Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected by postweaning multisystemic wasting syndrome

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Fifteen 8-week-old conventional pigs were selected from a farm where pigs were suffering from postweaning multisystemic wasting syndrome (PMWS). Ten of the animals were diseased pigs showing typical signs of PMWS (wasting and respiratory disorders) and positive for infection with porcine circovirus type 2 (PCV2), and the other five animals selected as controls were pen-mate, apparently healthy pigs. Blood samples and lymphoid tissues were taken from each animal for haematological, serological and histopathological studies. Also, cytokine mRNA expression of IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12p40 and IFN-γ from inguinal and bronchial lymph nodes, tonsils, spleen and thymus was determined by semi-quantitative RT-PCR. Pigs suffering from PMWS showed severe alterations of haematological parameters such as anaemia, lymphopenia with decrease of CD8+ and IgM+ cells, monocytosis and neutrophilia. Also, extensive lymphocyte depletion and altered cytokine mRNA expression patterns were seen in most of the examined lymphoid organs. Those cytokine mRNA alterations were characterized by an overexpression of IL-10 mRNA in thymus and IFN-γ mRNA in tonsils, and by decreases in the mRNA expression of several cytokines as IL-2 and IL-12p40 in the spleen, IL-4 in tonsils, and IFN-γ, IL-10, IL-12p40 and IL-4 in inguinal lymph nodes. Also, the IL-10 mRNA overexpression was histologically associated with the thymic depletion and atrophy observed in PMWS pigs. In conclusion, the cytokine mRNA imbalance, specially the increased mRNA levels of IL-10 in the thymus, jointly with the histopathological and haematological disorders, are highly indicative of a T-cell immunosuppression, enhancing the notion that the immune system of PMWS-affected pigs is severely impaired.

INTRODUCTION

Postweaning multisystemic wasting syndrome (PMWS) is a new condition originally described in nursery pigs of Canadian herds in 1991 (Harding, 1997). The clinical signs of this syndrome include progressive weight loss, unthriftiness, paleness of the skin and dyspnoea, and less frequently diarrhoea and jaundice. Grossly, the main features of PMWS are generalized lymphoadenopathy (with the superficial inguinal, submandibular, mesenteric and mediastinal lymph nodes the most affected ones) and tan-mottled, non-collapsed lungs (Rosell et al., 1999). Histopathological findings are characterized by lymphocyte depletion and histiocytic infiltration of lymphoid tissues, interstitial pneumonia, hepatitis and nephritis (Rosell et al., 1999). There is also recent description of granulomatous lesions and atrophy of the thymus (Ladekjaer-Mikkelsen et al., 2002; Kim et al., 2003), though such lesions have not usually been considered as a main feature of PMWS. The disease has also been reported in many countries of Europe, America and Asia (Allan et al., 1998; Choi et al., 2000; Kiupel et al., 1998; Madec et al., 2000; Onuki et al., 1999; Sato et al., 2000; Segalés et al., 1997).

Histological studies have demonstrated the association between porcine circovirus type 2 (PCV2), a member of the family Circoviridae, and the PMWS lesional pattern (Ellis et al., 1998; Hamel et al., 1998; Meehan et al., 1998; Rosell et al., 1999). Porcine circovirus type 2 genome or antigen can be detected in several tissues and cell types such as macrophage/monocyte lineage cells (histiocytes, multinucleate giant cells, alveolar macrophages, Kupffer cells), dendritic cells, and sporadically respiratory, renal and intestinal epithelial cells, hepatocytes, enterocytes, vascular endothelium and lymphocytes (Kiupel et al., 1999; Rosell et al.,...
1999; Shibahara et al., 2000). The main target cells are thought to be the monocyte/macrophage lineage cells and other antigen-presenting cells (Clark, 1997; Kennedy et al., 2000; Rosell et al., 1999). However, it is not yet fully understood how the virus penetrates those cells and whether or not all the cellular types that have PCV2 genome in the cytoplasm support virus replication. In addition, there are controversial results regarding the experimental reproduction of PMWS. Thus, some authors could reproduce the syndrome by inoculation of PCV2 alone (Allan & Kennedy, 1999; Bolin et al., 2001; Harms et al., 2001), while others claim the need for concomitant agents or immunomodulatory compounds to reproduce the syndrome or to get a higher number of diseased animals and more severe clinical signs (Allan et al., 2000; Balasch et al., 1999; Ellis et al., 2000; Kennedy et al., 2000; Krakowka et al., 2000, 2001; Rovira et al., 2002).

It is known that the immune system is involved in the pathogenesis of PMWS. Two facts support this idea: the development of lymphocyte depletion in lymphoid tissues (Darwich et al., 2002; Quintana et al., 2001; Rosell et al., 1999; Sarli et al., 2001; Shibahara et al., 2000), which correlates with a specific downshift of CD8\(^+\), CD4\(^+\) and double-positive cells and IgM\(^+\) cells (Darwich et al., 2002; Segalés et al., 2001), and the association of PMWS with several secondary or opportunistic infections (Carrasco et al., 2000; Clark, 1997; Ellis et al., 1998). All these facts suggest a serious disorder of the immune system in PMWS-affected animals.

Currently, there are a number of questions that remain to be solved as regards the role of the immune response on development of PMWS in PCV2 infection and why, if most or all the animals in a farm are infected (Rodríguez-Arrioja et al., 2000), only some of them become diseased. In order to obtain more detailed information about the immunological alterations in PMWS-affected pigs, we analysed the cytokine expression patterns in different lymphoid tissues of naturally PCV2-infected pigs that had developed PMWS and correlated these data with haematological and histopathological findings.

**METHODS**

**Animals.** Fifteen conventional 8-week-old pigs were selected from a farm free of infection with porcine reproductive and respiratory syndrome (PRRSV) and pseudorabies virus (PRV). The vaccination programme for the sows included an inactivated porcine parvovirus (PPV) vaccine. Ten of the 15 pigs were selected because they were farm free of infection with porcine reproductive and respiratory syndrome (PRRSV) and pseudorabies virus (PRV). The vaccination programme for the sows included an inactivated porcine parvovirus (PPV) vaccine. Ten of the 15 pigs were selected because they were

**Serological analysis.** Commercially available ELISAs for the detection of antibodies against PPV (Ingezim PPV; Ingenasa, Madrid, Spain), Mycoplasma hyopneumoniae (Hipra, Gerona, Spain), swine influenza virus (SIV) (Hipra, Gerona, Spain), PRV (HerdChek Anti-ADV gpI; IDEXX, Schiphol, The Netherlands) and PRRSV (IDEXX, Schiphol, The Netherlands) were used. Antibodies against PCV2 were determined by an immunoperoxidase monolayer assay (IPMA) technique using PCV2-infected SK (swine kidney) cells as previously described (Rodriguez-Arrioja et al., 2000).

**Haematological determinations.** Blood samples were collected from all pigs in commercial vacuum tubes containing tripotassium EDTA anticoagulant by puncturing the cranial vena cava. A complete haemogram was done using a semi-automatic electric impedance blood cell counter (Sysmex F-800; Toa Medical Electronic Europa). The analyses include red blood cell (RBC) and white blood cell (WBC) counts and haematocrit value indexes (mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH)), as well as platelets and total protein parameters. The leucocyte differential count was done by identification of 100 cells on a blood smear by means of a light microscope. Serum iron concentrations were analysed using an automated Cobas (Mira, Roche) system.

**Flow cytometry analyses.** Heparinized blood samples (100 μl) from each animal were lysed using NH\(_4\)Cl (0-155 M) and examined by flow cytometry as previously described (Darwich et al., 2002) to determine B- and T-cell subsets, using the appropriate monoclonal antibodies: anti-CD4 (b38c6, IgG1, Labor, Germany), anti-CD8 (295/33-25, IgG2a, Labor, Germany) and anti-IgM (K52-1c, IgG1, LabGen, Spain), and as secondary antibodies goat F(ab\(^-\))\(_2\)-mouse IgG2a R-phycocerythrin (R-PE)-conjugated and goat F(ab\(^-\))\(_2\)-mouse IgG1 fluorescein (FITC)-conjugated (Southern-Biotechnology). The analyses were performed using an EPICS XL-MLC cytometer (Coulter) to 488 nm excitation and with 580 and 630 nm filters.

**Tissue samples.** Pigs were killed by means of a sodium pentobarbital overdose. Immediately, approximately 1 cm\(^2\) of tissues from superficial inguinal and bronchial lymph nodes, thymus, tonsils and spleen were taken, suspended in 1 ml of Trizol (Life Technologies) and frozen in dry ice for transporting to the laboratory, where they were stored at −80 °C. The remaining portion of the above organs, as well as samples from lung, liver and kidney, were preserved and fixed by immersion in 10 % neutral-buffered formalin for subsequent histological examinations.

**Histopathological studies.** The formalin-fixed samples were dehydrated, embedded in paraffin wax and sectioned at 4 μm. Tissue sections were stained with haematoxylin and eosin (HE). Characteristic PMWS lymphoid lesions were scored as slight (+), moderate (+++) or severe (++++) grade based on the degree of lymphocyte depletion and histiocytic infiltration as previously described (Rovira et al., 2002). The same pathologist did all the evaluations, and samples were masked to ensure a blind fashion study.

**In situ hybridization (ISH) technique to detect PCV2 infection.** The ISH technique was done on sections of lung, liver, kidney, thymus, tonsil, spleen and lymph nodes of each animal using a PCV2-specific, single-stranded, 41 nucleotide probe (5’-CCTCCCT CATTACCCCTCCTGG CCACATAAAATAC AAAAAA-3’) end-labelled with digoxigenin. The DNA probe was complementary to nucleotides 168–208 of open reading frame 1 (ORF1) from the PCV2 genome (Hamel et al., 1998; Meehan et al., 1998). The hybridization procedure was done as previously described (Rosell et al., 2000) with a pre-hybridization heating step of 5 min at 105 °C followed by hybridization at 37 °C for 60 min. Positive controls were obtained from sections of previous PMWS diagnosed cases from Spain (Segalés et al., 1997) and negative ones corresponded to lymph node tissues of pigs from an experimental farm negative for PCV2. Finally, the results were classified from slight (+) to high (++++) based on the amount of labelled PCV2 DNA observed in the tissues.

**RNA extraction.** Tissue sections in Trizol were homogenized by using a homogenizer and total RNA was extracted regarding the manufacturer’s recommendations. After that, the total RNA was suspended

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in 60 µl of ultrapure water containing 0-02% (w/v) DEPC (Sigma) and 1 mM EDTA. The quantification of total RNA was carried out by spectrophotometry at an absorbance of 260 nm (λ260) and the purity of this RNA was assessed determining the A260/A280 ratio. All of the samples had an A260/A280 ratio ranging between 1-7 and 1.9.

RT-PCR detection of mRNA cytokine expression and quantification of PCR products. Semi-quantitative determination of IFN-γ, IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12p40 and cyclophilin was carried out using an RT-PCR procedure performed as previously described (Dozois et al., 1997) with minor modifications. In the first step, 1 µg of total RNA was reverse transcribed using murine Moloney leukemia virus reverse transcriptase (Point Mutant; Promega) and suspended to a total volume of 100 µl. After reverse transcription, 5 µl of cDNA for cyclophilin and 10 µl for the other tested cytokines was amplified by PCR using deoxynucleoside triphosphates (2 mM each) (Eurobio), 0.2 pM 5’ and 3’ primers, 2 mM MgCl2, and 2.5 U of DNA Taq polymerase enzyme (Invitrogen) in a final volume of 50 µl. Primer sequences, annealing temperatures and number of PCR cycles used for the amplification are summarized in Table 1. In all cases denaturation was done at 94 °C for 45 s and extension at 72 °C for 45 s. Initial denaturations were carried out for 5 min and final extensions for 10 min.

Semi-quantitative analysis of all PCR products was done by hybridization of 32P-labelled specific oligonucleotide probes to PCR products immobilized on nitrocellulose membranes by dot blotting as previously described (Pié et al., 1996). Pre-hybridization and hybridization were carried out with Rapid-hyb buffer (Amersham). The DNA probes used for hybridization of the different cytokines are listed in Table 1. The relative amounts of each product were determined by measuring radioactivity with a Phosphor Imager (Molecular Dynamics). For each cytokine, the amounts of RT-PCR products were normalized to the values obtained with cyclophilin, used as an internal standard for each sample.

Statistical analysis. Statistical comparisons of cytokine expression results between PMWS pigs and healthy animals were done using a Chi-square trial and Student’s unpaired t-test by means of the Stats direct program. One-way analysis of variance with the Turkey–Kramer multiple comparison method was also used to compare cytokine expression and degree of lymphocyte depletion. Results with a P value ≤0.05 were considered statistically significant.

RESULTS

Serological status

Serological determinations showed that all animals were seronegative for PRV, PRRSV, SIV and Mycoplasma hyopneumoniae. Only three pigs, two of them healthy, had detectable antibodies against porcine parvovirus (<1:400). Antibodies against PCV2 were detected in all the pigs with a titre ranging from 1:80 to 1:20 480. The mean values for healthy and diseased pigs were 1:368 and 1:5384 respectively.

Haematological parameters

Animals suffering from PMWS showed signs of microcytic hypochromic anaemia but with a higher concentration of RBC compared to healthy animals (P<0.05). Haemoglobin concentration and the MCV and MCH values were significantly lower in wasted animals as well as the serum iron concentration (P<0.05) (Table 2). Furthermore, monocytosis, neutrophilia and absolute lymphopenia were observed in the PMWS pigs with an inversion of the lymphocyte/neutrophil ratio in those animals (Table 2). However, the leukocyte total counts were not significantly different in

Table 1. Oligonucleotide sequences and DNA probes designed for the detection of different porcine cytokines, with the respective annealing temperatures and number of PCR cycles

<table>
<thead>
<tr>
<th>Gene specificity</th>
<th>Primer*</th>
<th>Oligonucleotide sequences (5’–3’)</th>
<th>Annealing temp. (°C)</th>
<th>No. of cycles</th>
<th>DNA probe (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>S</td>
<td>AAAAAAGGACCCAGTTGAAAGAG</td>
<td>54</td>
<td>28</td>
<td>ATCTTGACTCTGCTTTGTGATGAA</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTGCTTGGAGGTGTCTGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2†</td>
<td>S</td>
<td>GATTATCAAGTGTTGGTTGGAA</td>
<td>54</td>
<td>45</td>
<td>AGGAATCAATGAAACAATTCAACGT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GTTGAGTAGATGCTTTTGACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4†</td>
<td>S</td>
<td>TACCAAGCAACTTCGTCCCAC</td>
<td>54</td>
<td>45</td>
<td>ACAAGAGATCATCAAAAAACCTGAAAC</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>ATCGTCTTGTTGTTGCTTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8†</td>
<td>S</td>
<td>TTTCTGACGGCTTCTCTGTTGAAG</td>
<td>60</td>
<td>45</td>
<td>ATGTGAAGCTTTGCAATGGAAAAG</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTGCTGTTGTTGTTGCTTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10†</td>
<td>S</td>
<td>GCAATCCATCTCCACCA</td>
<td>54</td>
<td>40</td>
<td>TAGGCTTTCTATGTTGATGAAAGATG</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTTCCTGTATCTGCATGTCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12p40†</td>
<td>S</td>
<td>GATGCGTCAGGCTAGCAGAC</td>
<td>54</td>
<td>32</td>
<td>ACTCCGGACGTTCCACCT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>TACGAGGAGACGCCTAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>S</td>
<td>GAAGAAAGGGCCGAGCCACAGG</td>
<td>54</td>
<td>38</td>
<td>CCAATTTGTCTTTCTACTTCAA</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GCTGTATGATCATCTGCATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin†</td>
<td>S</td>
<td>TAACCCGACCGCTTCCTT</td>
<td>50</td>
<td>26</td>
<td>TGTTGACCTCCACAGCCATA</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>TGCCATCCAGACACACTCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*S, sense primer; AS, antisense primer.
†Oligonucleotide sequences previously described (Fournout et al., 2000; Marin et al., 2002).
PMWS-affected pigs compared to the healthy ones. No statistical differences were observed in eosinophil and platelet counts or in the total protein values (Table 2). Flow cytometry analyses showed significant decreases in the absolute and relative number of IgM$^+$ and CD8$^+$ cell subsets ($P < 0.05$). Specifically, the diseased pigs presented IgM$^+$ counts of 60±100 cells $\mu l^{-1}$ (0.8±1.4) and CD8$^+$ values of 490±600 cells $\mu l^{-1}$ (7.5±5.4), in comparison to the IgM$^+$ and CD8$^+$ cell numbers of the healthy pigs, which were 700±500 cells $\mu l^{-1}$ (7.7%±2.4) and 1890±900 cells $\mu l^{-1}$ (19.6%±4.1) respectively.

### Pathological findings

The most relevant macroscopic feature found in all the diseased pigs was thymus atrophy. In three of these ill animals it was not possible to find this organ during necropsy because of the advanced degree of involution. For that reason evaluation of cytokine mRNA expression in thymus was only performed in seven diseased pigs. Other gross lesions found in the affected pigs were the usual signs of PMWS cases [enlargement of lymph nodes ($n=7$), non-collapsed lungs ($n=5$), pulmonary consolidation ($n=5$) and serous atrophy of the fat ($n=5$)]. However, no gastric ulcerations were detected in any of the diseased and healthy animals. As expected, no gross lesions and normal thymic size were observed in the five healthy pigs.

Histological lesions were characterized by mild to severe generalized lymphocyte depletion in the lymphoid organs of the diseased pigs (Table 3). Other microscopic findings were interstitial pneumonia ($n=10$), lymphoplasmacytic hepatitis ($n=7$) and interstitial nephritis ($n=6$). No lesions were observed in bone marrow in any of the examined pigs. Only one animal of the healthy group had a very slight degree of depletion in lymph nodes, tonsils and spleen, but no lymphocyte depletion was observed in the thymus of that pig (data not shown).

### PCV2 in situ hybridization (ISH) technique

Most of the examined tissues from the diseased pigs were positive for PCV2, corroborating the definitive diagnosis of PMWS in those animals. Moreover, the amount of virus present in lymphoid tissues was from moderate to high level, except in the spleen where the number of PCV2-positive cells was lower (Table 3). On the other hand, ISH of tissues from the five apparently healthy pigs showed that four of them were negative for PCV2, and only one of these animals (the one that also had a slight degree of lymphocyte depletion in the histopathological studies) had very low amounts of PCV2 in inguinal lymph node, tonsils and spleen, but neither lymphocyte depletion nor PCV2 genome was observed in the thymus of that pig.

### RT-PCR detection of cytokine mRNA expression and quantification of PCR products

Cytokine mRNA expression was analysed in different lymphoid organs (tonsils, thymus, spleen and lymph nodes).

In tonsils of diseased pigs there was a significant increase in

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**Table 2. Mean (standard deviation) values of the haematological parameters in PMWS and healthy pigs**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PMWS group ($n=10$)</th>
<th>Healthy group ($n=5$)</th>
<th>Reference values $^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell count (10$^6$ $\mu l^{-1}$)</td>
<td>9.18 (2.8)</td>
<td>7.1 (0.5)$^*$</td>
<td>5.89 (1.15)</td>
</tr>
<tr>
<td>Haemoglobin concentration (g $dl^{-1}$)</td>
<td>8.31 (1.4)</td>
<td>10.04 (0.8)$^*$</td>
<td>11.6 (1.1)</td>
</tr>
<tr>
<td>Haematocrit value (%)</td>
<td>29.2 (7.0)</td>
<td>32 (1)</td>
<td>37.3 (3.1)</td>
</tr>
<tr>
<td>MCV ($fl$)</td>
<td>31.58 (9.7)</td>
<td>44.96 (3.7)$^*$</td>
<td>60 (8.0)</td>
</tr>
<tr>
<td>MCH ($pg$)</td>
<td>9.39 (2.1)</td>
<td>14.12 (1.5)$^*$</td>
<td>19.0 (2.0)</td>
</tr>
<tr>
<td>Serum iron ($\mu g dl^{-1}$)</td>
<td>52.32 (26.7)</td>
<td>120.8 (64.2)$^*$</td>
<td>111.7</td>
</tr>
<tr>
<td>White blood cell count (10$^3$ $\mu l^{-1}$)</td>
<td>22.81 (15.9)</td>
<td>15.08 (6.1)</td>
<td>13.03 (0.45)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute count (10$^3$ $\mu l^{-1}$)</td>
<td>4.43 (4.3)</td>
<td>10.13 (5.0)$^*$</td>
<td>8.0 (4.5)</td>
</tr>
<tr>
<td>Relative count (%)</td>
<td>23.1 (11.3)</td>
<td>64.2 (13)$^*$</td>
<td>68.1 (1.6)</td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Absolute count (10$^3$ $\mu l^{-1}$)</td>
<td>0.77 (0.5)</td>
<td>0.20 (0.09)$^*$</td>
<td>0</td>
</tr>
<tr>
<td>Relative count (%)</td>
<td>4.1 (3)</td>
<td>1.4 (0.5)</td>
<td>0</td>
</tr>
<tr>
<td>Segmented neutrophils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute count (10$^3$ $\mu l^{-1}$)</td>
<td>16.25 (11.7)</td>
<td>4.43 (1.1)$^*$</td>
<td>5.5 (3.0)</td>
</tr>
<tr>
<td>Relative count (%)</td>
<td>72.1 (11.1)</td>
<td>32.2 (12.2)$^*$</td>
<td>25.1 (1.4)</td>
</tr>
<tr>
<td>Platelet count (10$^6$ $\mu l^{-1}$)</td>
<td>0.29 (0.1)</td>
<td>0.27 (0.1)</td>
<td>0.52 (0.4)</td>
</tr>
<tr>
<td>Total protein (g $dl^{-1}$)</td>
<td>5.41 (0.9)</td>
<td>5.78 (0.5)</td>
<td>7.0 (1.0)</td>
</tr>
</tbody>
</table>

$^*$Statistically significant ($P < 0.05$).

$^\dagger$Porcine haematology reference values established for 2-month-old pigs (Jain, 1993; Leman, 1986).
IFN-γ mRNA expression and a decrease in IL-4 mRNA compared to tonsils of healthy pigs (P<0.05) (Fig. 1).

In the thymus, an interesting finding was the increase in expression of IL-10 mRNA in PMWS-affected pigs in comparison with the healthy group (P<0.05) (Fig. 1). Comparison of interleukin mRNA expression and degree of lymphocyte depletion showed a significant correlation between IL-10 mRNA expression and thymus depletion in pigs suffering PMWS. As a result, pigs presenting the syndrome had more cellular depletion and higher IL-10 mRNA levels (1.13±0.5) in the thymus than the healthy animals, which did not show any degree of thymic depletion (n=5) and lower IL-10 mRNA levels (0.32±0.05). In addition, in the diseased group, the higher the lymphocyte depletion the higher the expression of IL-10 in thymus. Thus, PMWS-affected pigs (n=4) with severe cellular depletion (+++) in thymus expressed higher IL-10 mRNA levels (1.7±0.1) than those PMWS pigs (n=3) with slight (+/+ +) depletions (0.7±0.2) (P<0.05).

In the spleen, a decrease in mRNA expression of both IL-2 and IL-12 was observed (P<0.05) (Fig. 1). When the IL-8 values were analysed in comparison with the degree of lymphocyte depletion in the spleen, animals could be differentiated in two groups: one comprising healthy

Table 3. Grade of lymphocyte lesions and amount of PCV2 by ISH in different lymphoid organs of PMWS-affected pigs

Animals not showing any lymphocyte depletion and/or viral load are not included in the table.

<table>
<thead>
<tr>
<th>No. of PMWS pigs showing any degree of lymphoid depletion and viral load</th>
<th>Inguinal lymph node (n=10)*</th>
<th>Tonsils (n=10)</th>
<th>Spleen (n=10)</th>
<th>Thymus (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Score†</strong></td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Lymphocyte depletion</strong></td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><strong>PCV2 load</strong></td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

*Total number of diseased pigs examined for each lymphoid organ.
†Slight (+), moderate (+++) and high (+++).

Fig. 1. Quantification of regulatory and inflammatory cytokine mRNA levels (▲) in different lymphoid tissues of healthy and PMWS-affected pigs. Vertical bars represent the mean (±SEM) of these results for both healthy and diseased groups. *P<0.05.
PCV2-uninfected animals and PCV2-infected pigs with severe lymphocyte depletions, and a second one comprising PCV2-infected animals with slight or moderate lymphocyte depletions. Thus, regarding IL-8 mRNA expression, healthy pigs with no lesions (5.22 ± 8.1) were statistically similar to severely depleted infected pigs (1.89 ± 2.0) while infected pigs with slight to moderate lesions showed higher IL-8 mRNA expression (28.05 ± 13.8) (P < 0.05). Corresponding values for the apparently healthy PCV2-infected pig were the same as for this latter group.

The cytokine mRNA profile was similar in inguinal and bronchial lymph nodes, but the difference between healthy and diseased animals was more pronounced in the inguinal lymph node (Fig. 2). In this organ, a decrease of mRNA encoding IFN-γ (P = 0.06), IL-4 (P < 0.001) and IL-10 (P < 0.05) cytokines was observed in PMWS pigs. Moreover, IL-12p40 mRNA expression was also decreased in lymph nodes of diseased animals compared to healthy pigs (P < 0.001) (Fig. 2).

**DISCUSSION**

This study provides the first description of an altered cytokine mRNA expression profile in lymphoid tissues of naturally PMWS-affected pigs. The histopathological and haematological features are highly suggestive of an immunosuppressive state in pigs. In addition, cytokine mRNA expression imbalance suggests a severe alteration, at least at a transcriptional level, of the examined cytokine profiles.

The pigs selected for this study were conventional animals from a farm with a high health status, free of common swine pathogens. Though it cannot be ruled out that these pigs were exposed to other swine pathogens, they represent a field situation of a common natural PMWS. To minimize extraneous effects, all animals included in the study were penmates of matched ages and, in consequence, the same environmental and microbiology factors were common to all of them.

Regarding haematological and immunological disorders, we found that the cellular composition of the blood was severely altered, comprising anaemia, lymphopenia, with a marked decrease of CD8+ and IgM+ cells, monocytosis and neutrophilia. Some of these features – anaemia and lymphopenia – have been reported in chicken anaemia virus infection (CAV), another immunosuppressive disease caused by a circovirus (Adair, 2000; Smyth et al., 1993). The PMWS-affected pigs studied suffered anaemia, but the characteristics of that anaemia were not compatible with bone marrow aplasia since red-blood cell counts were actually

![Fig. 2. Quantification of cytokine mRNA levels (△) in lymph nodes of healthy and diseased pigs. Vertical bars represent the mean (± SEM) of these values for each group. **P < 0.001, *P < 0.06.](image-url)
increased, not decreased, and thrombocytes were in a normal range. Interestingly, PMWS-affected animals had lower serum iron levels than their healthy counterparts, a fact that would suggest an iron deficiency. Iron deficiency is usually related to blood losses, as previously reported in PMWS pigs suffering gastric ulcerations (Segalès et al., 2000). Also, it could be attributable to deficient iron intake, impaired iron absorption, increased iron demands, or even to an acute phase response. However, the cause of anaemia in PMWS pigs deserves further study and may give clues to the pathogenesis of this syndrome.

Our flow cytometry analysis showed that lymphopenia is reflected in both T- and B-cell subsets, in agreement with previous reports (Darwich et al., 2002). An impairment of cellular and humoral responses could be expected in pigs suffering from this syndrome. Nevertheless, we did not perform functional analysis of these cells and a final conclusion cannot be drawn. The origin of this decrease in T- and B-cells might be attributed to the cellular depletion observed in lymphoid organs. The results of the histopathological examination of PMWS pigs included in our study showed that the thymus is one of the main targets of PCV2. Contrary to previous reports that mentioned thymus alteration as a minor feature of PMWS (Ladekjaer-Mikkelsen et al., 2002; Kim et al., 2003), our histopathological results indicate that thymus atrophy is much more evident and frequent in pigs suffering from PMWS than has been considered up till now. This organ suffered a marked destruction and atrophy associated with intense cell depletion and, in some animals the histological structure was entirely lost. This effect has also been reported in other circovirus infections like CAV and psittacine beak and feather disease virus (BFDV), where atrophy of the thymus has been correlated with severe lymphocyte depletion in peripheral blood and tissues (Todd, 2000).

When we examined cytokine mRNA expression profiles in the thymus we found a direct correlation between the degree of thymic depletion and IL-10 mRNA expression. The effects of IL-10 are diverse but mainly comprise an anti-inflammatory activity, mediating the negative feedback of Th1 and Th2 immune responses (Muraille & Leo, 1998), the regulation of NK- and B-cell growth and also the ability to rescue B-cells from apoptosis (Levy & Brouet, 1994). Also, IL-10 downregulates MHC-II expression in antigen-presenting cells (APC) (de Waal Malefyt et al., 1991; Moore et al., 2001) and can act on MHC-I expression by modulating the expression of the transporter protein associated with antigen processing (Salazar-Onfray et al., 1997). Experiments with transgenic BALB/c mice over-expressing IL-10 showed that this cytokine produces a specific blockage of the maturation of thymocytes at the pre-T-cell stage and induces severe aplasia of the thymus (Rouleau et al., 1999). Furthermore, it has been shown that HIV-1 infection causes severe parenchymal damage and involution of the thymus, which correlates with faster AIDS disease progression. HIV-1 also leads to induction of MHC-I expression on immature thymocytes by means of indirect mechanism involving IL-10 (Kovalev et al., 1999) and interferes with proper thymocyte maturation and survival, thus contributing to thymocyte depletion (Kovalev et al., 1999). Taking these facts into account, it is tempting to suggest that thymic depletion and atrophy in PMWS pigs could be caused by overexpression of IL-10 mRNA in the thymus. This observation can lead to speculation of some systemic impact on T-cells by means of involvement of T-cell development or regulation in thymus. However, with our data it is not possible to conclude if this thymic process is just local or not. Whether this mRNA overexpression is directly due to any viral protein, to dysregulation of cytokine production in dendritic cells or macrophages in thymic stroma or is a reactive response to counteract apoptosis remains unknown.

One of the criteria for selection of the PMWS-affected animals in our study was that they had only 1 week of clinical symptoms. Several authors (Bolin et al., 2001; Harms et al., 2001) have shown that, under experimental conditions, the PMWS incubation period varies from 14 to 21 days post-infection. We expected that pigs were infected within 2 or 3 weeks before the onset of clinical signs. However, this range of possible incubation periods may explain some differences in cytokine mRNA expression, especially for IL-8. This cytokine is considered to be a marker of ongoing infections, and thus its expression would be expected to be higher in the early phases of infection. Accordingly, moderate depletion lesions seen in the spleen of PMWS-affected pigs could correspond to very early phases with high levels of IL-8 mRNA, while severe lesions would correspond to more advanced stages with low levels of expression of the mRNA of this cytokine.

Cytokine expression patterns in spleen of PMWS pigs also showed a decrease of both IL-2 and IL-12. Similar features are seen in CAV infection where splenocytes show a decreased ability to produce IL-2 in response to concanavalin A and have impaired interferon production (Adair et al., 1991), suggesting an important impairment of cytotoxic T-cell responses. Furthermore, in inguinal lymph nodes, one of the most affected lymphoid structures in PMWS (Rosell et al., 1999), we observed a decrease in mRNA expression of several cytokines including IL-4, IL-12, IL-10 and IFN-γ. This reinforces the notion of extensive T-cell damage, at least at a transcriptional level. On the other hand, the decrease of IL-4 mRNA found in the different lymphoid tissues could interfere with B-cell activation and, consequently, contribute to suboptimal antibody responses.

In conclusion, PMWS-affected pigs present an altered cytokine mRNA expression profile in the different lymphoid organs examined. It was evident that key cytokines responsible for driving a specific immune polarization (IL-12, IL-10, IL-2, IL-4, IFN-γ) showed changes in their transcriptional phase in different tissues of diseased pigs. These changes could suggest inhibition of T-cell responses. Moreover, PMWS-affected pigs showed a severe disorder...
of the cellular composition of blood – anaemia and depletion of lymphocyte subsets – and had extensive cellular depletion that involved all lymphoid organs examined, particularly the thymus. Overexpression of IL-10 mRNA in this organ could contribute to the development of anaemia, as has been reported in human patients suffering from a chronic inflammatory state (Weiss, 2002). On the other hand, this IL-10 mRNA upregulation in thymus could be the origin of the generalized T-cell depletion and could have important consequences for T-cell functionality. However, further studies are required to confirm if the cytokine mRNA imbalance is also reflected at the protein level, a fact that would firmly demonstrate that there is a deep alteration in the immune system of PMWS-affected pigs.

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