

6. DISCUSSION

In this thesis we investigated the normal distribution of immune system cells by immunohistochemical techniques in paraffin-embedded tissues of conventional pigs, using four available antibodies and two mAbs, which reactivity in paraffin-embedded tissues had not been characterised previously. The results of these works permitted to study the immune system cell microscopic lesions in PMWS naturally affected pigs.

In the first study, we standardised two immunohistochemical protocols for BL2H5 and 3C3/9 antibodies to be used in paraffin-embedded tissues. We were not able to develop the methods for using the other tested antibodies. Further studies with other antigen retrievals or fixation methods are necessary to establish the utility of these antibodies in paraffin-embedded tissues.

In the second work, the normal distribution of immune system cells has been established. Polyclonal anti-human CD3 has been considered a specific pan T-cells antibody in paraffin-embedded tissue (Mason et al., 1989). In the second study, distribution of positive cells in normal lymphoid and non-lymphoid porcine tissues agreed with a previous description of other authors (Tanimoto and Ohtsuki 1996), confirming the specificity of this antibody for T cells in swine tissues. T cell areas in lymphoid organs were clearly depicted with this antibody. In our study, in addition to T-cell zones (Tanimoto and Ohtsuki 1996), scattered cells with lymphoid morphology appeared stained in the marginal zone of the spleen.

Immunohistological detection of B-cells has been done with anti-immunoglobulins antibodies (Ramos-Vara et al., 1992).

Recently, anti-CD79 , the B-cell equivalent to CD3, was found to be a useful marker for swine normal and neoplastic B cells in formalin-fixed, paraffin-embedded tissues (Tanimoto and Ohtsuki 1996). As expected, the majority of CD79 -positive cells observed in our study were identified as B-lymphocytes for its shape and location. In the germinal centres, stained cells with a larger amount of cytoplasm could correspond to immunoblasts. In addition, few CD79 -positive cells were also found in the thymic medulla, and in the marginal zone of the spleen.

Polyclonal anti-human lysozyme and monoclonal anti-human MAC387 have been described as useful markers to stain cells of the monocyte/macrophage series in swine paraffin sections (Evensen 1993). In the second study, lysozyme identified resident macrophages in tissues (Kupffer cells, alveolar macrophages, intraglomerular mesangial cells, tingible-body macrophages in the cortex of the thymus, spleen sinus macrophages, etc.) and other non-lymphoid cells (renal proximal tubular cells, epithelial lining cells of the tonsillar crypts, epithelial cells of gastric glands and endothelial cells of high endothelial venules) which may also contain this enzyme. On the contrary, MAC387 failed to detect macrophages, staining only polymorphonuclear granulocytes. A possible explanation to this fact might be that the L1 protein is not consistently expressed in swine macrophages. Other authors (Whyte et al., 1996, Smith et al., 1998) have also reported inconsistency of results of MAC387 staining.

In a previous study, 3C3/9 has been shown to stain different subpopulations of lymphocytes, particularly follicular lymphocytes and a small number of cells scattered in the interfollicular areas of swine frozen lymphoid sections (Bullido et al., 1997a). These results were confirmed in the present study, in paraffin-embedded tissues. Furthermore, in the present investigation, other organs were studied with this marker for the first time, as the spleen, the stomach and the liver. This antibody reacted with two different cellular types. One corresponded to lymphocytes for shape and location. A part of these cells could be considered B lymphocytes, on the basis of their location in primary follicles and mantle zones, while the other part could be B or T lymphocytes. In fact, it has been demonstrated that, in addition to B cells, naïve T cells also express the high molecular weight isoform of CD45 (CD45RA) (Mackay, 1993, Bullido et al 1997a). The other cellular type, with a more abundant cytoplasm and located in the germinal centres corresponded to immunoblasts.

Histocompatibility class II antigens are present in a limited number of cell types. In the swine they are expressed on all B cells, on APCs and in a variable number of resting and activated T cells (Saalmuller et al., 1991; Bullido et al., 1997b). In the present investigation, it was possible to identify these cells, and to study their distribution by using an anti-SLA II DQ, the BL2H5 molecule. This antibody was used for the first time in an immunohistochemical study. Positive cells with round central nuclei and a small rim of cytoplasm were considered lymphocytes, while large cells were recognized to be macrophages, dendritic cells or interdigitating cells,

depending on their distribution in the lymphoid organs. Lymphocytes that were observed in the mantle zone of the follicles in lymph node, tonsils, spleen and Peyer's patches were considered B-lymphocytes. Scattered small positive cells in the PALs, marginal zone and red pulp of the spleen could be considered T lymphocytes. In the dome region of the Peyer's patches and in the aggregated lymphoid follicles of the gastric tract mucosa both B and T cells are found. Positive large cells in follicular germinal centres of lymph nodes, tonsil, spleen and Peyer's patches were identify as follicular dendritic cells, while positive staining in the interfollicular areas was due to the presence of interdigitating cells. In the medulla of the thymus, positivity was attributed to the epithelial network and interdigitating cells. In the liver, staining was restricted to Kupffer cells and perivascular macrophages.

In the third study we described the changes of immune system cells distribution in different organs of pigs with naturally occurring PMWS using the immunohistochemical methods developed in the first studies.

These changes are mainly characterised by reduction or loss of B cells, diminution of T lymphocytes, increase of subcapsular and peritrabecular macrophages, and partial loss and redistribution of APCs throughout lymphoid tissues. The severity of the mentioned changes was strongly correlated with the severity of PMWS histological lesions and the higher presence of PCV2 antigen and/or nucleic acid.

Macroscopic lesions observed in lymph nodes, lungs, and kidneys in the present study had been previously reported by other authors (Clark, 1997; Rosell et al., 1999). The low incidence of renal macroscopic lesion found in this work may be due to the low number of pigs studied. However, a few number of pigs with macroscopic renal lesions was also reported in another study where a larger number of animal was used (Quintana et al 2001).

Characteristic microscopic lymphoid lesions, as lymphocyte depletion, histiocytic infiltration, presence of MGCs, and cytoplasmic inclusions, are considered typical findings in PMWS affected pigs (Clark, 1997; Rosell et al., 1999). We observed a varying degree of lymphocytic depletion and histiocytic infiltration in all pigs studied; however a relevant number of MGCs was only observed in stage II affected cases. The activation of the immune system is considered crucial for the formation of such cells in tonsils and adenoid tissues of human immunodeficiency virus affected patients (Orenstein and Wahl, 1999). Our results suggest that a similar phenomenon may occur in PCV2 infected pigs, with formation of MGCs only in more immunologically responsive animals. Further studies on PCV2 pathogenic mechanisms and a larger number of pigs are necessary to clarify this point.

Among non-lymphoid tissues, interstitial pneumonia was the main finding, whereas suppurative bronchopneumonia observed in stage II and III was associated with concomitant bacterial infections (data not shown). Hepatic and renal lesions associated with PCV2, as described in other reports (Rosell et al., 2000a, b), were only

sporadically observed in our study; the selection methods and the larger number of pigs used by these authors might explain the different results.

Concurrent ISH for PCV nucleic acid (without differentiation between PCV1 and PCV2) and immunohistochemistry for several cell markers confirmed macrophages to be the predominant virus containing cells in PMWS affected pigs (Kiupel et al., 1999). In our study, the application of a larger panel of cell markers, and the use of a PCV2-specific probe for ISH (Rosell et al., 2000a) demonstrated that APCs in general, and not only macrophages, are target cells for PCV2 infection. On the other hand, we found that lymphocytes are only sporadically infected, similarly to other authors' results (Kiupel et al., 1999). The relation between severity of lesions in secondary lymphoid tissues and presence of PCV2 nucleic acid has been already documented (Darwich et al., 2002). In the present study, thymus, a primary lymphoid organ, was also investigated. In this organ, PCV2 nucleic acid or antigen was detected only in a very low number of histiocytic cells of the medulla, suggesting that thymocytes and T cells might be more resistant to PCV2 infection.

The decrease of CD79⁺, and CD45RA positive lymphocytes observed in our study, agreed with the results of other immunohistochemical studies in tissues (Kiupel et al., 1999; Shibahara et al., 2000; Sarli et al., 2001) and in peripheral blood leukocytes using flow cytometry (Segalés et al., 2001; Darwich et al., 2002) of PMWS affected pigs. These studies described CD4⁺, CD8⁺, and CD4⁺/CD8⁺ lymphocyte changes in frozen tissues or in

peripheral blood of PMWS affected pigs (Sarli et al., 2001; Segalés et al., 2001; Darwich et al., 2002). In our study, T cell depletion was documented with the CD3 antibody, a pan-T lymphocyte marker, making impossible to compare our results with those of other authors. However, T naïve lymphocytes, which are positive for both CD45RA and CD3 antibodies observed in the study II, were still observed in the marginal zone of the spleen of all studied pigs, suggesting that this cell subpopulation is not apparently affected in PMWS affected pigs when compared with healthy animals.

PCV2 have been described to induce apoptosis of B lymphocytes, which would explain B cell areas depletion in PMWS affected pigs (Shibahara et al., 2000). Recent studies, including the present one, have now demonstrated that depletion also occurs, to a lesser degree, in T cell areas (Sarli et al., 2001). Death for apoptosis is a fundamental process in regulating T a B cell populations and also in regulating cell viral infection (Alcami and Koszinowski, 2000; Krammer, 2000). Therefore, apoptosis might be a reasonable cause of lymphocyte depletion observed in PMWS. Whether and how PCV2 might play a role in apoptosis up-regulation is still unknown.

Histiocytic cells infiltrating immune system organs in PMWS have been identified to be macrophages (Kiupel et al., 1999; Shibahara et al., 2000; Sarli et al., 2001). In our study a considerable infiltration of lysozyme positive macrophages was observed in stage II and III, with a similar pattern of distribution of PCV2 infected cells. Recent reports on viral immune evasion

mechanisms explain how DNA virus are able to acquire immune response genes of the host, to block or subvert the host anti-virus immune and inflammatory responses (Alcami and Koszinowski, 2000; Haig, 2001). PCV2 could inhibit one of these host anti-virus responses, as for example apoptosis, chemokine production, or interferon production allowing a longer survival to macrophages. Future studies *in vivo* and *in vitro* are needed to explain the pathogenesis of lymphocyte depletion, and increase of infiltrating macrophages characterising PMWS.

Lysozyme and SLA-II-DQ (BL2H5) staining of MGCs allowed to establish the macrophage origin of these cells. The BL2H5 antibody labelled the cytoplasm of these cells; this is not a surprising finding since MHC II is firstly assembled to the antigen in the endoplasmic reticulum before transportation to the cell surface (Calafat et al., 1994). The BL2H5 antibody demonstrated that APCs and PCV2 infected cells had a very similar pattern of distribution, fortifying the observation that all types of APCs could be PCV2 infection target cells (Rosell et al., 1999), and not only macrophages as previously reported (Kiupel et al., 1999). In stages I and II, the APCs number, but not their distribution, was very similar to control cases, whereas in stage III a lesser number of these cells was observed. This finding can be explained with the substitution of B cells by infiltrating macrophages, since B-lymphocytes are also APCs. In our study, only the most severely affected pigs showed a decreased number of APCs; however, because these animals were killed is not possible to know which changes would have occurred if they had

lived longer. Actually, APCs loss could occur in the last stage of the disease.

Increase of MAC387 stained polymorphonuclear granulocytes observed in almost all tissues in stages II and III might be due to secondary infections occurring in the lung (data not shown). In this work, the MAC387 antibody faintly stained some macrophages in suppurative foci in the lung, whereas in the normal pigs of study II no staining was ever observed in macrophages. These macrophages could have phagocytosed granulocyte material, being the responsible for positive stain; however a stronger macrophage stimulation can also be a reason for L1 protein expression recognised by the MAC387 antibody.

Differently from other viral diseases that provoke transitory suppression of the immune system, such as hog cholera (Summerfield et al., 2000) or PRRS (Drew, 2000), PMWS shows typical unique severe lymphoid lesions (Rosell et al., 1999). PCV2 have been demonstrated to be the causal agent of these lesions (Kennedy et al., 2000) and that can reproduce the disease when used as the only virus in the inoculum (Bolin et al., 2001; Harms et al., 2001). The characterisation of lymphoid lesions of the present study fortify the hypothesis of the immunosuppressive status suffered from PMWS affected pig suggested by other authors (Sarli et al, 2001; Segalés et al., 2001; Darwich et al., 2002). The stages I, II, and III described in this work could correspond to three different clinical stages of the disease (initial, intermediate and final), but it is also probable that could be due to different individual

immunological response of the pigs to PCV2 infection. Further studies with experimentally PCV2 infected pigs should be performed to better understand PMWS pathogenesis.

In conclusion, this thesis represent a detailed study of the distribution of the most important subpopulations of immune system cells in conventional, healthy pigs using immunohistochemical methods. These tools allowed defining and characterising the histological alterations of the immune system cells in PMWS.

7. CONCLUSIONS

1. BL2H5 and 3C3/9 are useful markers in formalin-fixed, paraffin-embedded swine tissues, to typify APC and CD45RA lymphocytes, respectively.
2. B lymphocytes, macrophages, dendritic cells, and interdigitating cells, are APC assessed with the BL2H5 antibody in swine paraffin-embedded tissues.
3. CD45RA porcine lymphocytes show the same distribution in paraffin-embedded tissues than in frozen tissues.
4. The MAC387 antibody is a useful marker to stain polymorphonuclear granulocytes in paraffin-embedded tissues of healthy pigs, but not macrophages.
5. The degree of histological and immunohistochemical lesions observed in PMWS affected pigs is sistematically correlated to PCV2 presence.
6. Depletion of lymphocytes expressing CD79 and, in a lesser degree lymphocytes expressing CD3, is a constant finding of PMWS affected pigs.
7. Infiltrating MGC in PMWS affected pigs have a macrophage origin since they are stained with lysozyme and BL2H5.
8. An altered distribution of APC, stained with BL2H5, is a constant finding in follicular lymphoid tissues of PMWS affected pigs.

9. Histiocytic cells infiltrating lymphoid tissues of PMWS affected pigs are stained with BL2H5 in a higher number than lysozyme, indicating that other non-macrophage APC may participate in the histiocytic infiltration characteristic of PMWS.

10. APC, stained with BL2H5, are main target cells for PCV2 infection in PMWS affected pigs.

11. Histological and immunohistochemical changes observed in the immune system cell populations of lymphoid tissues suggest an immunosuppressive status of the pigs suffering from PMWS.

6. REFERENCES

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