Changes in CD4⁺, CD8⁺, CD4⁺ CD8⁺, and Immunoglobulin M-Positive Peripheral Blood Mononuclear Cells of Postweaning Multisystemic Wasting Syndrome-Affected Pigs and Age-Matched Uninfected Wasted and Healthy Pigs Correlate with Lesions and Porcine Circovirus Type 2 Load in Lymphoid Tissues

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Forty-one 8- to 12-week-old wasted pigs were selected from several conventional farms with histories of postweaning multisystemic wasting syndrome (PMWS) and classified into two groups according to their porcine circovirus type 2 (PCV2) infection status, as determined by in situ hybridization (ISH). Twenty-four pigs tested positive for PCV2 (PCV2-positive group), while 17 pigs tested negative for PCV2 (PCV2-negative group). In addition, eight uninfected healthy pigs from an experimental farm were used as controls. Hepatinized blood samples were taken to obtain peripheral blood mononuclear cells. The CD4⁺, CD8⁺, CD4⁺ CD8⁺ (double-positive [DP]), and immunoglobulin M-positive (IgM⁺) cell subsets were analyzed by flow cytometry with appropriate monoclonal antibodies. Histopathological studies were done to evaluate the apparent degrees of lymphocyte depletion in different lymphoid organs (superficial inguinal and mesenteric lymph nodes, Peyer’s patches, tonsils, and spleen) and to determine the viral load of the PCV2 genome by using an ISH technique. Animals of the PCV2-positive group showed a significant downshift of the CD8⁺ and DP cell subsets compared to the other groups (P < 0.05). Moreover, in PCV2-positive pigs, the amount of PCV2 genome in lymphoid tissues was related to the degree of cell depletion in those tissues (P < 0.05) as well as to the relative decrease in IgM⁺ and CD8⁺ cells in peripheral blood. These data support the notion that PCV2-positive pigs might have an impaired immune response.

Since its first description in 1996 by Clark and Harding (Abstr. Proc. West Can. Assoc. Swine Pract., abstr. 21 to 25, 1996), postweaning multisystemic wasting syndrome (PMWS) has been reported worldwide (2, 4, 9, 11, 13, 22; S. C. Kyriakis, S. Kennedy, K. Saoulidis, S. Lekkas, C. C. Miliotis, G. C. Balkamos, and P. A. Papoutsis, Abstr. 16th Int. Pig Vet. Soc. Congr., p. 633, 2000). This syndrome, which affects weaned and unweaned pigs (8), is characterized by progressive weight loss or unthriftiness, dyspnea, enlarged lymph nodes, and, less frequently, pallor, jaundice, and diarrhea (5, 8). Viral detection techniques and nucleotide sequence analyses have shown an association between porcine circovirus type 2 (PCV2) and the presence of PMWS. Particularly, PMWS-affected pigs show histiocytic infiltration and lymphocyte depletion of both follicle centers and parafollicular zones, symptoms associated with the presence of PCV2 (18). Consequently, members of this viral family have been supposed to be immunosuppressive (1, 10, 14).

The effects of PCV2 on the pig immune system are not yet fully known, but it has been reported that the main target cells for PCV2 replication are the monocyte/macrophage lineage cells as well as other antigen-presenting cells such as follicular dendritic cells (18). In addition, PCV2 antigen in the nuclei of some lymphocyte subsets has been described (23). Recent studies have suggested that PCV2 infects dividing cells, macrophages, and B lymphocytes, inducing apoptosis of the B cells that leads to the depletion of the lymphoid organs (23). Moreover, studies of lymphocyte subsets showed that pigs suffering from PMWS had lower proportions of CD4⁺ and immunoglobulin M-positive (IgM⁺) cells in blood than healthy, uninfected pigs did (20). Taken together, these facts have led some to suggest that PCV2 infection might cause immunosuppression (20, 23). However, little is known regarding the immunological response of pigs suffering from PMWS and the mechanism by which PCV2 infection might result in the development of PMWS.

The aim of this study was to evaluate by means of flow cytometry the changes in lymphocyte subsets of peripheral blood of naturally PCV2-infected pigs suffering from PMWS as...
In order to detect porcine reproductive and respiratory syndrome virus (PRRSV) infection, an immunohistochemical (IHC) technique was carried out on lung tissue sections by using an avidin-biotin-peroxidase method described previously (21). Briefly, tissue sections were placed on silane [3-(triethoxysilyl)-propylamine]-coated slides. Endogenous peroxidase activity was inhibited by immersing the tissue sections in a 3% solution of hydrogen peroxide in methanol for 30 min. Antigen retrieval was done by enzymatic treatment (protease type XIV) in Tris-buffered saline (TBS; pH 7.4) for 10 min. Blocking was carried out for 1 h with 10% normal goat serum in TBS. Tissue sections were mounted on slides and covered with the monoclonal antibody (MAb) anti-PRRS SDOW17 (dilution, 1:1,000 in TBS), and the slides were incubated overnight at 4°C. Then, the secondary antibody (biotinylated goat anti-mouse linking antibody) and peroxidase-conjugated avidin were applied (at dilutions of 1:200 and 1:100, respectively) for 1 h at room temperature. Sections were incubated in diaminobenzidine-hydrogen peroxide solution for 8 min, counterstained with Harris’s hematoxylin, dehydrated, covered with a coverslip, and examined microscopically. Negative control procedures included the omission of the primary antiserum.

**MATERIALS AND METHODS**

**Animals and study design.** Forty-one 8- to 12-week-old pigs were randomly selected from those that were submitted to the Veterinary Faculty of the Universitat Autònoma de Barcelona because of their evident chronic wasting and unhealthy status. To be included in the study, the pigs had to be from conventional farms with historical records of PMWS and had to present clear signs of wasting and labored breathing. Eight healthy PCV2-uninfected pigs obtained from an experimental herd were also included to provide standards for the immune parameters. Before the pigs were euthanatized, blood samples were aseptically taken from the jugular veins, and after the animals were killed, they were necropsied and histopathological analyses were performed.

**Postmortem and histopathological studies.** All pigs examined were euthanatized by means of a sodium pentobarbital overdose, and detailed necropsies were performed. Samples of the superficial inguinal and mesenteric lymph nodes, Peyer’s patches, tonsils, and spleen were obtained from pigs from Spain that had been previously diagnosed with PMWS (22), and negative control samples were lymph node tissues from pigs that tested negative for PCV2 and that came from an experimental farm with no record of PMWS. The apparent levels of viral genome stained by ISH in the different lymphoid tissues were evaluated as described previously (15). Accordingly, three categories were considered: high (Fig. 2A), low (Fig. 2B), and absent.

**ISH technique to detect PCV2 infection.** The in situ hybridization (ISH) technique was performed according to a previously described protocol (17). Briefly, sections were hybridized to a PCV2-specific, single-stranded, 40-nucleotide DNA probe (5’-CAGTAATACGGACGACTACATATATCGTGTACCCATG-3’) that was complementary to nucleotides 1,085 to 1,124 of open reading frame 2 of the PCV2 genome. The DNA probe was end labeled with digoxigenin, and the prehybridization heating was carried out for 5 min at 105°C followed by the hybridization process at 37°C for 60 min. Positive control samples were obtained from pigs from Spain that had been previously diagnosed with PMWS (22), and negative control samples were lymph node tissues from pigs that tested negative for PCV2 and that came from an experimental farm with no record of PMWS. The apparent levels of viral genome stained by ISH in the different lymphoid tissues were evaluated as described previously (3). These evaluations were always done by the same pathologist, and samples were masked to ensure a blind study.

**IHC technique to detect PRRSV infection.** In order to detect porcine reproductive and respiratory syndrome virus (PRRSV) infection, an immunohistochemical (IHC) technique was carried out on lung tissue sections by using an avidin-biotin-peroxidase method described previously (21). Briefly, tissue sections were masked to ensure a blind study. The in situ hybridization (ISH) technique to detect PCV2 infection. Well as to correlate these changes with histopathological findings for lymphoid organs such as the superficial inguinal and mesenteric lymph nodes, Peyer’s patches, tonsils, and spleen.
10^6 cells/mL, washed with PBS supplemented with 0.01% sodium azide and 1% fetal calf serum (flow cytometry buffer [PBS-Flow]), and sedimented by centrifugation at 500 × g for 4 min. After the supernatants were discarded, the remaining cell pellets were resuspended with 50 µL of MAB against CD4 or CD8 (two-color cytofluorometry) and IgM (single-color) membrane antibodies and incubated in ice for 30 min. Then, 1 mL of PBS-Flow was added, and the PBMC were washed three times. In the last wash, the supernatant fluid was removed and pelleted cells were incubated with 50 µL of the corresponding conjugate for 30 min in ice and in the dark. Finally, the PBMC were washed three more times and fixed in PBS-Flow with 0.5% formaldehyde before analysis with an EPICS XL-MCL cytometer (Coulter) to an excitation wavelength of 488 nm and with 580- and 630-nm filters.

**Statistical analysis.** Statistics were calculated with Statsdirect and Epi-Info V 6.01. Comparison between groups regarding the proportion of cells in each subset was done by means of the Kruskal-Wallis test. Further pairwise comparisons were done by the Conover-Iman method. Additional statistical analyses (2-by-2 tables) were calculated by using the chi-square test, and regression analyses were carried out by the method of Draper and Smith (4a) with the Epi-Info program. A P value of ≤0.05 was considered statistically significant.

**RESULTS**

**ISH technique for PCV2 infection and IHC technique for PRRSV infection.** Regarding the results of the ISH, 24 wasted pigs were positive for PCV2 (PCV2-positive group) and 17 wasted pigs were negative (PCV2-negative group). All healthy animals were also negative for PCV2. The IHC analysis showed that six pigs were positive for PRRSV. Four of them were coinfected with PCV2, and the other two animals were PCV2-negative wasted pigs. The eight healthy pigs were also negative for PRRSV infection. The sex distributions for the different groups were 12 males and 12 females for the PCV2-positive group, 11 males and 6 females for the PCV2-negative group, and 4 males and 4 females for the healthy group.

**PPV status.** Eight out of 24 PCV2-positive pigs were seropositive for PPV, while 9 out of 17 PCV2-negative pigs were positive (insignificant differences). None of the healthy control pigs was seropositive.

**Flow cytometry.** Phenotypic analysis of PBMC subsets was done by flow cytometry with the following MABs: anti-CD4 (b38c6, IgG1; Labor, Reutlingen, Germany), anti-CD8 (295/33-25, IgG2a; Labor), and anti-IgM (k52-1c, IgG1; LabGen, Barcelona, Spain). For secondary antibodies, goat F(ab')2 anti-mouse IgG1, R-phycocerythrin-conjugated antibody and goat F(ab')2 anti-mouse IgG, fluorescein-conjugated antibody (Southern Biotechnology, Birmingham, Ala.) were used. Irrelevant isotype-matched antibodies were used as background controls. Optimal dilutions of MABs were standardized in previous experiments (data not shown). Mononuclear cells were dispensed at

**Serological techniques to detect PPV.** A commercially available enzyme-linked immunosorbent assay (Ingezim PPV; Ingenasa, Madrid, Spain) was used according to the manufacturer's recommendation.

**Isolation of PBMC.** Ten milliliters of blood was collected from each pig, with heparin used as an anticoagulant. Blood samples were processed to separate peripheral blood mononuclear cells (PBMC) by using gradient density centrifugation with Histopaque 1.077 (Sigma, Barcelona, Spain) at 500 × g for 30 min. Once the PBMC were recovered from the plasma-Histopaque interphase, they were washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (Life technologies, Barcelona, Spain). Cells were quantified with a hemocytometer, and a trypan blue-dye exclusion test was performed to assess viability.

**FIG. 2.** High (A) and low (B) levels of PCV2 nucleic acid in tissue from the tonsils of a pig with PMWS. Results were obtained by PCV2 ISH. All panels show fast green counterstaining. Magnification, ×400.
subgroups according to the ISH results. With this analysis, it was evident that pigs with higher levels of PCV2 had lower proportions of IgM\(^+\) cells in their blood (mean = 6.5\%), results that were statistically different from the IgM\(^+\) cell proportions in the pigs in the healthy group (mean = 12.5\%; \(P = 0.05\)). In contrast, pigs with low levels of PCV2 were similar to the healthy ones with respect to IgM\(^+\) cells (Table 1). In addition, PCV2-positive pigs with lower proportions of IgM\(^+\) cells (\(<5\%) also had lower proportions of CD8\(^+\) cells (\(r = 0.43; P < 0.05\)). As for DP cells, PCV2-infected animals had lower proportions of those cells than animals in all other groups (\(P < 0.05\)).

![Distribution of lymphocyte subsets](http://cvi.asm.org/Downloaded from)

**FIG. 3.** Distribution of individual results (percentage of cells for each animal) with respect to the different lymphocyte subsets. The large open circles comprise the clusters of pigs with the lowest relative proportions of peripheral blood lymphocyte subsets, pigs that thereby differ from the other pigs in the study. +ve, positive; −ve, negative.
Histopathological studies. The evaluation of cellular depletion in the different lymphoid tissues showed significant differences between groups. The PCV2-positive group had the highest proportion of pigs with any degree of depletion in most lymphoid tissues (Table 2), and the pigs in this group had a more severe degree of depletion than the pigs in the PCV2-negative group did; although there were some PCV2-negative animals with lymphocyte depletion, that depletion was always much slighter than that of the PCV2-positive pigs. Moreover, PCV2-positive pigs that had moderate to severe depletions of lymphocytes in both T- and B-cell-dependent zones of a tissue also had similar degrees of depletion in all of the other examined tissues \((P < 0.05)\). On the other hand, the degree of cellular depletion in superficial inguinal lymph nodes or tonsils was inversely correlated to the IgM\(^+\) cell proportions in blood \((P < 0.05)\) (Table 3). Thus, the greater the amount of PCV2, the more severe the depletion, except in periarteriolar lymphoid sheaths \((P = 0.07)\) (Table 3).

DISCUSSION

In our study, flow cytometric analyses showed that PCV2-infected animals suffered a shift in the relative counts of lymphocyte subsets in PBMC. Some of these changes have been described in other reports \((19, 20, 23)\), in which the reduction of CD4\(^+\) cells was associated with a decrease or shift in the CD4\(^+\) cell subset \((19, 20)\). In those reports, only PCV2-positive or healthy pigs were examined. In our report, we included PCV2-positive and PCV2-negative wasted pigs. Thus, the statistical evaluation of our results showed that the CD4\(^+\) cell shift occurred in both wasted groups, regardless of whether or not the pigs were PCV2 infected. This might suggest that the changes observed for CD4\(^+\) cells are related to the wasted state, regardless of the PCV2 status. By contrast, when the CD8\(^+\) and DP cell subsets were analyzed, the relative decreases in these cells were detected only in PCV2-infected pigs. In consequence, these results might suggest that CD8\(^+\) and DP cells are specifically affected during the development of PMWS. Other studies of experimental PCV2 inoculation in conventional pigs showed that animals infected with PCV2 which did not develop clinical signs of PMWS had, in the initial stages of infection (approximately 21 days postinfection), a downshift of

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**TABLE 2.** Distribution of pigs according to the histopathologic stage of cellular depletion in different lymphoid tissues\(^{a}\)

| Group (no. of pigs\(^{b}\)) | Superficial inguinal LN\(^{c}\) | Mesenteric LN | Peyer’s patches | Tonsil | Spleen (PALS\(^{d}\)) |
|-----------------------------|-------------------------------|--------------|----------------|--------|-------------------|
|                             | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) |
| PCV2 negative (17)          | 5    | 1    | 10   | 4    | 7    | 8    | 3    | 2    | 3 |
| PCV2 positive (24)          | 19\(^{g}\) | 17\(^{g}\) | 19   | 17\(^{g}\) | 18\(^{h}\) | 19\(^{h}\) | 17\(^{h}\) | 14\(^{h}\) | 13\(^{h}\) |

\(^{a}\) Since healthy control pigs showed no lesions, results for them have been excluded from the table, and the results of the statistical analysis reflect a comparison, between the PCV2-negative and PCV2-positive wasted-pig groups. \(^{b}\) Total number of pigs in each group, including those not showing lymphocyte depletion. \(^{c}\) LN, lymph node. \(^{d}\) PALS, periarteriolar lymphoid sheaths. \(^{e}\) B zone, B-cell-dependent area. \(^{f}\) T zone, T-cell-dependent area. \(^{g}\) \(P < 0.001\). \(^{h}\) \(P < 0.05\).

**TABLE 3.** Comparison of the degrees of lymphocyte depletion of different lymphoid tissues with respect to the apparent amount of PCV2 DNA genome observed after ISH

| Level of PCV2 DNA (no. of pigs\(^{b}\)) | Superficial inguinal LN\(^{c}\) | Mesenteric LN | Peyer’s patches | Tonsil | Spleen (PALS\(^{d}\)) |
|-----------------------------------------|-------------------------------|--------------|----------------|--------|-------------------|
|                                         | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) | B zone\(^{e}\) | T zone\(^{f}\) |
| Low (7)                                 | 4    | 0    | 0    | 0    | 3    | 0    | 1    | 0    | 1 | 1 |
| High (17)                               | 0    | 15   | 8    | 9    | 0    | 17   | 1    | 16   | 0 | 16 |

\(^{a}\) Score of lymphocyte depletion degree: 1, slight; 2, moderate; 3, severe. \(^{b}\) Total number of pigs, including those not showing lymphocyte depletion. \(^{c}\) LN, lymph node. \(^{d}\) PALS, periarteriolar lymphoid sheaths; \(P = 0.07\). \(^{e}\) B zone, B-cell-dependent area. \(^{f}\) T zone, T-cell-dependent area. \(^{g}\) \(P < 0.01\); for other groups, \(P < 0.001\), except as indicated. \(^{h}\) \(P < 0.05\).
CD8+ cells that recovered later on (L. Darwich, J. Segalés, A. Resendes, C. Rosell, M. Domingo, M. Balasch, J. Plana, and E. Mateu, Abstr. 6th Int. Vet. Immunol. Symp., abstr. 146, 2001). Since the CD8+ cell subset is mainly responsible for the cytotoxic responses, it would be advisable to focus future studies on the general and virus-specific cytotoxic responses of PMWS-affected animals.

The fact that DP cells were also downshifted is difficult to explain and deserves further study. However, DP cells are mature lymphocytes with properties of memory and effector cells (26). If that shift in DP cells represents a true decrease, it can be inferred that memory responses could also be altered.

Changes in the proportion of IgM+ cells in PBMC of PCV2-infected pigs have been reported (19, 20, 23). It was suggested that this decrease could be due to the apoptotic effect that PCV2 infection might have on B cells (23). The results of our analysis of IgM+ cell levels showed two different clusters of animals within the PCV2-positive group: pigs with proportions of IgM+ cells of <5% and pigs with proportions of >5%. Though this 5% cutoff was obviously identified after the analysis, it clearly correlates with the amount of virus in the tissues and the severity of the lesions. Thus, this proportion of 5% of IgM+ cells might be a good candidate for a marker of severe progression of the PCV2 infection, particularly since PCV2-negative wasted animals were not clinically distinguishable, while alive, from PMWS-affected pigs.

The histopathological analysis showed that the intensity of depletion in B- or T-cell-dependent areas of the examined lymphoid tissues was related to the apparent amount of viral DNA in those tissues. Also, when severe cellular depletions were found in one lymphoid organ, it became more likely that similar lesions would be found in others. We think that these results indicate that lymphoid cell depletion occurs generally in all secondary or peripheral lymphoid organs affected by PMWS. On the other hand, present knowledge supports the theory that in the pathogenesis of PMWS, the first replication of PCV2 takes place in local lymphoid organs such as the tonsils and lymph nodes (18). This is thought to cause lymphocyte damage that leads to the typical histopathological pattern of PMWS—absence of follicles and lymphoid tissue atrophy (18, 19)—that we found in PCV2-infected pigs.

In relating the histopathological observations to the flow cytometry results, it is difficult to determine whether the downshift of lymphocyte subsets in the blood of PMWS-affected pigs represents a depletion or a relative change with respect to other cell types (T-γδ cells or monocytes) that were not examined. However, since the most severe depletions in lymphoid tissues were found in pigs with lower blood counts, we think that changes in the blood probably reflect general lymphocyte depletion. Unfortunately, we did not examine primary organs like the thymus or bone marrow, nor did we further characterize the phenotype of existing lymphocytes to ascertain whether they were naïve or effector or memory cells (LFA-3, CD45RO/RA, CD69, etc.). Thus, additional studies are needed to clarify whether these decreases are general or specific to a given type of lymphocyte subset.

The results of the present report reinforce the notion that in natural cases of PMWS, there is an evident change (absolute or relative) in the proportions of the different lymphocyte subsets. This change seems to be related to the levels of PCV2 in lymphoid tissues and to the extent of depletion in both B- and T-cell-dependent areas of these tissues. Taken together, these facts could suggest the existence of damage to the immune systems of PMWS-affected pigs. However, it remains unclear how PCV2 acts upon the immune systems of infected pigs.

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