day 14, all inoculated pigs had seroconverted (ELISA and immunoperoxidase monolayer assay). Virus-neutralizing antibodies were undetectable until day 56 p.i. and, by day 70 p.i., two inoculated pigs still were negative. Flow-cytometry assays using peripheral blood mononuclear cells (PBMC) showed an upshift in CD8⁺ cells (day 7 p.i.) and a downshift of CD21⁺ cells (days 7 and 28 p.i.). Regarding cell-mediated responses, development of PRRSV-specific gamma interferon-secreting cells (IFN- γ -SC) and interleukin 4-secreting cells (IL4-SC) in PBMC was examined by ELISPOT assay. IFN- γ -SC were not detected significantly until day 14 p.i., whereas, for IL4-SC, no differences between groups were seen. Concurrently with the onset of viraemia and the development of clinical signs, serum haptoglobin levels and interleukin 10 (IL10) in PRRSV-stimulated PBMC-culture supernatants increased significantly. These differences disappeared later on. For IL2, IL4, IL8 or transforming growth factor beta, no differences were seen among groups. These results are compatible with a model in which the immune response does not fully control the outcome of the infection.

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INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) was first identified in 1991 by Dutch researchers (Wensvoort *et al.*, 1991) as the causative agent of a new disease of pigs. Since then, PRRSV has become one of the leading causes of economic losses in swine operations worldwide. At present, this virus is classified in the family *Arteriviridae* together with *Equine arteritis virus*, *Simian hemorrhagic fever virus* and *Lactate dehydrogenase-elevating virus* (Meulenbergh *et al.*, 1994). Two genotypes are recognized (American and European). Both are thought to derive from a common ancestor, but genetic similarity between these two genotypes is about 55–65% (Meng *et al.*, 1995; Murtaugh *et al.*, 1995; Gagnon & Dea, 1998; Dea *et al.*, 2000). In addition, genetic diversity of strains within a given genotype is high (Drew *et al.*, 1997; Forsberg *et al.*, 2002; Goldberg *et al.*, 2003; Mateu *et al.*, 2003).

Immune response to PRRSV is poorly understood but, in spite of this, some vaccines made from European- or American-type strains are being commercialized. Several studies have reported controversial results about the efficacy of vaccination (Meng, 2000). It seems that American-type vaccines are more effective to protect against infections caused by American-type strains than against European-type infections (van Woensel *et al.*, 1998; Lager *et al.*, 1999). However, some degree of protection against the heterologous genotype is observed (Lager *et al.*, 1999; Labarque *et al.*, 2003). This fact indicates that critical determinants of the immune response against each type of strains are similar, but not equal. As a consequence, at present there is no single vaccine that can claim full protection against all strains of PRRSV.

One of the reasons for the lack of development of newer and more efficacious vaccines against PRRSV is the scarce

knowledge on the immune response of pigs after infection by field strains. Available data have mostly been obtained by using American-type strains and have indicated that the adaptative immune response against American-type PRRSV is unique in its features. Infected piglets can be viraemic for up to 6–12 weeks, but circulating antibodies can be easily detected much earlier (Yoon *et al.*, 1995; Batista *et al.*, 2004; Johnson *et al.*, 2004). Nevertheless, neutralizing antibodies (NA) do not appear until 8–10 weeks post-infection (p.i.) and their role in protection is not clear (Murtaugh *et al.*, 2002). Besides, cell-mediated immune responses, measured as virus-specific gamma interferon-secreting cells (IFN- γ -SC), are erratic during the first weeks p.i. Later, there is a sudden increase in IFN- γ -SC that is maintained, despite a progressive fading of PRRSV-specific antibodies. However, the IFN- γ -SC response seems to be delayed, compared with other pathogens (Meier *et al.*, 2003).

To date, there has been no report showing the humoral and cell-mediated evolution of the immune response after infection with a wild PRRSV strain of the European genotype. The aim of the present study was to characterize the immune responses of pigs after infection with a European-type field strain of PRRSV.

METHODS

Animals and housing. Ten healthy 4-week-old Landrace pigs were selected in a high-health farm belonging to the Institut de Recerca i Tecnologia Agroalimentària (IRTA, Barcelona, Spain) that has historically been free of all major pig diseases, including PRRS. Animals were transported to the experimental facilities and raised until they were 16 weeks old, being examined periodically for antibodies against porcine circovirus type 2, Aujeszky's disease virus, *Porcine parvovirus*, swine influenza and *Mycoplasma hyopneumoniae*. All animals were seronegative for all of the above-mentioned pathogens. Also, animals were confirmed to be free of PRRSV, as determined by ELISA (HerdChek PRRS 2XR; IDEXX Laboratories).

Virus and challenge. The virulent PRRSV VP21 strain used for the challenge was isolated in porcine alveolar macrophages (PAM) from sera of naturally infected pigs during an outbreak of PRRS affecting a breeding farm located in the north of Spain in December 1991. That outbreak was characterized by abortions, stillbirths and, later on, piglet mortality. The virus was shown to be of the European genotype by sequencing of ORFs 2–7. Viral stock was done by passage ($n=3$) in PAM and titrated by means of the immunoperoxidase monolayer assay (IPMA). Before use, the viral stock was checked for bacterial contamination and for the presence of other viruses. Challenge was done when pigs were 17 weeks old. Pigs were divided randomly in two groups (A and B). Pigs in group A were inoculated intranasally with 2 ml viral suspension containing 10^6 TCID₅₀ PRRSV VP21 strain ml⁻¹, whilst pigs in group B (controls) received 2 ml sterile PBS.

Clinical examination and production parameters. Experimental pigs were clinically examined daily and rectal body temperatures were recorded from days 0 to 14 p.i. Every week, pigs were weighed and the amount of feedstuff consumed was recorded.

Samples. Blood samples were collected in duplicate (heparinized and siliconized blood-collecting tubes) immediately before challenge and then at days 7, 14, 21, 28, 56 and 70 p.i. Sera were used for PRRSV-specific RT-nested PCR (nPCR), determination of humoral

responses and evaluation of haptoglobin and interleukin 8 (IL8), IL10 and transforming growth factor beta (TGF- β) levels. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood samples and used in flow-cytometry assays and for *in vitro* experiments.

Detection of viraemia. Serum samples were inoculated on MARC-145 and PAM cultures and incubated at 37 °C in 5% CO₂ for 90 min. Then, sera were removed and minimal essential medium (Sigma) was added. Cultures were incubated for 3 days at 37 °C in 5% CO₂. Infection of inoculated cells was determined by IPMA.

Besides viral isolation, sera were examined by nPCR. Briefly, total RNA was extracted from 150 μ l serum by using a commercial system (Nucleospin RNA virus; Macherey-Nagel). Total RNA was transcribed and cDNA was used in a first PCR round directed to viral ORF5 (Mateu *et al.*, 2003). This first-round PCR had a sensitivity of about 10^2 TCID₅₀ (ml serum)⁻¹. PCR products were used in an nPCR (forward primer, 5'-TCTTGCTTCTGGTGGCTTTT-3'; reverse primer, 5'-CATGTTT-GATGGTGACGAGG-3') that produced a 499 bp amplicon. Cycling parameters for both PCRs were: 94 °C for 45 s; 55 °C for 45 s; 72 °C for 45 s for a total of 35 cycles. Under these conditions, the nPCR was considered to be able to detect <10 viral copies (ml serum)⁻¹.

Humoral immune response. A commercially available ELISA (HerdChek PRRS 2XR; Idexx Laboratories) was used to measure PRRSV-specific antibodies. According to the manufacturer, sample to positive control (S/P) ratios of >0.4 were considered positive. PRRSV antibody titres were also determined in sera by using IPMA as described by Wensvoort *et al.* (1991), using MARC-145 cells infected with the PRRSV VP21 strain. Tests were done in duplicate.

NA were measured by the technique described by Yoon *et al.* (1994) and Jusa *et al.* (1996). Tests were done in triplicate by using whole serum, inactivated serum and serum plus 10% fresh guinea-pig complement. Briefly, 50 μ l of each serum to be tested was diluted serially from 1/20 to 1/160 in cell-culture medium. Dilutions were mixed with 50 μ l viral suspension containing 200 TCID₅₀ of the PRRSV strain VP21 (with or without complement). Virus-serum mixtures were incubated for 1 h at 37 °C and then added to MARC-145 cultures in duplicate (96-well plates) and incubated for 3 days at 37 °C in 5% CO₂. Infection of cell cultures was revealed by using IPMA.

Total serum antibodies (IgG and IgM) were analysed by means of a commercial capture ELISA (Pig IgG ELISA quantification kit and Pig IgM ELISA quantification kit; Bethyl Laboratories), done according to the recommendations of the manufacturer. Serum-antibody concentrations were calculated by using a regression line calculated after measurement of ODs of the standards provided with the kit.

Isolation and culture of PBMC. PBMC were separated from whole blood by density-gradient centrifugation with Histopaque 1077 (Sigma). For PBMC cultures, RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen), 1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 mM 2-mercaptoethanol (Sigma), 50 000 IU penicillin I⁻¹ (Invitrogen), 50 mg streptomycin I⁻¹ (Invitrogen) and 50 mg gentamicin I⁻¹ (Sigma) was used. Trypan blue was used to assess viability.

Flow-cytometry assays. Phenotypic analysis of PBMC subsets was done by flow cytometry using the following mAbs: anti-CD4-PE (phycoerythrin) (clone 74-12-4, provided by Dr J. Domínguez, INIA, Madrid, Spain), anti-CD8-FITC (fluorescein isothiocyanate) (clone 76-2-11, INIA), anti-CD21-FITC (4530-02; Southern Biotechnologies), anti-CD25 (K231-3B2, INIA), anti-SWC3-PE (4525-09, Southern Biotechnologies) and anti-SLA-II DR (clone 1F12, INIA).

When needed, goat F(ab')₂ anti-mouse IgG_{2a} R-PE-conjugated

antibody and goat F(ab')₂ anti-mouse IgG₁ FITC-conjugated antibody (Southern Biotechnologies) were used as secondary antibodies. Irrelevant, isotype-matched antibodies were included as background controls. Analyses were done by using an EPICS XL-MLC cytometer (Coulter) to an excitation wavelength of 488 nm and with 580 and 630 nm filters.

ELISPOT: PRRSV-specific IFN- γ -SC and IL4-secreting cells (IL4-SC). Frequencies of PRRSV-specific IFN- γ -SC and IL4-SC in PBMC were analysed by an ELISPOT assay using commercial mAbs (Swine IFN- γ and Swine IL4 Cytosets; Biosource Europe) according to a previously reported method (Díaz & Mateu, 2005). Briefly, for IFN- γ , ELISA plates (Costar 3590; Corning) were coated overnight with 8.3 μ g IFN- γ capture antibody ml⁻¹ diluted in carbonate/bicarbonate buffer (pH 9.6). Plates were then washed and blocked for 1 h at 37 °C with 150 μ l PBS with 1% BSA. After removal of the blocking solution, 5 \times 10⁵ PBMC were dispensed per well (50 μ l) and stimulated with the VP21 strain at an m.o.i. of 0.1 as the recall antigen. After 20 h incubation at 37 °C in a 5% CO₂ atmosphere, cells were removed and the biotinylated detection antibody was added at 2.5 μ g ml⁻¹ (50 μ l) and incubated for 1 h at 37 °C. The reaction was revealed by sequential incubation of plates with streptavidin-peroxidase (1 h) and insoluble TMB blue (Calbiochem). For IL4, the protocol was similar, but working dilutions of the mAbs were 9.6 μ g ml⁻¹ (capture antibody diluted in PBS, pH 7.2) and 5.0 μ g ml⁻¹ (biotinylated detection antibody). In both cases, unstimulated cells and phytohaemagglutinin (PHA)-stimulated controls (10 μ g ml⁻¹) were also included.

To calculate the PRRSV-specific frequencies of IFN- γ -SC and IL4-SC, counts of spots in unstimulated wells were subtracted from counts in virus-stimulated wells. Frequencies of cytokine-producing cells were expressed as responding cells in 10⁶ PBMC.

Cytokine (IL2, IL4, IL8, IL10 and TGF- β) and haptoglobin ELISAs. PBMC were seeded at a density of 2 \times 10⁶ cells per well (250 μ l) in 96-well plates and were mock-stimulated or stimulated with either PRRSV strain VP21 (m.o.i. of 0.1) or PHA (10 μ g ml⁻¹). After 24 h incubation at 37 °C in 5% CO₂, cell-culture supernatants were collected and frozen at -80 °C until needed. Capture ELISAs for IL2, IL4 and IL10 were performed as reported previously (Darwich *et al.*, 2003; Díaz & Mateu, 2005), using commercial pairs of mAbs (Swine IL2, IL4 and IL10 cytosets; Biosource Europe). For IL8, an ELISA kit was used (IL8 Immunoassay kit; Biosource Europe). For TGF- β , an ELISA was developed by using commercial antibodies (TGF- β cytosets; Biosource Europe). The cut-off point of each ELISA was calculated as the mean + 3SD OD of negative controls. Cytokine concentrations were calculated by using the linear-regression formula from ODs of the cytokine standards provided by the manufacturer. IL8, IL10 and TGF- β were also measured in sera of experimental animals by using the above-mentioned ELISAs. Serum-haptoglobin levels were determined by means of ELISA (Haptoglobin assay phase range; Tridelata) according to the manufacturer's instructions.

Statistical analysis. The regression line of cytokine standards in ELISA was calculated by using SPSS v. 12.0 (SPSS Inc.). Statistical comparisons between groups (Mann-Whitney test) were done by using StatsDirect v. 2.4.1. All tests done in the study, as well as the statistical analysis, were performed blind.

RESULTS

Clinical course and viraemia

In infected animals, the first signs of disease were seen at 24 h p.i. Pigs were dull, anorectic and had a fever

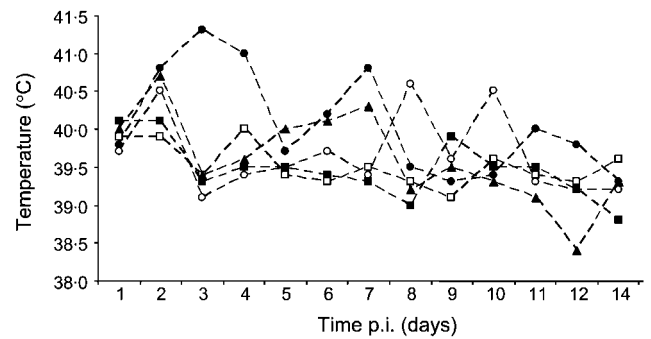


Fig. 1. Rectal body temperatures were measured in all pigs (five infected and five controls) at 24 h intervals during the first 2 weeks p.i. The graph only shows the results of PRRSV-infected pigs, as control pigs always had normal body temperatures. Each symbol (●, ○, ▲, ■ and □) corresponds to one of the five pigs of the challenge group.

(> 39.5 °C). During the first week, all infected pigs had a fever but, by day 7 p.i., rectal temperatures had returned to normality. Between days 7 and 11 p.i., a second peak of fever was seen in three of five challenged animals (Fig. 1). Significant differences in weight gain were observed during the first 2 weeks of infection. From days 0 to 7, challenged pigs gained 5.1 kg less than uninfected controls and, from days 7 to 14, 2.6 kg less ($P < 0.05$). On the third week, challenged pigs showed a compensatory growth and, by day 35 p.i., weight gains were equal in infected and uninfected pigs (Table 1).

All challenged animals were positive by nPCR at least once during the first 2 weeks p.i. However, viraemia was probably low as, with a single-round RT-PCR, only one animal was detected as positive and the remaining viraemic pigs were detected only after nPCR. Viral isolation was less sensitive than nPCR, only being isolated from four pigs. None of the uninoculated pigs yielded nPCR-positive results or viral isolation.

Table 1. Weekly weight gain of PRRSV-infected pigs and uninfected controls

From 35 days p.i. to the end of the experiment, both groups showed the same weight gain.

Time p.i. (days)	Mean \pm SD weekly weight gain (kg)	
	Uninfected	Infected
0–7	11.1 \pm 1.08*	6 \pm 4.60
7–14	7.9 \pm 1.38*	5.3 \pm 2.56
14–21	8.1 \pm 0.74	7.1 \pm 1.08
21–28	4.8 \pm 0.75	7.5 \pm 1.27
28–35	1.1 \pm 0.74	1.1 \pm 0.75

* $P < 0.05$.

Humoral immune response

All pigs were seronegative to PRRSV at day 0. By using ELISA, two infected pigs seroconverted by day 7 (mean S/P ratio, 0.51) and all were clearly positive by day 14 (mean S/P ratio, 2.12). ELISA titres remained high throughout the 70 days of observation and a slow decline was seen from day 56 p.i. onwards (mean S/P ratio at day 70 p.i., 1.81). IPMA was slightly less sensitive than ELISA. With IPMA, positive results were only obtained from day 14 p.i. onwards. The highest mean IPMA titres were seen at day 56 p.i. [$\log_{10}(\text{titre}) = 3.05$].

NA were first detected at day 56 p.i. and only in three of five infected pigs. The remaining two pigs had not developed NA by day 70. By using whole serum, the highest neutralizing titre was $\log_{10}(\text{titre}) = 1.80$. Addition of fresh complement increased titres by one dilution. All un inoculated controls remained seronegative to PRRSV in all tests throughout the study. Table 2 summarizes these results.

Total antibodies were determined by using specific pig IgG and IgM ELISAs. Although great individual variability was seen in both groups for both IgG and IgM, comparison of mean values between groups did not show significant differences.

Flow-cytometry assays

At day 7 p.i., infected pigs had a significant increase in the proportion of peripheral blood CD8⁺ cells and a decrease in CD21⁺ cells ($P < 0.05$). At day 14 p.i., these differences disappeared. Infected pigs again had a decrease in CD21⁺ cells at 28 days p.i. No differences were seen among groups regarding expression of CD4, CD25, SWC3 or SLA-II in PBMC. Table 3 shows the complete results of the flow-cytometry analysis.

ELISPOT

In contrast to anti-PRRSV-antibody values, the frequencies of PRRSV-specific IFN- γ -SC had some variability in all samples of the infected group. Notwithstanding, a similar evolution of PRRSV-specific IFN- γ -SC was observed in all infected animals. PRRSV-specific IFN- γ -SC were detected for the first time at day 14 p.i. (49 IFN- γ -SC in 10^6 PBMC, $P < 0.05$). Then, counts of PRRSV-specific IFN- γ SC showed highs and lows until day 70 p.i., when they reached a peak (62 IFN- γ -SC in 10^6 PBMC) (Fig. 2). Regarding IL4-SC, values were low (< 10 in 10^6 PBMC) and no significant differences were seen among groups.

Cytokine ELISAs

Examination of PRRSV-stimulated PBMC-culture supernatants showed no significant differences among groups for IL2, IL4 or TGF- β (data not shown). In contrast, at 1 week p.i., levels of IL10 were significantly higher ($P < 0.05$) in culture supernatants of PRRSV-stimulated PBMC (> 80 pg ml⁻¹) obtained from infected pigs. This difference was not observed later in the course of infection, although PBMC from challenged pigs always produced more IL10 against PRRSV than did cells from control animals (Fig. 2). IL8, IL10 and TGF- β secretion was also examined by ELISA in sera. None of the sera yielded positive results for these cytokines.

In PRRSV-challenged pigs, serum-haptoglobin levels were increased significantly at days 7 and 14 p.i. ($P < 0.05$), returning to values similar to those of uninfected pigs by day 21 p.i. (Fig. 3).

DISCUSSION

The immune response of pigs against PRRSV is not yet fully understood and some phenomena, such as the delayed development of NA and the role of T cell-mediated

Table 2. Serological evolution of PRRSV-infected pigs by ELISA (HerdChek PRRS 2XR; Idexx Laboratories), IPMA and viral-neutralization test (VNT)

Results are expressed as the mean \pm SD titre. Uninfected animals were negative in all assays.

Test	Development of specific PRRSV-antibodies (days p.i.)						
	0	7	14	21	28	56	70
ELISA							
No. positive	—	2/5	5/5	5/5	5/5	5/5	5/5
S/P ratio		0.51 \pm 0.14	2.12 \pm 0.20	2.23 \pm 0.24	2.17 \pm 0.26	1.76 \pm 0.3014	1.81 \pm 0.25
IPMA							
No. positive	—	—	5/5	5/5	5/5	5/5	5/5
Mean $\log_{10}(\text{titre})$			2.57 \pm 0.33	2.75 \pm 0.13	2.99 \pm 0.16	3.05 \pm 0.13	2.69 \pm 0.27
VNT							
No. positive	—	—	—	—	—	3/5	3/5
Mean $\log_{10}(\text{titre})$						1.80 \pm 0.28	1.60 \pm 0.42

Table 3. Flow-cytometry results for PBMC of PRRSV-infected pigs and uninfected controlsResults are expressed as mean \pm SD (%) for five PRRSV-infected and five control pigs.

Marker	Proportion of PBMC \pm SD (%) (days p.i.)				
	0	7	14	21	28
CD4					
Uninfected	18.9 \pm 0.5	19.8 \pm 2.8	13.3 \pm 4.6	14.8 \pm 1.9	17.2 \pm 1.8
Infected	20.8 \pm 3.4	18.9 \pm 2.5	18.0 \pm 4.3	16.2 \pm 3.2	14.3 \pm 5.7
CD8					
Uninfected	31.3 \pm 7.7	24.0 \pm 4.7	23.9 \pm 5.1	25.3 \pm 6.5	31.9 \pm 3.4
Infected	23.0 \pm 3.0	34.6 \pm 6.6*	25.4 \pm 2.0	27.2 \pm 3.8	27.4 \pm 3.6
CD21					
Uninfected	10.1 \pm 2.2	8.4 \pm 2.7	6.9 \pm 2.7	5.5 \pm 2.6	9.0 \pm 1.4
Infected	14.7 \pm 5.1	3.9 \pm 1.9*	5.4 \pm 1.8	5.7 \pm 1.7	7.4 \pm 0.9*
CD25					
Uninfected	5.6 \pm 1.3	8.2 \pm 1.8	7.3 \pm 1.2	9.4 \pm 4.6	10.6 \pm 1.2
Infected	6.5 \pm 1.2	6.7 \pm 0.6	6.9 \pm 0.9	9.9 \pm 2.0	10.6 \pm 1.2
SWC3					
Uninfected	6.1 \pm 2.7	9.1 \pm 2.8	7.1 \pm 1.7	14.2 \pm 1.9	8.5 \pm 1.6
Infected	9.5 \pm 3.0	9.1 \pm 4.0	7.8 \pm 5.4	13.1 \pm 1.3	12.2 \pm 6.6
SLA-II					
Uninfected	50.7 \pm 7.1	50.4 \pm 9.5	47.3 \pm 9.5	53.3 \pm 10.9	53.8 \pm 12.5
Infected	51.9 \pm 3.9	55.1 \pm 3.9	48.8 \pm 5.1	54.3 \pm 8.6	55.7 \pm 6.8

* $P < 0.05$.

immunity, remain poorly explained. Most immunological studies have been done by using American-type strains (Osorio *et al.*, 2002; Meier *et al.*, 2003; Royae *et al.*, 2004) and, as genetic diversity is thought to play a role in the immune escape of the virus, differences might arise between the immune responses in European- and American-type

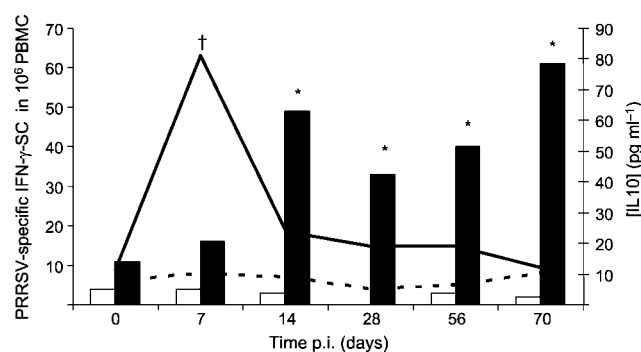


Fig. 2. IFN- γ -SC (ELISPOT) and IL10 (ELISA) levels in PBMC-culture supernatants after recall of PRRSV in pigs infected with the VP21 strain. For IL10, results are expressed as the mean of secreted IL10 (pg ml^{-1}), whereas specific virus IFN- γ -SC are expressed as the mean frequency of the specific virus IFN- γ -SC in 10^6 PBMC. Empty bars, IFN- γ -SC in control pigs; filled bars, IFN- γ -SC in infected pigs; dashed line, IL10 in control pigs; solid line, IL10 in infected pigs. *, $P < 0.05$; †, $P < 0.05$.

PRRSV infections. Some studies analysed the role of immunity after the infection or vaccination of pigs with a European-genotype PRRSV (Nielsen & Bøtner, 1997; López Fuertes *et al.*, 1999; Samsom *et al.*, 2000; Aasted *et al.*, 2002; Suradhat & Thanawongnuwech, 2003), but a global immunological picture is still lacking.

Our experiment was deliberately conducted with relatively aged pigs (17 weeks of age). This selection was done firstly because we wished to have a clear model of infection in which maturation of the immune system was not a source of confusion and, secondly, because we tried to avoid

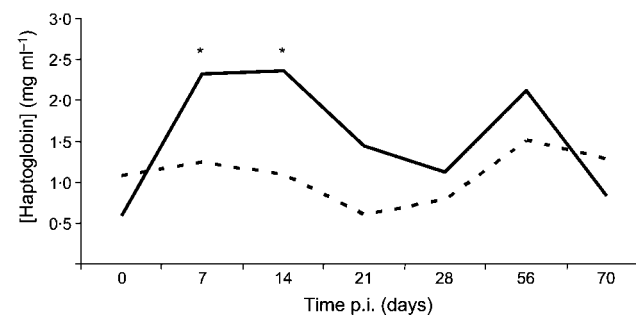


Fig. 3. Kinetics of serum haptoglobin as measured by ELISA after infection with a PRRSV European-type strain. Dashed line, control pigs; solid line, infected pigs. *, $P < 0.05$.

development of a severe disease that could kill animals before the planned end of the experiment.

After experimental infection with the PRRSV VP21 strain, all infected pigs developed only mild signs of disease in the first 24 h p.i. These signs, mainly fever, anorexia and weight loss, lasted until 14 days p.i., when viraemia ceased. This course of infection is similar to that described in other reports using European-type strains (van der Linden *et al.*, 2003).

Interestingly, viraemia was low and was only detectable after nPCR was used. These facts suggest that clinical signs are related to the presence of virus in blood. As PRRSV does not replicate in monocytes or other blood cells (Duan *et al.*, 1997), it would be expected that viraemia reflected the intensity of virus replication in tissues. Other experimental PRRSV infections or vaccinations have also shown short viraemias, even in pigs younger than ours (López Fuertes *et al.*, 1999; Meier *et al.*, 2003; Sipos *et al.*, 2003).

By using ELISA, development of specific antibodies could be seen as early as 7 days p.i. and lasted until the end of the study at detectable levels. The performance of IPMA was somewhat poorer but, by day 14 p.i., all infected animals were detected as seropositive. It is known that detection of early antibodies is useful as a diagnostic tool, but it seems that they do not play a role in protection against the infection (reviewed by Murtaugh *et al.*, 2002; Lopez & Osorio, 2004).

In our experiment and as reported by others (Vezina *et al.*, 1996; Meier *et al.*, 2003), NA appeared late (from day 56 p.i. onwards) and not in all animals. The existence of decoy immunodominant epitopes in GP5 has been suggested as the cause of such delayed production (Ostrowski *et al.*, 2002).

The role of NA in protection against PRRSV is controversial. Osorio *et al.* (2002) showed that NA could be protective against PRRSV if administered exogenously at high doses. However, most studies showed that the production of NA after an experimental infection or vaccination is low and sporadic, with considerable individual variability (Loemba *et al.*, 1996; Meier *et al.*, 2003). In our case, clearance of viraemia was observed before NA could be detected. We cannot rule out the possibility that very small amounts of NA, undetectable in neutralization assays, could have existed at that time and played a role in the cessation of viraemia but, as three different methods of examining serum NA were used, it seems more reasonable to think that viraemia disappeared in the absence of NA. In immunodeficient mice infected with *Lactate dehydrogenase-elevating virus*, a related arterivirus, viraemia can also disappear without noticeable NA (Onyekaba *et al.*, 1989).

One of the effects attributed to PRRSV is the induction of a polyclonal B activation (Lamontagne *et al.*, 2001). In our case, neither total IgM nor IgG levels rose significantly after

infection. Also, the proportion of neither CD21⁺ nor CD25⁺ cells increased. Moreover, the proportion of CD21⁺ cells in blood decreased at 1 week p.i. Mobilization of B cells to tissues could explain these results and would be consistent with the suggestions of other authors, which have indicated that, in PRRS, polyclonal activation of B cells occurs in lymphoid organs, but not in blood (Kawashima *et al.*, 1999; Lamontagne *et al.*, 2001).

In contrast, CD8⁺ cells increased in blood in the first week p.i. Samsom *et al.* (2000) observed that CD8⁺ cells also increased in bronchoalveolar fluid of pigs after a PRRSV infection. However, this increase is not likely to be attributable to a clonal expansion of PRRSV-specific T cells. If this was the case, other evidence, such as IL2 production, IFN- γ increase or an upshift of CD25⁺ cells, should have been observed. Other reports (Shimizu *et al.*, 1996; Albina *et al.*, 1998) indicated that this was not a mitogenic effect of the virus, but a response to some physiological stimulus. As we will discuss later, this increase in CD8⁺ cells correlated with the detection of IL10 in PBMC-culture supernatants and with the raise of haptoglobin levels in serum. Moore *et al.* (2001) showed that IL10 can promote growth and differentiation of CD8⁺ cells.

Regarding the cellular immune response, our results showed that PRRSV-specific IFN- γ -SC appeared at week 2 p.i. Levels of IFN- γ -SC were undulant in the first 70 days p.i. This evolution agrees with the results of Meier *et al.* (2003) although, in our case, the strength of the response, in terms of the number of IFN- γ -SC, was lower. Whether these differences were caused by the use of different mAbs in ELISPOT or reflect a true difference cannot be known at this time.

Interestingly, although the initial raise of PRRSV-specific IFN- γ -SC was delayed for 14 days after the challenge, the last detection of viraemia in infected pigs corresponded to the appearance of these cells. This fact may be incidental, but suggests some role of IFN- γ -SC in the control of the infection. Regarding IL4-SC, no significant responses against PRRSV were detected in infected pigs. This is surprising, considering the fast and high humoral response against the virus, but might be an explanation for the delayed development of NA.

In our study, results for IL2, IL4 and IL8 did not show significant differences between control and infected animals. These observations reinforce the notion that some type of immunomodulation takes place in the course of infection (Molitor *et al.*, 1997; Lager & Mengeling, 2000). We also evaluated IL10 responses of infected pigs. Other authors showed that this cytokine can play a significant role in PRRS (Chung & Chae, 2003; Suradhat & Thanawongnuwech, 2003; Suradhat *et al.*, 2003). In our case, the ability of PBMC to produce IL10 in response to viral stimulation was a significant fact in the first week p.i., but IL10 was not detected significantly in cell-culture supernatants afterwards. This pattern is difficult to explain.

It is known that IL10 is produced mainly by cells of the monocyte/macrophage lineage, by regulatory T cells or, less frequently, by B cells. At this time, none of these populations can be ruled out as the source of this cytokine. Besides, TGF- β levels were not significantly different in serum or cell-culture supernatants of infected and uninfected pigs and, as we discussed before, IL4 was not detected in response to PRRSV. Natural regulatory T cells are thought to belong to the CD4⁺CD25⁺ subset (McGuirk & Mills, 2002). We did not see any change in the relative proportion of these two markers in PBMC throughout the study and, under this perspective, it seems unlikely that natural regulatory T cells were involved in IL10 production. It is tempting to think of monocytes as being responsible for this production. However, this needs further research to be clarified.

The increased ability of PBMC to produce IL10 against PRRSV at the first week p.i. correlated with the rise of serum-haptoglobin levels. Haptoglobin is an acute-phase protein produced by the liver and related to IL6 (Asai *et al.*, 1999). It seems to play a major role in modulating immune responses through a complex network of interactions. Thus, haptoglobin release has been related to the secretion of anti-inflammatory cytokines, particularly IL10, through the interaction with CD163, a haemoglobin-scavenger receptor that is solely present in cells of the monocyte/macrophage lineage (Moestrup & Moller, 2004; Philippidis *et al.*, 2004). Interestingly, other pig viruses, such as *African swine fever virus*, replicate mainly in CD163⁺ macrophages (Sánchez-Torres *et al.*, 2003).

In summary, comparison of our results with previous studies performed with American strains (Meier *et al.*, 2003; Royae *et al.*, 2004) suggests that the immune response in all PRRSV infections is similar. Main traits are a strong, non-neutralizing humoral response with a delayed development of NA, a low IFN- γ -SC frequency, very low or undetectable IL2 and IL4 responses and, in the first stages of infection, a significant involvement of IL10 and haptoglobin.

As suggested by Xiao *et al.* (2004), the results presented in this work are consistent with a hypothetical model in which the outcome of PRRSV infection is related more to the dynamics of permissive macrophages and the early events of the natural response than to the development of specific immunity. In such a model, the earliest events in the course of the infection would involve release of IL10 and haptoglobin. This IL10, either released by infected macrophages as an anti-inflammatory response or induced by viral proteins, would probably impair, but not abolish, the development of cell-mediated immunity (Royae *et al.*, 2004). This fact would be reflected in the erratic levels of virus-specific IFN- γ -SC during the first weeks p.i. (Meier *et al.*, 2003) and in the lack of virus-specific IL2- and IL4-producing cells. As infection progresses, the cytolytic cycles of virus replication would decrease the number of permissive macrophages (Xiao *et al.*, 2004). As seen by others (Duan *et al.*, 1997), permissive cells should account for only a small proportion

of total tissue macrophages. The stronger the cytolytic cycle, the faster the resolution of the viraemia. At this point, the weak cell-mediated immunity would be able to confine PRRSV to certain tissues (i.e. lymph nodes) where the proportion of permissive cells could be still relatively high (Xiao *et al.*, 2004). In consequence, viraemia should cease or become inconstant and of low level. This would occur regardless of whether or not NA had developed. However, as long as the virus persisted in tissues, local IL10 release (or other cytokines) would keep T-cell responses low. As far as the infection can be confined to certain tissues and permissive macrophages are not replaced at a high rate, the number of infected cells will decline steadily and the immune response will finally be able to clear the infection. This moment would correspond with the rise of blood IFN- γ -SC and the final development of NA. From then onwards, the pig would be protected against homologous challenge (Mengeling *et al.*, 2003). Such a model would explain why no clear correlation between the immune response and the clearing of infection can be determined, as well as making sense of the delayed T-cell responses observed in PRRSV infection. In our opinion, this hypothesis deserves further study, as it can give a whole image of the events taking place in PRRSV infection and can contribute to understanding how to develop new and more efficient vaccines.

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