FACULTAT DE VETERINÀRIA

DEPARTAMENT DE SANITAT I ANATOMIA ANIMALS

IMMUNOHISTOCHEMICAL CHARACTERISATION OF MICROSCOPIC LESIONS IN POSTWEANING MULTISYSTEMIC WASTING SYNDROME NATURALLY AFFECTED CONVENTIONAL PIGS

Francesca Chianini

Memòria presentada per optar al grau de doctora en Veterinària

Bellaterra, febrer del 2002

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CERTIFIQUEN:

Que la tesi doctoral titulada IMMUNOHISTOCHEMICAL CHARACTERISATION OF MICROSCOPIC LESIONS IN POSTWEANING MULTISYSTEMIC WASTING SYNDROME NATURALLY AFFECTED CONVENTIONAL PIGS, de la que és autora la llicenciada en Veterinària Francesca Chianini, s'ha realitzat als laboratoris de la Unitat d'histologia i Anatomia Patològica de la facultat de Veterinaària de la Universitat Autònoma de Barcelona sota la nostra direcció.

I perquè així consti, a tots els efectes, firmem el present certificat a Bellaterra 1 de Febrer del 2002

Natàlia Majó i Masferrer | Joaquim Segalés i Coma

The author of this thesis, Francesca Chianini has been a doctoral Marie Curie fellow (FMBICT983417) from 1st September 1998 to 31st August 2001. This work was partly funded by the QLRT-PL-199900307 Project from the European Commission's Fifth Framework Programme 1998-2002, and the 2-FEDER-1997-1341 project, I+D National Plan (Spain)

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1. INTRODUCTION

1.1. Postweaning multisystemic wasting syndrome (PMWS)

1.1.1 History

Since 1994 a new disease affecting post-weaning and growing pigs, firstly observed by Harding in a Saskatchewan herd in 1991, was seen in several "high health" fattening pig herds in Western Canada (Harding, 1997; Clark, 1997). Characteristic clinical findings were wasting, dyspnea, and icterus. Gross lesions were observed in lungs, lymph nodes, liver, stomach and intestine, while microscopic lesions were mainly observed in lungs and lymphoid tissues. Due to the macro and microscopic affectation of several organs, and the age of incidence, the authors proposed the name of "postweaning multisystemic wasting syndrome" to describe the disease.

In 1996, in California (USA), Daft et al. reported a single case of a six week-old pig with pneumonia and lymphadenopathy associated to porcine circovirus (PCV). It was the first time that PCV, previously known as a non-pathogenic contaminant of pig kidney cell lines (Tischer et al. 1974, 1982), was associated to PMWS. In the same year, in Brittany (France), a syndrome characterised by wasting and high mortality was observed in 2-3 month-old pigs. The syndrome was referred as "*Maladie de l'Amaigreissement du Porcelet*" (LeCann et al., 1997). In 1997, Segalés et al. reported the first description of PMWS in Spain. Afterwards several studies have been done in tissues from the Canadian, French and Californian affected pigs (Allan et al., 1998a;

Ellis et al., 1998) describing PCV as the only agent isolated from the observed lesions.

The sequencing of the PCV associated to PMWS revealed substantial nucleotide differences in identity respect the previously known PCV. Since then, two different PCV (PCV types 1 [PCV1] and 2 [PCV2]) have been described and classified in the International Committee for the Taxonomy of Viruses (ICTV), and the association of PCV2 with PMWS has been observed and described in several countries all around the world (Allan and Ellis, 2000). Recently, PCV2 has been experimentally demonstrated to be the etiological agent of PMWS (Bolin et al., 2001; Harms et al., 2001).

1.1.1 Aetiology

1.1.2.1 Porcine circoviruses

In 1974 a picornavirus-like was reported as a contaminant of a continuous pig kidney cell line (PK/15) (Tischer et al., 1974). Some years later it was described as a new, small, icosahedral, non-enveloped virus, and due to its circular, covalently-closed single-stranded DNA it was called "circovirus" (Tischer et al., 1982). Subsequently the ICTV classified this virus within the *Circoviridae* family (Lukert et al., 1995). Prior to the PMWS description, PCV was considered non-pathogenic, as it was not related to any disease, and

for the failure of experimental infection to produce clinical signs or gross lesions in pigs (Tischer et al., 1986; Allan et al.; 1995). Since 1996, PCV was constantly reported associated to PMWS (Harding, 1997; Clark, 1997; LeCann et al., 1997; Segalés et al., 1997; Allan et al., 1998a; Ellis et al., 1998). Further studies demonstrated antigenic and genomic differences between the PCV contaminant of PK/15 and the PCV isolated from PMWS affected pigs. PCV1 was the name proposed for the non-pathogenic PK/15 contaminant and PCV2 for the potential pathogenic PMWS-related circovirus (Allan et al., 1998b; Meehan et al., 1998).

1.1.2.2. Taxonomy

The circular, single-stranded DNA genome observed in the members of the *Circoviridae* family is a unique characteristic among vertebrates' viruses (Tischer et al., 1982). This family comprises two genus: *Circovirus*, whose members are PCV1, PCV2, psittacine beak and feather disease virus (PBFDV), and columbid circovirus (CoCV) (Mankertz et al., 2000b) and *Gyrovirus* with the chicken anaemia virus (CAV) (Todd, 2000). This last virus is similar to two recently isolated virus of humans: TT virus (TTV) (Miyata et al, 1999; Mushahwar et al. 1999; Handa et al., 2000; Kazi et al., 2000; Lin et al., 2000; Biagini et al., 2001) and TTV-like mini virus (TLMV) (Takahashi et al., 2000; Biagini et al., 2001). All viruses within this family are small, icosahedral, and non-enveloped. In the pig only two of the reported

viruses have been described: PCV1 and PCV2. When the sequences of these viruses have been compared, a homology lower than 75% has been observed (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Mankertz et al., 2000a). On the other hand, homology between PCV2 isolates from several cases of PMWS all around the world is equal or higher than 96% (Meehan et al., 1998), even though a phylogenetic difference may exist among PCV2 strains (Mankertz et al., 2000a).

1.1.2.3. Morphology and structure

Porcine circoviruses are icosahedral, non-enveloped viruses with a diameter of 17 nm, containing a covalently closed circular single-stranded DNA (Tischer et al., 1982). The PCV1 complete nucleotide sequence of different isolates consists of 1759 nucleotides (Mankertz et al., 1997; Niagro et al., 1998), while PCV2 have been reported to contain 1767 or 1768 nucleotides (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998; Mankertz et al., 2000a). The difference between the two circoviruses, apart from the nucleotide number, consisted in small deletions and insertions located throughout the genomes (Todd, 2000). Up to 11 open reading frames (ORFs) have been identified (Hamel et al., 1998); however, only 6 of them are able to encode proteins larger than 5 kDa (Meehan et al., 1997; Morozov et al., 1998; Mankertz et al., 1998). The largest ORFs are represented by ORF1 and ORF2, which encode respectively a 35.7 kDa protein,

involved in virus replication (Mankertz et al., 1998), and a 28-30 kDa protein, probably involved in viral capsid formation (Nawagitgul et al., 2000). Different isolates of PCV1 and PCV2 showed a considerable homology for ORF1 nucleotide (83%) and protein (86%), while nucleotide and protein homology for ORF2 was 67% and 65%, respectively (Meehan et al., 1998; Morozov et al., 1998; Gibbs and Weiller, 1999).

1.1.2.4. Biological and physiochemical properties

PCV1 have been reported to replicate in several pig-derived cell cultures and Vero cell cultures (Tischer et al., 1987). This virus does not hemagglutinate erythrocytes from a range of animals and it is resistant to inactivation to pH 3, chloroform, 56ºC, and 70ºC (Allan et al., 1994). PCV1 buoyant density is of 1.37 gm/ml CsCl, and it has a sedimentation coefficient 57S (Tischer et al., 1982; Allan et al., 1994). Specific reports on biological and physiochemical properties of PCV2 have not been published, however a study on susceptibility of PCV2 to commercial and laboratory disinfectants has been recently done. The result of this work demonstrated the capacity of some investigated disinfectants, such as potassium peroxymonosulfate (plus sodium chloride) and sodium hydroxide, to reduce PCV2 titres under controlled laboratory conditions (Royer et al., 2001).

1.1.3. Epidemiology

1.1.3.1. Geographic distribution

Since the first description in Canada (Harding, 1996), PMWS has now been reported worldwide (Allan and Ellis, 2000). The first descriptions in France (LeCann et al., 1997) and Spain (Segalés et al., 1997) were followed by many reports in Europe and other places of the world: the UK (Kennedy et al., 1998; Allan et al., 2000; Gresham et al., 2000; Thomson et al., 2000), Ireland (Spillane et al., 1998), Denmark (Allan et al., 1999b), Italy (Marcato et al., 1999), Korea (Choi and Chae, 1999), Japan (Onuki et al., 1999), the Czech Republic (Celer and Carasova, 2001), Hungary (Kiss et al., 2000), Greece (Kyriakis et al., 2000), Germany (Pesch et al., 2000), the Netherlands (Wellenberg et al., 2000), Mexico (Trujano et al., 2001), Taiwan (Chen, 2000), and Switzerland (Borel et al., 2001). Retrospective studies in paraffin wax embedded tissues showed that histopathological lesions suggestive of PMWS were associated to PCV2 genome in Spanish cases of 1986 (Rosell et al., 2000c) and to PCV2 viral protein in Japanese cases of 1989 (Mori et al., 2000).

1.1.3.2. Transmission

PCV2 in experimentally infected pigs has been detected in nasal and ocular secretions, saliva, faeces, urine, and semen (Krakowka et al.,

2000; Larochelle et al., 2000; Resendes et al., 2001). All these routes can be considered potential routes of viral excretion. Based on these statements, field studies to detect PCV2 genome have been performed by polymerase chain reaction (PCR) on nasal, tonsillar, faecal, urinary, and bronchial swabs (Calsamiglia et al., 2001). In this study, PCV2 DNA was detected in a considerable number of urinary swabs (67%), and in a decreasing percentage on nasal (59%), tonsillar (54%), bronchiolar (54%), and faecal (35%) specimens from PMWS affected pigs. These results suggest the existence of different possible transmission routes. On the other hand, a recent contact-challenge study with PMWS pigs and specific pathogen free (SPF) pigs showed failure of SPF pigs to present typical lesions of the syndrome or to seroconvert against PCV2 (Madec et al., 2001). The vertical transmission can be another route playing a role on the spread of the viral infection. In fact, in one clinical case PCV2 has been detected as the only pathogen on aborted pig foetus lesions (West et al., 1999). For this reason, experimental SPF sow infections through tracheal and intramuscular routes, and intrauterine route have been performed (Cariolet et al., 2001a, b), as well as foetus inoculations at different stages of gestation (Sanchez et al., 2001). The results of these studies showed that PCV2 produced severe consequences on reproduction, without crossing the placenta barrier, and that the virus replicated in inoculated foetuses at different stages of gestation, without spreading from inoculated to non-inoculated foetuses. Due to the detection of PCV2 on semen in experimental conditions, a longitudinal PCR study on semen and serum of boars naturally infected was done (Le Tallec

et al., 2001). Preliminary results indicated that initially seronegative boars became viremic and excreted virus in semen for a longer period than initially seropositive boars. Nevertheless, additional studies are needed to clarify which are the most common routes of transmission.

1.3.3.3. Morbidity and mortality

The nursery and growing periods are the most critical regarding PMWS expression. The disease has been reported in 5 to 6-week-old pigs in Canada (Harding and Clark, 1997) and 8 to 13-week-old in France (Madec et al., 2000). Morbidity and mortality rates associated with PMWS vary depending on examined herds, and reached 10% in acute outbreak in Canada (Harding et al., 1998) and 14.6% in France (Madec et al., 2000). In the latter work a strong litter effect on disease susceptibility was observed. In a recent report a higher affectation of castrated males has been described, as well as a higher predisposition to develop clinical signs of lighter pigs at birth, weaning and fattening periods (Corrégé et al., 2001). A higher mortality in castrated males was also observed in a recent study in which no significant correlation between syndrome and pig weight was noted (Rodríguez-Arrioja et al., 2002).

1.1.3.4. Porcine circovirus serological prevalence

The ubiquitous presence of both porcine circoviruses throughout the world has been confirmed (Allan and Ellis, 2000). Indeed, serological studies for the detection of antibodies against PCVs in swine sera showed a widespread presence of these antibodies in fattening and adult pigs. Antibodies against PCV1 were reported in Germany (Tischer et al., 1986; Tischer et al., 1995), Canada (Dulac and Afshar, 1989), New Zealand (Horner, 1991), the UK (Edward and Sands, 1994; Allan et al., 1994), the USA (Hines and Luckert, 1995), and antibodies against PCV2 were detected in Belgium (Mesu et al., 2000), Spain (Rodríguez-Arrioja et al., 2000) and Italy (Martelli et al., 2001). Retrospective serological studies demonstrated that antibodies against PCV2 in swine serum were already present in 1973 in the UK (Walker et al., 2000), and in 1985 in Canada (Magar et al., 2000), Belgium (Vyt et al., 2000), and Spain (Rodríguez-Arrioja et al., 2001). Contrasting data exist about the detection of serum antibody to PCV in species other than pig. Some authors detected PCV1 seropositivity in humans, mice, and cattle (Tischer et al., 1995), not detected in previous studies (Tischer et al., 1982; Allan et al., 1994). Serum samples of sheep, chicken, turkey, goat, rabbit and duck were also examined and found negative (Allan et al., 1994). Furthermore, several recent reports described the absence of antibodies against PCV2 in humans, cattle and horse (Allan et al., 2000d; Ellis et al., 2000b; Ellis et al., 2001).

1.3.3. Pathogenesis

The bulk of available evidence in field and experimental cases have established that PCV2 is the principal pathogen involved in PMWS (Krakowka et al., 2000), whilst the viral mechanism triggering the syndrome is still unknown. It has been suggested that some cofactors may be implicated in the clinical expression of PMWS (Allan and Ellis, 2000). In fact, several reports have described the association of PCV2 with other pathogen agents, such as porcine parvovirus (PPV) (Allan et al., 1999a; Ellis et al., 2000a), porcine reproductive and respiratory syndrome virus (PRRSV) (Sorden et al., 1998; Ellis et al., 1999b), or Aujeszky's disease virus (ADV) (Rodriguez-Arrioja et al., 1999). Furthermore, other studies pointed out that immune system stimulating products (Allan et al., 2000c; Krakowka et al., 2001), as well as environmental factors (Madec et al., 2000) may promote PMWS expression. The two first experimental inoculations of conventional (Balasch et al., 1999) and gnotobiotic (Ellis et al., 1999a) pigs with PMWS pig tissue homogenates, demonstrated the transmissibility of PCV2 to naïve pigs but they could not reproduce the severe lesions and clinical symptoms of naturally occurring PMWS cases. Since then, many experimental infections have been performed to better understand the pathogenesis of PMWS, to identify the role of infectious and non-infectious cofactors in its diffusion, and to fulfil Koch's postulates confirming the ability of PCV2 alone to cause the syndrome. Co-infections with PCV and PPV of colostrum-deprived (CD) (Allan et al., 1999a), conventional (Kennedy et al., 2000), and

gnotobiotic swine (Krakowka et al., 2000) reproduced PMWS; however, in the conventional pigs, typical syndrome signs were also reproduced with PCV2 alone. PRRSV has been reported to enhance PCV2 replication in infected CD swine (Allan et al., 2000c). Furthermore, double inoculation with PRRSV and PCV2 was reported to induce severe PMWS in CD/CD (colostrum-deprived, caesarean-derived) pigs (Harms et al., 2001), and in a proportion of double inoculated conventional pigs (Rovira et al., 2002). The activation of the immune system, and the infection of PCV2 have been reported as the pivotal events in the development of PMWS in gnotobiotic pigs (Krakowka et al., 2001). On the other hand, in another experiment, both immunostimulated and non-immunostimulated seronegative conventional pigs showed typical PMWS signs following PCV2 inoculation (Bøtner et al., 2001). Furthermore, co-inoculation of PCV2 and a vaccine adjuvant failed to reproduce the disease (Resendes et al., 2001), but produced a higher number of viremic pigs compared to only PCV2 inoculated. *In vivo* and *in vitro* additional studies are necessary to find out which pathogenic mechanism leads PCV2 to develop PMWS.

1.3.5. Clinical signs

PMWS clinical signs are restricted to nursery and early growing pigs, and consist of wasting, dyspnea, enlarged inguinal superficial lymph nodes, diarrhoea, pallor, and jaundice (Harding and Clark, 1998)

Haematological data showed that the microcytic and hypocromic anaemia observed in PMWS natural cases is usually associated to the presence of ulcers in the oesophagic pars of the stomach (Segalés et al., 2000).

Nevertheless it is dutiful to remember that PCV2 has been also associated to other diseases with clinical signs other than the previously described (Harding, 2001). These conditions are porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000b), reproductive failure (West et al., 1999; Ohlinger et al., 2000), proliferative and necrotising pneumonia (PNP) (Pesch et al., 2000), and congenital tremors type A2 (Stevenson et al., 2001), and porcine respiratory disease complex (Harms et al., 2002).

1.3.6. Pathological findings

1.3.6.1. Macroscopic lesions

Dr E.G. Clark, at the American Association of Swine Practitioners of 1997, reported the first description of PMWS lesions (Clark, 1997). Enlargement of lymph nodes, showing a homogeneous white surface when cut, and non-collapsed lungs, with atelectatic or consolidated areas in the cranial and middle lobes were the most relevant lesions. Half of the PMWS cases had lesions in liver and kidney, and consisted mainly of small to large patchy areas of hepatic discoloration with prominent interlobular connective tissue, and white foci of various

sizes visible in the subcapsular surface of the kidney. Gastric ulceration is another lesion that has been found in a relatively high percentage of PMWS natural cases (Segalés et al., 2000).

1.3.6.2. Microscopic lesions

The most frequent histopathological lesions are observed in lymphoid organs and lungs (Clark, 1997; Segalés et al., 1997; Rosell et al., 1999). Lymphoid organs and tissues showed early loss of B cell follicles and infiltration of T cell areas by large histiocytic cells and multinucleated giant cells (MGC). Furthermore in B cell-dependent areas clusters of round, various-sized, basophilic inclusion bodies were seen in the cytoplasm of histiocytic cells. The lungs show varying degrees of interstitial pneumonia, with lympho-histiocytic or granulomatous infiltration, and thickening of interalveolar septa. Bronchopneumonia associated with secondary bacterial infection has also been reported. Hepatic lesions were firstly reported to affect 75% of PMWS pigs, but in a more recent study lymphoplasmacytic periportal hepatitis was found in all pigs studied (Rosell et al., 1999). Mild to severe multifocal interstitial nephritis was observed in 50-75% of cases (Clark, 1997; Rosell et al., 1999). Virtually, all tissues may show lympho-histiocytic inflammatory infiltration in severely PMWS affected pigs (Segalés et al., 2001).

1.3.7. Diagnosis

Definitive diagnosis of PMWS is based on three criteria: evidence of clinical signs, presence of microscopic lesions in lymphoid tissues, and PCV2 detection in these histopathological lesions (Sorden, 2000; Quintana et al., 2001).

1.3.7.1. Histology

Since the first description of PMWS lesions, histopathologic examination was considered determinant to establish the diagnosis (Clark, 1997). In fact, observation of characteristic lesions in lymphoid organs, such as depletion, histiocytic and MGC infiltration, and intracytoplasmic inclusions is strongly suggestive of PMWS (Rosell et al., 1999).

1.3.7.2. PCV2 detection in tissues

The most used tests to detect PCV2 in tissues are ***in situ*** hybridisation (ISH), immunohistochemistry (IHC), and indirect immunofluorescence (IIF) (Rosell et al., 1999; Allan and Ellis, 2000). Recently, some authors compared the results of three different methods for the detection of PCV2 (IHC, IIF, and PCR), concluding that the best test was Mab-based IIF for being as sensitive as IHC, highly specific, cheap, simple and quick to perform (Alborali et al., 2001). Nevertheless, this method produces a stain that is visible only with an

ultraviolet microscope, interfering on viral distribution detection (Haines and Chelack, 1991). Furthermore, IIF has to be performed on frozen tissues, making this method unsuitable for the majority of archive specimens. On the contrary, ISH and IHC can be used on formalin-fixed paraffin-embedded tissues, which maintain their characteristic structure for long time, and are applicable on retrospective studies (Segalés et al., 1999). Both methods have been reported in studies of PCV2 detection and distribution (Rosell et al., 2000c; McNeilly et al., 2001). Even though ISH and IHC are very different methods, detecting genomic material, and viral protein, respectively, they usually give similar results when used in studies of PCV2 distribution (McNeilly et al., 1999; Rosell et al., 1999). The increased signal intensity, sometimes observed in these reports following ISH or IHC, may be due to viral infection stage. In fact during active infection, replication of microorganisms is accompanied by high protein production, facilitating PCV2 IHC detection, while in other phases ISH could be equally or more sensitive depending on nucleic acid amount present on tissues studied (Segalés et al., 1999). Several ISH probes have been used to detect both PCV1 and PCV2 (Allan et al., 1998b; Rosell et al., 1999; Choi and Chae, 1999), and other more specific for PCV2 (Rosell et al., 2000a; Morvan et al., 2001). The nucleic acid have been observed within cytoplasm and sometimes nuclei of cells that have been morphologically recognised as dendritic cells, histiocytic cells, and MGC in most lymphoid tissues. Occasionally, small round cells resembling lymphocytes have been described to show sporadic cytoplasmic reaction. In the lung, PCV2

location depend on the grade of lesion, and in severe cases it has been observed in macrophage cytoplasm as well as in alveolar, bronchial or bronchiolar epithelial cells. Kupffer cells have been reported as target cells of PCV2 infection in liver. PCV2 genetic material has been only sporadically seen in renal tubular epithelial and endothelial cells in kidney. PCV2 antigen had a very similar pattern of distribution when studied with polyclonal antibodies (Rosell et al., 1999; Choi et al., 2000). Recently, monoclonal antibodies to PCV2 have been developed representing a more specific alternative for IHC used in routine diagnosis as well in studies of PMWS pathogenesis (McNeilly et al., 2001).

1.3.7.3. PCR

The use of PCR for PCV detection in tissues was initially reported in 1997 by Nayar et al. Since then, several PCR methods to detect porcine circovirus have been reported. Some of them detected PCV, without differentiation of PCV1 and PCV2 (Onuki et al., 1999; Hamel et al., 2000; Mankertz et al., 2000a; Rosell et al., 2000b). Other PCR techniques reacting with PCV1 or PCV2 have been developed (Allan et al., 1999b; Mankertz et al., 2000a; Quintana et al., 2001). Multiplex PCR has been applied to differentiate PCV1 and PCV2 in the same reaction (Larochelle et al., 1999a; Ouardani et al., 1999). Furthermore, PCR used together with restriction fragment length polymorphism (RFLP) enabled PCV characterisation and subclassification (Onuki et al., 1999; Fenaux et al., 2000; Hamel et al., 2000; Rosell et al.,

2000b). The possibility to be applied to various specimens, such as serum, fresh and frozen tissues (Quintana et al., 2001) and semen (Hamel et al., 2000) represent the main advantage of PCR compared to other methods. In fact, nested PCR, whose high specificity was reported (Hamel et al., 2000), showed similar results to ISH when performed in formalin-fixed paraffin-embedded tissues (Kim and Chae, 2001). It is dutiful to point out that PCR allowed the whole amplification of PCV1 and PCV2 genomes (Meehan et al., 1997; Hamel et al., 1998).

1.3.7.4. Serology

Immunoperoxidase monolayer assay (IPMA) (Balash et al., 1999; Rodriguez-Arrioja et al., 2000), IIF (Cottrell et al., 1999; Allan and Ellis, 2000), and competitive enzyme-linked immunosorbent assay (c-ELISA) (Walker et al., 2000) have been developed to detect antibodies against PCV2. Serological studies in PMWS affected farms, have demonstrated a larger number of PCV2 positive pigs than PCV1 positive pigs, and in double seropositive cases, PCV2 titres were higher than PCV1 titres (Magar et al., 2000; Rodríguez-Arrioja et al., 2000). These authors have suggested a cross-reaction between PCV2 and PCV1 antibodies, which has also been observed in experimental conditions (Bolin et al., 2001). A 35-kd protein, produced by ORF1, a high homologous region in both PCVs, has been considered the responsible for the serological cross-reaction (Pogranichnyy et al., 2000). It has also been reported that high titres against PCV2 are

equally detectable in PMWS affected farms, and in apparently healthy farms (Rodríguez-Arrioja et al., 2000; Sibila et al., 2001). All these data suggest that serological techniques can not represent a good tool to diagnose PMWS.

1.3.8. Immunology

It has been suggested that PMWS may induce immunosuppression in pigs (Domingo et al., 2001). Lymphoid lesions observed in PMWS affected tissues (Rosell et al., 1999) and the association of the disease with opportunistic pathogens, commonly associated with immunosuppression (Clark, 1997; Carrasco et al., 2000), are good arguments to support this hypothesis. Furthermore, some studies demonstrated that the stimulation of the immune system or a routine vaccination of PCV2 infected pigs, can promote PCV2 replication and trigger PMWS (Allan et al., 2000b; Krakowka et al., 2001). Flow cytometry and immunohistochemical studies have been performed to assess the changes on leukocyte populations of conventional pigs affected with PMWS (Shibahara et al., 2000; Sarli et al., 2001; Segalés et al., 2001; Darwich et al., 2002). The most relevant findings reported in these works were a decrease of circulating B-cells (Segalés et al., 2001; Darwich et al., 2002) and loss of lymphocytes in B-cell areas (Shibahara et al., 2000). In addition, diminution of CD4+ (Sarli et al., 2001; Segalés et al., 2001) or CD8+ T lymphocytes (Darwich et

al., 2002) were also observed. Further studies are needed to understand the immunopathogenesis of PMWS.

1.2. Swine immune system cells

Swine lymphoid tissues have morphological and functional differences respect to other domestic mammals (Binns, 1982). In the pig, cortical region including follicular and interfollicular areas are located in the central part of lymph nodes, while medullar tissue is located in the periphery (Chievitz, 1881 cited in Ramis et al., 1991). Medulla is formed by a uniform and diffuse network of cells, and not by cellular cords and sinus as in other species (Binns, 1982), and is believed to support an efficient system filter to cell traffic (Banks, 1993). Furthermore, in porcine lymph nodes, lymphocytes recirculate from post-capillary venules to blood vessels, making possible a rapid antigen recognition and immune response (Banks, 1993).

1.2.1. Characterisation of swine immune system cells

During the last decade, new emergent viral diseases such as PMWS and PRRS, causing important lesions in the lymphoid tissues have been described in pigs (Molitor et al, 1997; Rosell et al, 1999). The swine also has become an increasingly interesting experimental model to investigate transplant rejection, and a potential donor for

xenografts (Sundt et al., 1992; Bennet et al., 2000, Yoo and Giulivi, 2000). Detailed knowledge of the normal distribution of cell populations of the immune system is required to understand pathogenesis of changes in these processes.

Immunohistochemistry is a useful technique for characterisation of immune system cells in tissues in pathogenic studies of diseases in humans and animals. Many antibodies have been developed that recognise cell populations in lymphoid organs. In fact, since 1982, three International Swine Differentiation Cluster (CD) Workshops have been organised. In these Workshops, a large panel of antibodies raised to pig cells and others to human cells cross-reacting on pig cells, have been tested and characterised by flow cytometry, immunohistology and molecular weight analysis (Haverson, 2001). Results of the three Workshops are summarised in table 1. However, the majority of these antibodies work only on frozen sections (Bianchi et al., 1992; Denham et al., 1998; Haverson et al., 1994). In such sections, the evaluation of morphological details is more difficult than in paraffin-embedded sections (Falk et al., 1994). For this reason, detailed studies of the distribution of the different cell populations in the swine immune system are hampered by the lack of antibodies that allow the detection of these cells in formalin-fixed, paraffin-embedded tissues. The cross-reactivity of some antibodies to human differentiation markers has allowed immunostaining of a few swine immune cell populations with protocols standardised in formalin-fixed, paraffin-embedded tissues.

Table 1. CD Workshops results

Sections	CD/SWC[a]	I workshop mAb[b]	II workshop mAb	III workshop mAb
B cells	wCD1	76-7-4		
	wCD21	1AH-CC51	BB6-11C9; C35	huCD21; C35
	SWC7	1AH-CC55	2F6/8	2A10/8
T cells	CD2a	MSA4; MAC83; MAC80;PG168A; 1038-8-31	PGBL6A; PGBL23A	huCD2
	CD3a		FY2A11; BB23-8E6	
	CD3b		FY1H2	
	CD4a	74-12-4; b38c6; 10.2H2; PT90A	STH293	
	CD5	b53b7; PG71A, PG75A; PG99A PG11A4; PG116A	1H6/8; BB6-9G12	
	wCD6	a38b2; PG90A	MIL8	
	wCD8a	76-2-11; PT36B, PT81B	STH101	PPT20; PPY21
	wCD8b	11/295/33; 11/122/28; PG93A		
	wCD8c		PG164A; UCP1B2	PPT23
	CD25	K231-3B2	PGBL25A	
	SWC1a	11/8/1; K263; MUC13A; PT15A; PT80A		
	SWC1	PT91A,PT37A; 11/305/44; 76-6-7	335-2	
	SWC2	b30c7; PG124A		
Myeloid cells	SWC3a	74-22-15; 6F3; DH59B		
	CD14	My4	TUK4; biG10; biG13; G7	CAM36A
	CD16			
CD44/CD 45	WCD44	ZO62; PORC/24a; H22A		
	CD44	BAG40A; BAT31A; MAC325; 25-32		
	wCD45R	MAC326; 3a56; MAC327, -a2	6E3/7; MIL5; STH267; FG2F9	
	wCD45	K252.1E4; MAC323; 74-9-3; 2A5		
	CD45RA			PG167A; PG77A; PG96A; PGB78A; 3C3/9; MIL13; NHT101
MHC II	MHC II DR			2E9/13; 1038H-12-34
	MCH II DQ			BL2H5; BL4H2; TU39

[a] SWC: Swine Workshop Cluster; [b] mAb: monoclonal antibody.

1.2.1.1 Lymphocytes

Cross-reacting polyclonal anti-human CD3 antibody is considered an excellent marker for swine T lymphocytes when used on paraffin sections (Mason et al., 1989; Tanimoto and Ohtsuki, 1996).

The identification of B-lymphocytes is a more complex issue. Antibodies against several molecules, such as immunoglobulins (Ramos-Vara et al., 1992), CDw75, CD79 , CD79 and HLA-DR have been tested (Tanimoto and Ohtsuki, 1996). Among all these molecules, CD79 , one of the two polypeptide chains forming the CD79 molecule, has been considered the B cell equivalent of CD3 (Mason et al., 1995). In swine, mAb anti-CD79 has been described as a useful pan-B marker in paraffin-embedded tissues, although it also stains cells of non-lymphoid origin (Tanimoto and Ohtsuki, 1996). More recently, a mAb to the largest isoform of porcine CD45 (clon 3C3/9) has been used for phenotypic and functional analyses of porcine lymphocyte subpopulations (Bullido et al., 1997a). The 3C3/9 antibody recognises all B-lymphocytes and a subpopulation of T lymphocytes, and it has been applied only in frozen sections (Bullido et al., 1997a).

1.2.1.2. Macrophages and granulocytes

Antibodies exclusively detecting cells of the swine monocyte/macrophage series are rare, probably because of the high heterogeneity of this cell population. However, two antibodies have

been described which stain these cells in paraffin-embedded tissues: polyclonal human anti-lysozyme (Falk et al., 1994) and monoclonal human anti-L1 (MAC387) (Evensen, 1993). Furthermore, in swine and other species, it has been shown that MAC387 also stains polymorphonuclear granulocytes (Evensen 1993, Christgau et al. 1998).

1.2.1.3. Antigen presenting cells

Studies on the distribution of antigen presenting cells (APC) in swine tissues have not been reported. Recently, Bullido et al. (1997b) have developed a monoclonal antibody (BL2H5) recognising swine leukocyte antigens class II (SLA-DQ). This antibody has allowed the study of APC distribution in swine by flow cytometry (Bullido et al., 1997b), but it has not been used for immunohistochemistry in formalin-fixed paraffin-embedded tissues.

OBJECTIVES

The main objective of the present thesis is to characterise the histological lesions of naturally PMWS affected pigs. To accomplish this principal aim, three specific objectives were assessed:

1. To develop immunohistochemical techniques to detect immune system cells in normal formalin-fixed, paraffin-embedded porcine tissues.

2. To describe the normal distribution of different immune system cell populations using immunohistochemical techniques in formalin-fixed paraffin-embedded tissues of conventional, healthy pigs.

3. To characterise the lymphoid, renal, pulmonary, and hepatic lesions of naturally PMWS affected pigs by means of immunohistological methods on formalin-fixed, paraffin-embedded tissues.