THE CRYPT LYMPHOEPITHELIUM OF THE PALATINE TONSILS OF PIGS: A STUDY OF ITS STRUCTURE, FUNCTION AND ROLE IN THE PATHOGENESIS OF *STREPTOCOCCUS SUIS* INFECTION

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ABSTRACT

The crypt epithelium of the palatine tonsils, which is the first barrier between external environment and lymphoid tissue, was investigated in 6-month-old healthy market weight pigs and in 3 week-old piglets experimentally infected with *Streptococcus suis* serotype 2. This pathogen enters via and persists in the palatine tonsils, and the pathogenesis of this infection is still unclear.

First, the subpopulations of intra-epithelial leukocytes were characterized and quantified using immunohistochemistry and monoclonal antibodies to porcine lymphocyte markers. Different lymphocyte subpopulations, namely CD4, CD8, and γδ T lymphocytes, and B lymphocytes, were identified. Next, the alterations in the intra-epithelial leukocyte subpopulations in response to experimental infection with *S. suis* were described. Myeloid cells, CD4 and CD8 T lymphocytes, and B lymphocytes were increased in the first 72h post-infection.

The transport of *S. suis* serotype 2 across the crypt epithelium was investigated by transmission electron microscopy. *S. suis* was observed within neutrophils, macrophages, and also in occasional epithelial cells as early as 18h post-infection, indicating that participation of the innate immune response and epithelial cellular invasion are early steps in this infection. Macrophages containing bacteria were noticed straddling the basal lamina, suggesting that they can transport *S. suis* from the epithelium to the subepithelial lymphoid tissue and subsequently may disseminate the bacteria systemically.

Last, the ability of epithelial cells to engulf *S. suis* and their ultrastructural morphology were correlated to determine whether cells compatible with M (microfold/membranous) cells were involved in bacterial uptake. Although many of the features described for M cells were observed in epithelial cells of the crypt epithelium, those containing *S. suis* had no specific common feature to permit classification as M cells.

Taken together, these data suggest that the tonsillar crypt epithelium actively participates in the early phase of *S. suis* infection and likely plays an important role in the initiation of immune response.

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ABC	avidin biotin complex immunoperoxidase
Ab	antibody
APC	antigen-presenting cell
BBB	blood-brain-barrier
BALT	bronchus-associated lymphoid tissue
BMEC	brain microvascular endothelial cells
BVD	bovine viral diarrhea
CALT	conjunctiva-associated lymphoid tissue
CD	cluster of differentiation
CFU	colony-forming units
CK(s)	cytokeratin(s)
CMIS	common mucosal immune system
CSF	colony stimulating factor
DALT	duct-associated lymphoid tissue
DC(s)	dendritic cell(s)
DP	CD4/CD8 double positive T cells
EBV	Epstein-Barr virus
EF	extra-cellular factor
FACS	fluorescence activated cell sorter
FAE	follicle-associated epithelium
Fas	apoptosis-inducing receptor
Fc	Fraction crystallized, constant region of the immunoglobulin heavy chain
Galα1-4Gal	galactosyl-α1-4-galactose
HEV	high endothelial venule
HIV-1	human immunodeficiency virus 1
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
kDa	kilo-Dalton
LPS	bacterial lipopolysaccharide
MALT	mucosa-associated lymphoid tissue
M cells	microfold/membranous cells
MAdCAM-1	mucosal addressin cell adhesion molecule 1
MHC - I	major histocompatibility complex class I
MHC - II	major histocompatibility complex class II
M. leprae	*Mycobacterium leprae*
MRP	muramidase-released protein
NK	natural killer
NALT	nasal-associated lymphoid tissue
PAMP	pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline

PBSO	PBS with 0.1% ovalbumin
PBL	peripheral blood lymphocytes
PNA	peanut agglutinin
PP	Peyer's patches
PRRS	porcine reproductive and respiratory syndrome
RER	rough endoplasmic reticulum
rRNA	ribosomal RNA
SCID	severe combined immunodeficiency
SEM	scanning electron microscopy
SP	single positive T cells
SPF	specific pathogen free
S. suis	*Streptococcus suis*
TCR	T cell receptor
TEM	transmission electron microscopy
Tc1	T cytotoxic 1
Tc2	T cytotoxic 2
Th1	T helper 1
Th2	T helper 2
TNF	tumour necrosis factor
TNF-β	tumour necrosis factor beta
UEA-I	*Ulex europaeus* agglutinin I
VCAM-1	venule cell adhesion molecule 1

1 - Literature Review

1.1- Introduction

In mammals and in humans there is a concentration of mucosa-associated lymphoid tissues (MALT) in the upper digestive and respiratory tracts, of which the palatine tonsils are the main representatives. The tonsils form an important defence in these areas, are portals of entry, and sites of multiplication and persistence of numerous pathogens. The first physical barrier of the palatine tonsils is the crypt epithelium which is in close contact with both the external environment and lymphoid tissue. This specialized epithelium of the tonsillar crypts is highly infiltrated by lymphocytes and therefore forms the initial zone of potential interaction with the immune system. Knowledge of the morphology and lymphoid composition of the crypt epithelium is essential for understanding the function of the palatine tonsils and for the elucidation of their role in the early stages of the pathogenesis of numerous infectious diseases. Therefore, the present research focuses on the morphology and leukocyte composition of the crypt epithelium in healthy pigs and in piglets infected with *Streptococcus suis* (*S. suis*) serotype 2, an outstanding example of a bacterial pathogen utilising the palatine tonsils as portals of entry and as carrier site.

In this chapter, the relevant literature that forms the basis for this work is reviewed. Section 1.2 is a broad review of *S. suis* serotype 2 infection in pigs. Section 1.3 introduces the MALT, which include the palatine tonsils, and reviews the recent information on M cells, the specialized epithelial cells of MALT. In this section the structure and function of the palatine tonsils are reviewed. Section 1.4 gives an overview of the lymphocyte subpopulations and their respective functions. It also reviews the current information on lymphocyte subsets and functions in pigs, an area that

has been advancing rapidly in the last 15 years due to the development and characterization of monoclonal antibodies against porcine lymphocyte markers.

1.2- *Streptococcus suis* Infection in Pigs

1.2.1- History and Importance of the Disease

Streptococcal meningitis in pigs has been described since 1912 and experimentally reproduced in 1951. In 1954, this disease was first described in Great Britain and the species isolated did not fit into any group of Lancefield's classification for *Streptococcus* species described until then (Field et al., 1954). In 1963, de Moor, in The Netherlands, reported that the streptococci isolated from the cases of meningitis and arthritis in pigs were different from the known streptococcal species and designated them as two new groups: Lancefield's group S, the strains isolated from newborn pigs, and group R, the strains isolated from older pigs. Later it was determined that these bacteria belonged to the Lancefield's group D and the names *Streptococcus suis* (*S. suis*) serotype 1 and *S. suis* serotype 2 were proposed to replace de Moor's groups S and R, respectively (Elliott, 1966; Windsor and Elliott, 1975). Since then, disease caused by *S. suis* has been described worldwide, with cases reported in the United States, The Netherlands, United Kingdom, Canada, Australia, New Zealand, Belgium, Brazil, Denmark, Norway, Finland, Spain, Germany, France, Ireland, Hong Kong, Japan and China (Staats et al., 1997; Berthelot-Hérault et al., 2000). In the last 20 years, *S. suis* has emerged globally as important pathogen of pigs, mostly in association with the intensification of the swine industry (Reams et al., 1993; Torremorell et al., 1998; Higgins and Gottschalk, 1999b). *S. suis* infection causes significant economic loss in the pig industry due to the necessity for control measures such as vaccination and addition of antibiotics in food and water, treatment of sick animals, death, and unthriftiness of recovered animals (Amass et al., 1995; Staats et al., 1997). In the United States, the economic loss of the swine industry due to *S. suis* infection is estimated at over 300 million dollars (Staats et al., 1997). *S. suis* serotype 2 is zoonotic and septicaemia, meningitis, deafness, arthritis, endocarditis, endophthalmitis and death have occurred in abattoir workers and others such as farmers, butchers and, less commonly, housewives, coming into close contact with pigs or raw

pork meat (Cheng et al., 1987; Clarke et al., 1991; Maher, 1991; Perseghin et al., 1995; Francois et al., 1998). More rarely, *S. suis* serotypes 4 and 14 are also involved in human infections (Higgins and Gottschalk, 1999b).

1.2.2 - Predisposing Factors

S. suis can cause disease at any age but it usually affects pigs between 3 and 16 weeks old, with a higher incidence at post-weaning and mixing of piglets. Factors that predispose to herd outbreaks include high population density, poor ventilation, sudden change in weather affecting temperature and ventilation in controlled environment houses, vaccination, weighing and movement of animals (Clifton-Hadley, 1984; Staats et al., 1997; Higgins and Gottschalk, 1999b). All these factors occur commonly in this intensive animal industry and have the potential to induce stress and consequent immunosuppression. Disease caused by *S. suis* is common in herds with high-health status, likely due to the poor stimulation of both innate and acquired immune systems in these animals (MacInnes and Desrosiers, 1999). Viral infections, such as porcine reproductive and respiratory syndrome (PRRS), are also known to predispose pigs to *S. suis* (Thanawongnuwech et al., 2000).

1.2.3 - Clinical Signs and Pathology

The incidence of clinical disease caused by *S. suis* is variable among herds and although it can reach 50%, it usually is less than 5% (Clifton-Hadley, 1984; Staats et al., 1997; Higgins and Gottschalk, 1999b). With proper treatment the mortality in the herds is usually low, ranging between 0 and 5%, but without treatment can reach 20% (Staats et al., 1997). The disease in pigs may manifest as respiratory, septicaemic and nervous forms. The clinical signs are variable and may include fever, coughing, sneezing, anorexia, depression, reddening of the skin, lameness, incoordination, opisthotonus, paralysis and convulsions. In the peracute form, animals can be found dead without premonitory signs (Reams et al., 1994; Higgins and Gottschalk, 1999b).

The lesions depend on the duration of the disease; animals that die acutely may have no lesions or have signs of septicaemia such as cutaneous reddening, congested organs and haemorrhages in the lung and myocardium. Often there is an excess of fluid

and presence of fibrin tags in the thoracic and abdominal cavities, and arthritis, usually involving the carpal and tarsal joints. Synovial fluid is increased in affected joints and varies from serous to fibrinopurulent in character. Meningitis is a very common finding and depending on the severity can be seen grossly or only microscopically. Usually the choroid plexus is involved and fibrinopurulent exudate accumulates in the ventricles (Staats et al., 1997). Different types of pulmonary lesions can occur such as fibrinous pleuritis, suppurative or fibrinous bronchopneumonia, fibrinohaemorrhagic pneumonia or interstitial pneumonia, and frequently a combination of these is seen in the same lung. In addition to *S. suis*, other bacteria e.g. *Pasteurella multocida*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Mycoplasma hyopneumoniae* and *Escherichia coli*, are frequently isolated from the lung lesions (Sanford and Tilker, 1982; Reams et al., 1994; Staats et al., 1997). In respiratory disease due to swine influenza virus, pseudorabies virus and PRRS virus, *S. suis* is often a secondary pathogen (Iglesias et al., 1992; Reams et al., 1994; Galina et al., 1994).

1.2.4 -General Features of *Streptococcus suis*

S. suis is a small ovoid Gram-positive coccus, less than 2 μm in diameter, that occurs singly, in pairs or in short chains, has a tendency to rod formation, is non-motile and is facultatively anaerobic. All strains of *S. suis* are α-haemolytic on sheep blood agar and many produce β-haemolysis on horse blood agar. Although *S. suis* reacts with Lancefield's group D antiserum and was classified in the past as belonging to this group, it is genetically different from the other group D streptococci, and thus it does not belong to this group (Kilpper-Balz and Schleifer, 1987; Bentley et al., 1991). *S. suis* is classified according to capsular polysaccharide antigens (Staats et al., 1997); to date there are 35 serotypes (1 to 34 and 1/2). In addition, untypable isolates are reported (Higgins and Gottschalk, 1999a). Multiple serotypes of *S. suis* may be isolated from the same healthy or sick animal. Disease is usually associated with serotypes 1 to 8 (Higgins and Gottschalk, 1999b). Worldwide, serotype 2 is the serotype most commonly associated with disease (Reams et al., 1993; Smith et al., 1997; Rasmussen et al., 1999; Higgins and Gottschalk, 1999b), with the exceptions of Belgium, The Netherlands and Germany, where *S. suis* type 9 is the most prevalent serotype, and the United Kingdom where

serotype 1 is the most frequently isolated (Wisselink et al., 2000). Not all strains of *S. suis* serotype 2 are pathogenic however, and virulent strains can be isolated even from clinically healthy animals (Staats et al., 1997; Smith et al., 1997; Torremorell et al., 1998; Rasmussen et al., 1999; Higgins and Gottschalk, 1999b).

All 35 serotypes of *S. suis*, including different strains of the serotypes 1, 2 and 1/2, were analysed for genetic differences by restriction fragment length polymorphism of genes encoding ribosomal RNA (rRNA), i.e. ribotyping. Except for serotypes 4 and 5 that had an identical ribotype pattern, genetic heterogeneity was noticed among all serotypes. Genetic heterogeneity was also observed among different strains within the same serotype for *S. suis* types 1, 2 and 1/2 (Okwumabua et al., 1995).

Attempts to make a correlation between the ribotype of *S. suis* and virulence have yielded contradictory results; in Denmark, the ribotype profile of avirulent strains of *S. suis* serotype 2 is very similar to the profiles of isolates from diseased animals (Rasmussen et al., 1999), while in The Netherlands and The United States of America, with a few exceptions, the ribotype profile of virulent strains of *S. suis* serotype 2 is different from the avirulent strains (Okwumabua et al., 1995; Smith et al., 1997; Staats et al., 1998).

1.2.5 - Resistance to the Environment and Modes of Transmission

S. suis is a contaminant of faeces, dust and water and, depending on the temperature, can survive for a few months in these environments. Transmission from the environment can occur via contaminated boots, needles and flies; the most common mode of transmission, however, is by direct contact between animals, either through contact of a carrier animal with an uninfected animal or from the carrier sow to the piglets (Amass et al., 1995; Staats et al., 1997; Higgins and Gottschalk, 1999b). Studies have shown that multiple serotypes of *S. suis* may be present in the saliva, milk, feces and vaginal fluids of sows and that in piglets the tonsils may be colonized by *S. suis* as early as 24 h after birth (Amass et al., 1995; Amass, 1997). It has been suggested that the piglets become infected with *S. suis* during parturition and that they then carry the bacteria into the nursery, transmitting it to other piglets. Once the acquired lactogenic

maternal immunity declines, at around 5 weeks of age, disease may develop (Amass, 1997).

1.2.6 - Virulence Factors

Several virulence factors have already been detected in *S. suis*; however, their roles in inducing infection and disease have not been well elucidated. Results of studies by different groups of researchers on *S. suis* virulence factors have not always been in agreement.

1.2.6.1 - Muramidase-released Protein and Extracellular Factor

In The Netherlands, two proteins of *S. suis* serotype 2 have been extensively studied: a 136 kilo-Dalton (kDa) cell wall-associated protein, known as muramidase-released protein (MRP), and a 110 kDa extracellular protein, called extracellular factor (EF). An amino acid sub-sequence of MRP has similarities to the fibronectin-binding protein of *Staphylococcus aureus*, suggesting that MRP is involved in binding to fibronectin (Smith et al., 1992). Fibronectin-binding protein is a molecule through which different pathogens, e.g. streptococci, can mediate attachment to the host (Finlay and Falkow, 1997). In a study involving 180 strains of *S. suis* serotype 2 isolated from organs of diseased pigs, tonsils of healthy pigs and from human cases of disease, 77% of the isolates from the diseased pigs were MRP+ EF+. In contrast, only 2% of isolates from healthy pigs, and 15% of isolates from human cases had this phenotype. The isolates from healthy pigs were mainly MRP-EF- (86%) and from human patients mostly MRP+EF- (74%), strongly suggesting that the MRP and EF proteins were linked to disease production; however, as the phenotype of a small percentage (11%) of the isolates from both diseased pigs and human patients was MRP-EF-, additional factors might be required for virulence (Vecht et al., 1991). Since the strains tested in this study came from diverse backgrounds, they were likely genetically distinct, and the role of virulence factors other than MRP and EF was not examined.

Experimental studies in newborn germfree pigs confirmed a correlation between MRP and EF and virulence; all germfree neonates experimentally infected with MRP+EF+ *S. suis* serotype 2 became sick, and 67% died with severe lesions of

meningitis and arthritis, compared to none of the piglets inoculated with MRP-EF-. Of those inoculated with MRP+EF- *S. suis*, only 5% became ill and the lesions developed were mild (Vecht et al., 1992). Similarly, strains of *S. suis* type 1 have either the phenotype MRP^{s} (reduced molecular mass of MRP) EF+, which is highly pathogenic, or a less virulent phenotype MRP-EF- (Smith et al., 1996). Other *S. suis* serotypes have also been demonstrated to possess MRP and EF (Wisselink et al., 2000; Berthelot-Hérault et al., 2000).

Despite this strong evidence that MRP and EF are virulence factors, experiments comparing a wild-type pathogenic *S. suis* strain with a isogenic mutant strain lacking these factors failed to confirm a role in disease. When inoculated into newborn germfree pigs, mutant strains of *S. suis* type 1 and 2 with impaired expression of MRP and EF, caused disease in the same way that the wild type strains of bacteria did. Taken together, these studies suggest that the factors MRP and EF are not an absolute requirement to cause the disease, but that they may be correlated with virulence. Another possibility is that the virulence of *S. suis* is multifactorial and a specific virulence factor can be replaced by a redundant or alternative factor (Smith et al., 1996).

Whereas the association of MRP and EF with virulence has been reported in the United States, Australia and several European countries, in Canada and France a different profile has been noticed (Gottschalk et al., 1998; Wisselink et al., 2000; Berthelot-Hérault et al., 2000). A study was performed with 98 Canadian field isolates of *S. suis* serotype 2 originating from diseased pigs and from tonsils of healthy pigs, and with 2 strains originating from affected humans. Most of the strains isolated from diseased pigs were MRP-EF- (72%) and only 1 strain was MRP+EF+; all strains isolated from healthy pigs were MRP-EF-; and the human strains were MRP-EF- (Gottschalk et al., 1998). In France, no clear association between MRP and EF and virulence was apparent, since the isolates from healthy animals were mostly (67%) MRP+EF- and the isolates from diseased animals were either MRP+EF- (46%) or MRP+EF+ (28%) (Berthelot-Hérault et al., 2000).

1.2.6.2 - Suilysin

Another widely studied virulence factor is a hemolysin, which was designated suilysin. This hemolysin is present in most of the serotypes of *S. suis*, including serotype 2 (Jacobs et al., 1994). Suilysin is a 54 kDa extracellular protein that belongs to a family of bacterial toxins known as thiol-activated toxins, which includes pneumolysin, streptolysin, listeriolysin, perfringolysin and alveolysin (Boulnois et al., 1991; Jacobs et al., 1994). These toxins are membrane-damaging proteins that cause cell lysis by interacting with membrane cholesterol. Some thiol-activated toxins also interfere with the immune response by activation of the classical pathway of complement in a non-immune fashion, i.e. without the need of antibody, by diverting and consuming the complement factors that would be used to clear the microorganism (Paton et al., 1984; Boulnois et al., 1991). Other mechanisms of interference with the immune response have been described, e.g. pneumolysin interferes with antimicrobial mechanisms by inhibition of chemotaxis, the respiratory burst, and lysosomal enzyme release from neutrophils (Paton and Ferrante, 1983) and macrophages (Nandoskar et al., 1986), and also is able to inhibit the lymphocyte response (Ferrante et al., 1984). The suilysin gene sequence and its translation product correspond to those of thiol-activated toxins. Phylogenetically, suilysin is most closely related to pneumolysin (Segers et al., 1998).

Although suilysin was present in 95% of *S. suis* field isolates from Europe and Asia (Segers et al., 1998), only 1 from a total of 98 field isolates of *S. suis* serotype 2 in Canada produced suilysin (Gottschalk et al., 1998). In a study involving 122 isolates of *S. suis* from diseased and healthy pigs in France, 37% of the isolates from diseased pigs were positive for suilysin, but some isolates from healthy animals also produced this protein (Berthelot-Hérault et al., 2000). In the United States, suilysin was present in 27% of field isolates analyzed and the correlation between virulence and suilysin activity was significant (Staats et al., 1998). Similar to MRP and EF factors, the association of suilysin with virulence varies according to the geographic area investigated. Consequently, involvement of other factors is likely required for virulence.

1.2.6.3- Surface Components

Surface components such as the capsule, fimbriae, adhesins and binding proteins have been investigated as potential virulence factors for *S. suis*.

1.2.6.3.1- Capsule

The capsule of bacteria is known to interfere in phagocytosis, and to permit a microorganism to evade the immune response of the host. Several studies have shown that the capsule of *S. suis* might play an important role in virulence. Encapsulated *S. suis* are more resistant to phagocytosis by neutrophils than non-encapsulated (Wibawan and Lammler, 1994); and while both encapsulated and non-encapsulated *S. suis* are engulfed by macrophages, only encapsulated have the ability to survive and multiply intracellularly (Brazeau et al., 1996). Additionally, non-encapsulated mutants were cleared more efficiently than the encapsulated wild-type strain by both murine and porcine macrophages, and these non-encapsulated mutants lacked virulence for mice and pigs (Charland et al., 1998). The thickness of the capsule of *S. suis* also correlates with the degree of virulence. Experimentally, virulent but not avirulent strains of *S. suis* grown in rats, developed a thicker capsule and marked resistance to *in vitro* phagocytosis by porcine neutrophils (Quessy et al., 1994).

In a study to evaluate the ability of *S. suis* isolates from diseased and from healthy pigs to adhere to frozen sections of porcine lung, adherence correlated with capsular thickness, suggesting involvement of the capsule in virulence; however, a non-encapsulated mutant maintained this ability, indicating that the capsule is not the only factor involved in adherence (Gottschalk et al., 1991). Despite the abundant evidence pointing to the capsule as an important virulence factor, most avirulent strains are encapsulated, indicating that additional virulence factors are essential (Gottschalk and Segura, 2000).

1.2.6.3.2 - Sialic Acid

Sialic acid, a sugar present in the capsule of *S. suis* and many pathogenic bacteria, has the ability to inhibit the activation of the alternative complement pathway, and consequently protects a sialylated microorganism from phagocytosis (Staats et al.,

1997). The role of sialic acid (N-acetyl neuramic acid) in *S. suis* serotype 2 virulence was evaluated. This sugar was found to be present in low concentration in both virulent and avirulent strains, a concentration one and a half to four times lower than in virulent strains from group B streptococci. In addition, neutralization of sialic acid did not affect virulence or susceptibility to phagocytosis of the strains tested. These findings suggest that sialic acid does not play a major role in the virulence of *S. suis* (Charland et al., 1996).

1.2.6.3.3- Fimbriae

Ultrastructurally surface fibrils or fimbriae are present on *S. suis* (Jacques et al., 1990). These structures are present in other bacterial species and mediate the attachment to host cells. While their function in *S. suis* is so far unknown, they probably have a role in colonization and cellular invasion.

1.2.6.3.4- Adhesins

Adhesins are microbial surface proteins with a high affinity for specific carbohydrates of glycoconjugates on cell surfaces and, in this way, can attach to and facilitate bacterial invasion of the host. The ability of bacteria to attach to and agglutinate erythrocytes *in vitro* is used as a tool for evaluation of the attachment mechanisms. Through agglutination of erythrocytes *in vitro*, it was determined that *S. suis* has an adhesin for a specific disaccharide, galactosyl-α1-4-galactose (Galα1-4Gal), which is present in the human blood group P. Galα1-4Gal is also expressed in many human and porcine tissues, and the binding of *S. suis* to sections of porcine pharyngeal epithelium was decreased by the addition of Galα1-4Gal, indicating that this sugar may function as a receptor for *S. suis* in the porcine pharyngeal epithelium (Haataja et al., 1993). The 18kDa adhesin that recognizes Galα1-4Gal has been purified and is present in all strains tested so far (Gottschalk and Segura, 2000). This adhesin is a potential target in the designing of drugs to inhibit the attachment of *S. suis* to its target cells (Haataja et al., 1994).

1.2.6.3.5 - Binding Proteins

Other potential virulence factors detected in *S. suis* are a 52 kDa immunoglobulin G (IgG)-binding protein and an albumin-binding protein. IgG-binding protein is reported in several species of *Streptococcus*, *Staphylococcus* and other bacterial genera (Serhir et al., 1993). This protein belongs to the family of heat shock proteins (Benkirane et al., 1998), also known as stress proteins. These are highly conserved and are present in different microorganisms, yeasts, plants and mammalian cells. Their production increases in response to different types of insults, such as increase in temperature, anoxia and reactive oxygen metabolites (Kaufmann, 1990).

IgG-binding protein has the ability to bind IgG by a non-immune mechanism, i.e. through the Fc portion (constant region of the immunoglobulin heavy chain) of an antibody. Thus, IgG-binding protein seems to allow a microorganism to evade the immune response either by interfering with opsonization and phagocytosis, or by complement consumption. IgG-binding protein is present in all 29 serotypes (1 to 28, and 1/2) of *S. suis* tested, including strains of *S. suis* serotype 2 isolated from clinically healthy animals. This protein in *S. suis*, as in other bacterial species, is associated with the cell surface but is also released in a soluble form during bacterial growth (Serhir et al., 1993). IgG-binding protein can also bind to human IgA (Serhir et al., 1995). If IgG-binding protein also binds to porcine IgA, it could possibly have a role in the colonisation and invasion of mucosal surfaces, such as the tonsil or respiratory tract, by *S. suis*. IgG-binding protein could allow *S. suis* to evade the immune exclusion mechanism performed by IgA at those surfaces.

A 39-kDa protein which binds to albumin, has been detected in virulent and avirulent isolates of *S. suis* serotype 2. The N-terminal sequence of this protein has 95.6% homology with a protein of group A streptococci, that binds to fibronectin, lysosyme, actin and myosin. The addition of albumin to cultures of virulent strains of *S. suis* enhances the virulence of these bacteria in mice, suggesting a role for the interaction of *S. suis* with albumin in the pathogenesis of *S. suis* infection. Since albumin-binding protein is also present in avirulent strains, still other factors may be involved in virulence. It has been suggested that binding of albumin may contribute to uptake of *S.*

suis and survival inside phagocytes (Quessy et al., 1997). Recently, *S. suis* serotype 2 mutant strains defective in expression of 39-kDa protein were generated. These mutants had reduced adherence to porcine tracheal rings and bovine embryonic tracheal cells when compared to the parent strain, suggesting that this protein may be involved in adherence and possibly play a role in the first steps of infection (Brassard et al., 2001).

Although all these many virulence factors above, namely MRP, EF, suilysin, capsule, sialic acid, fimbriae, Galα1-4Gal and binding proteins, are described for *S. suis*, to date none correlates well with the pathogenesis of the disease. Additionally, there are numerous conflicting results regarding virulence of specific strains, a possible consequence of the different criteria used for the designation of virulence and of differing experimental conditions. The strains used in the various experiments with *S. suis* are usually defined as virulent or avirulent based on factors such as the clinical status of the animal from which the strain was isolated, the presence of virulence factors, and the outcome of the experimental infection. However, this outcome depends on several factors, such as the immunologic status of the animals, the species of animal used as a model of disease, the presence of *S. suis* as a normal inhabitant of the upper respiratory tract before the experimental inoculation, the route of infection and size of the inoculum (Gottschalk et al., 1999a; Gottschalk and Segura, 2000).

Attempts have been made to immunize pigs with subunit vaccines containing suilysin (Jacobs et al., 1996) and EF (Wisselink et al., 2001); although protection was obtained, these virulence factors are not present in all strains of *S. suis*, limiting the use of such vaccines. Multiple virulence factors are likely involved in the progression of *S. suis* infection and disease and, in addition to that, factors unrelated to the pathogen, such as management, stress, the immune status of the animal and other infections, are very likely to influence the outcome of infection. All these aspects should be considered in the search for virulence factors and in the development of efficient vaccines.

1.2.7- Pathogenesis of *S. suis* Infection

Although numerous investigators have reported on potential virulence factors of *S. suis*, studies on the pathogenesis of the disease are still limited. *S. suis* is known to colonise the upper respiratory tract of pigs, especially the palatine tonsils, and it is

believed to gain access to other organs through the palatine tonsils, as demonstrated experimentally by oral infection of piglets with *S. suis*. Tonsillar invasion can occur without disease, as demonstrated by the isolation of *S. suis* from submandibular, retropharyngeal and parotid lymph nodes of experimentally infected animals that did not get sick (Williams et al., 1973).

Epithelial cell lines were used as models to study the interaction of *S. suis* and the epithelium to understand the initial steps of this infection. In a study using a human laryngeal epithelial cell line as a model for mucosal invasion, both pathogenic and non-pathogenic strains of *S. suis* had the ability to invade epithelial cells, but the former were more invasive and when present in high numbers, capable of cell lysis. A significant correlation between cell lysis and suilysin was observed in this study, suggesting that this virulence factor is important in epithelial invasion. It was postulated that when there is a reduction in the local immunity due to environmental stresses and viral infections, strains of *S. suis* that are more invasive could disseminate and cause systemic disease. Based on these findings, invasion and cell lysis seem to be possible mechanisms by which *S. suis* breaches the mucosa and disseminates to other tissues (Norton et al., 1999).

Using different cell lines derived from human lung and cervix, and from porcine and canine kidney, other authors were able to demonstrate epithelial cell adhesion by *S. suis*, but not invasion. It was suggested that suilysin-positive *S. suis* use adherence and cell lysis, as opposed to cellular invasion, to disseminate and cause disease (Lalonde et al., 2000). These results contrasted with the findings observed in the laryngeal epithelial cell line in which the bacteria invaded the epithelial cells. The differences in cell lines, *S. suis* strains and experimental techniques used could explain these contrasting results. Since not all virulent strains of *S. suis* possess suilysin, it is more likely that mechanisms other than cell lysis are involved in the dissemination from the palatine tonsils or upper respiratory tract to other tissues.

How *S. suis* disseminates from the palatine tonsils and upper respiratory tract to the bloodstream has still to be determined, but several mechanisms have been suggested. *S. suis* could be transported inside macrophages and neutrophils or could travel freely in

the bloodstream, to be taken up by monocytes later. The migration of *S. suis* inside monocytes was demonstrated experimentally following intravenous inoculation of pigs (Williams and Blakemore, 1990). Another suggested mechanism is that *S. suis* could travel in the bloodstream bound to but not inside macrophages (Gottschalk and Segura, 2000).

Macrophages seem to have an important role in the dissemination of *S. suis* and numerous studies have demonstrated the ability of virulent strains of *S. suis* to survive inside these cells and neutrophils (Williams, 1990; Wibawan and Lammler, 1994; Norton and Leigh, 1997). The mechanisms by which virulent strains of *S. suis* resist the killing stage of phagocytosis are unknown. Pathogenic and non-pathogenic strains of *S. suis* have similar levels of superoxide dismutase, an enzyme that protects bacteria against killing. Hence, this enzyme is unlikely involved in the pathogenesis of *S. suis* infection (Langford et al., 1991). Non-pathogenic *S. suis* is readily phagocytosed by porcine neutrophils, even in the absence of specific antibody and complement, while for phagocytosing pathogenic strains both are needed (Williams, 1990). Virulent strains of *S. suis* are able to survive and multiply in murine macrophages and also to cause degeneration of these cells; however, in the presence of complement and specific antibody there is bacterial killing (Williams, 1990).

Based on the evidence from *in vitro* studies that *S. suis* is able to survive inside murine macrophages (Williams, 1990), and considering that macrophages are likely involved in the dissemination of *S. suis* throughout the host (Williams and Blakemore, 1990), it is speculated that in natural *S. suis* infection of non-immune pigs that systemic dissemination is not controlled by hepatic and splenic tissue macrophages which typically are the most important cells responsible for clearance of circulating bacteria in non-immune animals. However, in the presence of specific antibody, limited progression of the infection is more likely (Williams, 1990). Other studies have demonstrated that the addition of serum either lacking specific antibody, or heat-inactivated, significantly increased uptake and intracellular killing of *S. suis*, illustrating that besides specific antibody and complement, unknown serum factors also have a role in the clearance of *S. suis* (Brazeau et al., 1996).

The pathogenesis of meningitis due to *S. suis* has been investigated (Williams and Blakemore, 1990). Based on the pattern of the central nervous system lesions, mostly confined to the choroid plexus and meninges, these authors suggested that *S. suis* gains access to the brain via the choroid plexus. Following intravenous inoculation of pigs with *S. suis* and examination of the central nervous system before the development of clinical signs of meningitis, it was observed that inflammation and associated extravascular, intracellular Gram positive organisms were present only in the choroid plexus, indicating that *S. suis* penetrated through this site. Additionally, *S. suis* was observed within monocytes from the blood and cerebrospinal fluid, implying that *S. suis* was carried by monocytes to the nervous tissue via choroid plexus (Williams and Blakemore, 1990).

The mechanisms by which *S. suis* invades the blood-brain-barrier (BBB) were investigated in a model using human brain microvascular endothelial cells (BMEC). *S. suis* did not invade BMEC but bound to these cells, and the suilysin-positive strains were cytotoxic for BMEC. It was proposed that *S. suis* binds to endothelial cells of the BBB and the secretion of suilysin leads to cell injury and increased permeability, allowing free or monocyte-associated bacteria to penetrate the nervous tissue. Alternatively, and a more likely mechanism involved in the suilysin-negative strains, the adherence of *S. suis* to endothelial cells could stimulate cytokine production, which would result in increased permeability and recruitment of inflammatory cells. Indeed, BMEC were demonstrated to release interleukin-6 when stimulated with *S. suis* (Charland et al., 2000; Gottschalk and Segura, 2000). Additionally, heat-killed *S. suis* induces the release of tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6) by murine macrophages, suggesting a possible role of these cytokines in the inflammation seen in *S. suis* meningitis (Segura et al., 1999).

1.2.8 - Palatine Tonsils as a Carrier Site for *S. suis*

S. suis is commonly part of the normal flora of porcine palatine tonsils (Devriese et al., 1994). Many serotypes of *S. suis*, both non-pathogenic and pathogenic strains, may be isolated from the palatine tonsils of healthy pigs. The rate of *S. suis* serotype 2 carrier animals is variable, with reports ranging from 20% to 90% in herds with a history

of disease and from 0 to 20% in herds without the disease (Clifton-Hadley et al., 1984). These wide variations in the carrier rate have been attributed to the number of animals sampled and to the sensitivity of the various tests used for *S. suis* detection (Robertson and Blackmore, 1989).

A positive correlation between the rate of carrier animals and disease status in a herd has been observed (Davies and Ossowicz, 1991), although one study found no correlation between the two (Clifton-Hadley et al., 1984). The association of carrier rate and disease is probably real, but seems to be dependent on the methods used for bacterial detection and on the number of animals tested in a herd (Davies and Ossowicz, 1991; Gottschalk et al., 1999b). Detection of *S. suis* in the palatine tonsils can be difficult due to the presence of other bacteria from the flora that can overgrow and obscure *S. suis*, and to the fact that *S. suis* reside deep in the tonsillar tissue, i.e. in the crypt lumen and epithelium (Williams et al., 1973; Arends et al., 1997). Several methods have been described to improve *S. suis* detection in the palatine tonsils (Davies and Ossowicz, 1991; Gottschalk et al., 1999b). The culture of frozen sections of tonsillar tissue, for example, was demonstrated to be more sensitive than the culture of swabs or deep scrapings of tonsils (Davies and Ossowicz, 1991). When these more reliable methods for *S. suis* isolation are used, a positive correlation between carrier rate and occurrence of clinical disease is observed (Davies and Ossowicz, 1991).

Since the carrier pig is considered the most important source of infection in a herd (Clifton-Hadley, 1984), considerable effort has been directed toward eliminating this state. Measures such as medicated early weaning, which has been successful in eliminating the carrier state of several other pathogens, and antibiotic medicated feed are inefficient in eliminating the *S. suis* carrier state (Clifton-Hadley and Alexander, 1980; Clifton-Hadley et al., 1984; Amass et al., 1996). Animals that recover from clinical *S. suis* disease may still carry *S. suis* in the tonsils, even in the presence of circulating antibodies (Staats et al., 1997). At 23 to 45 days after experimental intravenous or intranasal *S. suis* inoculation, the palatine tonsils were the only tissues from which *S. suis* was re-isolated in animals that recovered or did not get sick. Many of these animals

developed antibodies against *S. suis*, indicating that these antibodies did not result in the elimination of *S. suis* within the tonsil (Clifton-Hadley, 1984).

Given the importance of carrier pigs in the persistence of infection in a herd, their role in the transmission of *S. suis* to other animals, and the difficulties in the elimination of this state, studies on how *S. suis* interacts with cells in the palatine tonsils are essential in the development of methods to control this important pathogen of pigs.

1.3 -Tonsils as Part of the Mucosa-associated Lymphoid Tissue (MALT)

1.3.1 - MALT

1.3.1.1 - Structure and Function

The internal environment of an animal is in contact with the external environment through the skin and mucosal membranes. The large surface area of the intestine, for example, which is greater than 300 m^2 in the adult human, and constantly exposed to antigens and pathogens, illustrates the need for a specialized lymphoid tissue distributed at mucosal surfaces (McGhee et al., 1999). The mucosa-associated lymphoid tissue (MALT) fulfils this need; it is distributed widely at mucosal barriers between external and internal environments and limits the entrance of a large number of microorganisms and antigens. Components of MALT are found in alimentary tract, upper and lower respiratory tracts, conjunctiva of the eye, middle ear, minor salivary glands ducts, and nasolacrimal duct (Croitoru and Bienenstock, 1994; Gebert et al., 1996; Gebert and Pabst, 1999). MALT is composed of organized lymphoid tissue that is in intimate contact with mucosal membranes, e.g., tonsils, Peyer's patches (PP), appendix and bursa of Fabricius in birds (Gebert et al., 1996; Gebert and Pabst, 1999). Some authors also consider non-organized lymphoid elements in the mucosa and the mesenteric lymph nodes to be part of MALT. These non-organized elements include widespread intraepithelial and lamina propria lymphocytes, which are a common finding in the skin, urogenital tract and intestine (Croitoru and Bienenstock, 1994).

MALT consists of a specialized epithelium, named follicle-associated epithelium (FAE) or lymphoepithelium, which is highly infiltrated by leukocytes, and the lymphoid

tissue underneath. MALT is organized in B-cell follicles and interfollicular T cell areas that contain efferent lymphatic vessels and high endothelial venules (HEV) (Belz, 1998; Kelsall and Strober, 1999). There are no afferent lymphatics in MALT. Antigens enter this lymphoid tissue through the epithelial surface, and lymphocytes via the HEV. The organized MALT is the inductive site of the mucosal immune response, where antigens are sampled from the luminal surface by specialized epithelial cells (microfold/membranous cells - M cells) and delivered to antigen presenting-cells (APC), such as macrophages or dendritic cells (DCs), that process and present antigens to antigen-specific T and B lymphocytes. Memory B lymphocytes present in this specialized epithelium can operate as efficient APC and provide cognate stimulation of adjacent T lymphocytes, leading to a rapid and specific immune response in the epithelium. Alternatively, DCs can transport the antigen from the epithelium to the underlying lymphoid tissue and activate T and B lymphocytes. In the lymphoid follicle, B cell proliferation occurs in association with development of antibody affinity and isotype switching to mainly IgA production (McGhee et al., 1992; Kelsall and Strober, 1999; Brandtzaeg et al., 1999a).

IgA, the main immunoglobulin isotype at mucosal surfaces, has an important role in "immune exclusion" which is a non-inflammatory mechanism of surface protection impeding penetration of antigens and pathogens into the mucosa. Through immune exclusion, IgA prevents overt mucosal inflammation and immunopathologic processes, such as formation of immune complexes, which can result from ineffective attempts to eliminate pathogens that succeed in penetrating the mucosa. Unlike lymph nodes and spleen, the lymphoid follicles of MALT do not give rise to significant numbers of plasma cells locally. Instead, activated IgA^+ B lymphocytes and also T lymphocytes leave MALT through efferent lymphatics, reach the systemic circulation through the thoracic duct and migrate into the effector sites of the mucosal immune system, e.g. lamina propria of the respiratory or alimentary tracts. Under the influence of antigen, modulator T lymphocytes and cytokines, B lymphocytes in these sites clonally expand and differentiate into plasma cells secreting IgA antibodies. Activated T lymphocytes in addition to having a modulating function in these effector sites, can also act as cytotoxic

cells or remain dormant as memory cells (McGhee et al., 1992; Kelsall and Strober, 1999; Brandtzaeg et al., 1999a).

Specific terms denote MALT within the different systems in the body. The gut-associated lymphoid tissue (GALT) includes PP in small intestine, lymphoid aggregates in large intestine, solitary lymphoid nodules in the intestinal wall and the bursa of Fabricius in birds (Gebert et al., 1996; Gebert and Pabst, 1999). Bronchus-associated lymphoid tissue (BALT) is described in several species such as rats, rabbits, mice, sheep and chickens. Some species such as humans, cats and pigs have very little BALT (Pabst, 1996). The development of BALT is likely dependent on exposure to irritant substances that induce inflammation and to antigens, as illustrated by the higher number of these structures in the lungs of smokers (Richmond et al., 1993), and in pigs infected with *Mycoplasma hyopneumoniae* or *Actinobacillus pleuropneumoniae* (Pabst, 1996; Bienenstock et al., 1999). NALT is the acronym for the nasopharyngeal lymphoid tissue which includes the palatine and nasopharyngeal tonsils (McGhee et al., 1999) or for nasal-associated lymphoid tissue, a paired lymphoid organ situated in the nasal cavities of rats, mice, hamsters, rabbits and monkeys (Kuper et al., 1992; Gebert et al., 1996; Bienenstock et al., 1999). Other MALT components include DALT (duct-associated lymphoid tissue) found in the wall of minor salivary ducts of non-human primates and CALT (conjunctiva-associated lymphoid tissue) present in conjunctival epithelium, close to the nasolacrimal duct of rabbits, guinea pigs and sometimes humans (Gebert et al., 1996).

1.3.1.2 - The Common Mucosal Immune System

The different components of MALT are connected to each other in a way such that exposure to antigen at one mucosal surface can lead to dissemination of effector cells of an immune response to other mucosae. An immune response induced in the PP, for example, may be effective at distant sites such as the mammary gland. Indeed, a link between intestinal and mammary gland immune responses is illustrated by the finding of specific antibodies to enteric pathogenic and non-pathogenic microorganisms in colostrum and milk (Scicchitano et al., 1988). This led to the concept of a common mucosal immune system (CMIS), the features of which can be exploited in the

development of efficient methods of mucosal vaccination. Since the great majority of pathogens invade mucosal surfaces, a protective immune response at these sites is very important to prevent disease. IgA, the main immunoglobulin at mucosal surfaces, plays a major role in this protection by attaching to microorganisms and reducing their ability to attach to epithelium and penetrate the internal environment of the body (Mestecky et al., 1999). Mucosal IgA is mostly produced locally from plasma cells and only a small fraction is derived from serum. Hence, immune responses induced by parenteral vaccines are not as effective at reaching mucosal surfaces; however, if the individual being immunized parenterally has been previously exposed to the same antigen by mucosal route, an IgA response can be developed at the mucosal surfaces. This is well illustrated by the failure to induce efficient IgA production in salivary and milk secretions following parenteral immunization of lactating Swedish women with cholera vaccine; in contrast, the same immunization procedure in Pakistani women who had been naturally exposed to *Vibrio cholerae* previously, induced an efficient IgA response in both secretions (Mestecky, 1987).

The CMIS relies on the constant traffic of lymphocytes through the different components of MALT. In this way, inductive sites, such as PP and tonsils which are sites of antigen sampling, are constantly being supplied by a large repertoire of antigen specific lymphocytes, increasing the chances that naïve lymphocytes which are present in very low number, meet their cognate antigens. The lymphocyte traffic also provides a mechanism by which continuous surveillance against potential pathogens penetrating mucosae is accomplished by effector and memory lymphocytes, ensuring that all mucosal membranes are supplied with a large repertoire of specific antibody secreting cells. This constant migration of lymphocytes through the mucosal tissues is mediated by homing receptors. These are constitutive or inducible molecules present in the cytoplasmic membrane of lymphocytes and they interact with specific adhesion molecules, called vascular addressins, on the surface of endothelial cells of HEV in lymphoid tissues or in inductive sites of the immune response (Kraehenbuhl and Neutra, 1992; Brandtzaeg et al., 1999a).

The lymphocyte traffic throughout MALT components is tissue specific and shows a certain regionalization. In the GALT from humans and mice, for example, the endothelial cells of HEV and of the venules from the lamina propria of the other parts of the gut, express the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) which interacts with the homing receptor leukointegrin $\alpha_4\beta_7$ expressed on lymphocytes (Brandtzaeg et al., 1999b; Hathaway and Kraehenbuhl, 2000), whereas other tissues such as tonsils, lung and salivary glands, express these molecules in very low amounts. The lack of intestinal homing receptors in tonsillar lymphocytes seems to explain the preferential migration of effector lymphocytes from NALT to upper respiratory and urogenital tracts (Brandtzaeg et al., 1999b). Consequently, antigenic challenge in one inductive site of MALT leads to an immune response in a physiologically related effector site, e.g., while oral immunization results in a response preferentially in the intestine, nasal immunization induces an immune response in salivary glands, respiratory and genital tracts (Russel et al., 1996).

1.3.1.3 – M Cells

1.3.1.3.1 – M Cell Structure

M cells are specialized epithelial cells, found exclusively in the lymphoepithelium of MALT, that sample antigens from the lumen to the underlying lymphoid tissue. These cells have been largely described in the PP and other lymphoid aggregates of the intestine, such as in the appendix and isolated lymphoid follicles of rabbits (Jepson et al., 1993a), mice (Kohbata et al., 1986; Clark et al., 1994), rats (Owen et al., 1986a; Childers et al., 1990), cattle (Torres-Medina, 1981; Liebler et al., 1988; Liebler et al., 1991), pigs (Chu et al., 1979; Torres-Medina, 1981; Morfitt and Pohlenz, 1989), dogs (HogenEsch and Felsburg, 1990), horses (Gebert et al., 1996), guinea pigs (Marcial and Madara, 1986) and humans (Amerongen et al., 1991; Finzi et al., 1993; Kato and Owen, 1999). The presence of M cells in other components of MALT is not well defined and has not been widely investigated. Some authors believe that these cells are present only in MALT with simple epithelium such as GALT and BALT, whereas in MALT with stratified epithelium, e.g. palatine tonsil, other cells perform M cell

functions (Neutra et al., 1996b). The presence and number of M cells seem to vary according to the species and among different tissues of the same species. Approximately 50% of the epithelial cells in the FAE of PP in the rabbit are M cells, while in mice they comprise only 10% (Ermak et al., 1995). M cells are present in large number in the palatine tonsils of rabbits (Olah and Everett, 1975; Gebert, 1997a), but very few are thought to exist in the palatine tonsils of pigs (Belz and Heath, 1996) and dogs (Belz and Heath, 1995). In dogs there are more M cells in the ileal PP than in the duodenual and jejunual PP (HogenEsch and Felsburg, 1990). A similar situation is seen in cattle, where the epithelium of entire domes of the ileal PP consists of a sheet of M cells, while in the jejunal PP they are infrequent (Landsverk, 1988).

M cells are described in the nasopharyngeal tonsils of cattle (Schuh and Oliphant, 1992), sheep (Chen et al., 1991; Stanley et al., 2001), horses (Kumar et al., 2001), and humans (Claeys et al., 1996). However it is still controversial whether or not human palatine tonsils have M cells. Although they have been described in few studies (Howie, 1980; Finzi et al., 1993), further investigations have not confirmed their presence (Perry et al., 1988; Perry, 1994; Claeys et al., 1996). Cells with features of M cells are described in BALT of rabbits, but they are absent in the rat (Gebert and Pabst, 1999). It is not clear whether M cells exist in CALT; although they have been described morphologically in rabbits, further morphological and immunohistochemical studies have not confirmed their presence (Gebert and Pabst, 1999). Cells with morphological features of M cells were described in the CALT of guinea-pigs, however, whether these cells represent a separate cell type that transports antigens is still questionable (Gebert and Pabst, 1999). M cells have also been described in the bursa of Fabricius of chickens and turkeys (Olah and Glick, 1992).

Studies of M cells are hampered by the morphological diversity that these cells can assume, not only among different species but also different locations of MALT within the same species. Since M cells cannot be discerned by light microscopy, but their presence inferred only by their close association with lymphoid cells, they are usually identified morphologically by transmission or scanning electron microscopy. In the PP, where these cells were first described, the apical cytoplasmic membrane of M

cells is markedly different from that of the adjacent absorptive cells. While the apical cytoplasmic membrane of enterocytes has homogeneous and tightly packed microvilli (brush border) that increase the superficial area in contact with the luminal contents, M cells lack this surface specialization, and instead the apical membrane can assume different shapes. The apical processes of M cells differ in length and diameter from enterocyte microvilli; they are thicker, more widely spaced and irregularly distributed, can form long processes, or microfolds, or sometimes the apical membrane is devoid of any specialization. The apical specializations have generally no terminal web as seen in enterocytes, or when present, they are less developed. The cytoplasm is less electron-dense than in the adjacent enterocytes. The apical portion of the cytoplasm is rich in vesicles, tubular structures and mitochondria, but shows a paucity of lysosomes (Chu et al., 1979; Torres-Medina, 1981; Morfitt and Pohlenz, 1989; HogenEsch and Felsburg, 1990; Liebler et al., 1991; Gebert et al., 1996; Kato and Owen, 1999). The layer of glycocalyx and mucus on the apical surface of the M cells is sparser than on adjacent enterocytes (Kato and Owen, 1999). Other features of M cells include a prominent Golgi complex, rough endoplasmic reticulum (RER) and clumps of microfibrils dispersed in the cytoplasm (Owen and Jones, 1974). The basolateral cytoplasmic membrane of the M cells is usually indented by lymphocytes, macrophages, or less often neutrophils and plasma cells. By this means, M cells form a membrane-like structure separating lymphoid cells in the epithelial cell layer from the lumen, a feature which led to the term membranous (M) cell (Owen, 1977). In the same way as classical epithelial cells, M cells are connected to adjacent epithelial cells by lateral interdigitations and desmosomes. M cells can extend cytoplasmic processes into the underlying lymphoid tissue, and the basal lamina subjacent to them is discontinuous, facilitating the traffic of lymphoid cells (Neutra et al., 1996b).

1.3.1.3.2 – M Cell Function

Under normal conditions the intestinal epithelium is impermeable to macromolecules and particles, and only small molecules such as amino acids and saccharides can cross this barrier. The immune system however, needs to make contact with potential pathogens and food antigens in order to develop immunity and/or

tolerance. M cells fulfill this need by continuously sampling antigens, particulate material and pathogens (Gebert et al., 1996). The morphological features of the M cells illustrate that they are well suited for transport of antigens and particles from the lumen to the underlying lymphoid tissue. The smaller amount of mucus and glycocalyx seems to facilitate contact between antigens and cytoplasmic membrane domains in M cells. The absence of a rigid brush border allows more movement of the apical cytoplasmic membrane for antigen uptake. Additionally, the actin-associated protein villin that is confined to the core of microvilli in enterocytes is diffusely distributed in the cytoplasm of M cells, also reflecting their ability to take up and transport particles across the cytoplasm (Neutra et al., 1996b). The system of vesicles, which are mostly concentrated in the apical cytoplasm and at sites of close contact with lymphoid cells, also suggests a role in transport, such as of antigens and particulate material. The thin cytoplasmic processes of the M cell forming a pocket around lymphocytes facilitate a rapid transport of substances between these cells. Lymphocytes within these pockets can be as close as 0.3 μm to the lumen, and present features of active cells, such as a prominent Golgi apparatus and RER (Owen and Jones, 1974).

Many studies have demonstrated the capacity of M cells for uptake and transport of particulate and soluble substances to the lymphoid cells that lie in close contact with them. These include horseradish peroxidase (Owen, 1977), microspheres of copolymer (Ermak et al., 1995), latex (Landsverk, 1988; Pappo and Ermak, 1989; Jepson et al., 1993c) and polystyrene (Pappo et al., 1991; Jepson et al., 1993b), liposomes (Childers et al., 1990), lectins (Gebert and Hach, 1993) and IgA antibody (Weltzin et al., 1989). Many pathogens have been shown to be taken up and transported by M cells to lymphoid cells. These include: poliovirus in people (Sicinski et al., 1990), human immunodeficiency virus -1 (HIV-1) in mice and rabbits (Amerongen et al., 1991), reovirus in mice (Wolf et al., 1983), *Yersinia enterocolitica* in mice (Grutzkau et al., 1990), *Shigella flexneri* in rabbits (Wassef et al., 1989), *Salmonella typhi* in mice (Kohbata et al., 1986), *Escherichia coli* in rabbits (Inman and Cantey, 1983), *Vibrio cholerae* in rabbits and people (Owen et al., 1986b; Gebert et al., 1996), *Campylobacter jejuni* in rabbits (Walker et al., 1988), *Mycobacterium paratuberculosis* and *Brucella*

abortus in cattle (Gebert et al., 1996), *Giardia muris* in mice (Gebert et al., 1996) and *Cryptosporidium* sp in guinea pigs (Marcial and Madara, 1986). Certain pathogens, e.g., *Salmonella typhi*, are believed to exploit the M cell as a way of invading the host (Kohbata et al., 1986), while others like *Cryptosporidium* sp. and *Vibrio cholerae*, where the pathogenesis is independent of cellular invasion, are sampled by M cells for the development of an immune response (Marcial and Madara, 1986; Neutra et al., 1996a). These features of the M cells make them a valuable tool in the development of mucosal vaccines and for drug delivery.

In rabbits and mice, luminal secretory IgA, regardless of antigen specificity, and IgA-antigen complexes bind selectively to the apical membrane of intestinal M cells and are transported to the pocket containing lymphoid cells. Antigens coupled to IgA enhance the mucosal immune response, as demonstrated in mice that were intra-rectally inoculated with liposome-encapsulated ferritin. When liposome-encapsulated ferritin was coated with IgA, there was an increase in liposome uptake and in the secretory IgA anti-ferritin response in the rectum and colon (Zhou et al., 1995). This could represent a mechanism by which M cells boost the mucosal immunity against reinvading pathogens, i.e., if IgA against a particular pathogen is already available from a previous infection, there is an enhanced uptake of the pathogen by M cells and therefore a faster secondary immune response (Amerongen et al., 1992; Zhou et al., 1995; Neutra, 1998).

The absence or very small number of lysosomes in M cells coupled with the delivery of intact microorganisms to the pocket containing lymphoid cells (Liebler et al., 1988; Amerongen et al., 1991; Neutra et al., 1996a) suggests that these cells act only in transcellular transport, i.e. trancytosis, and do not digest or process ingested substances (Allan et al., 1993). However, M cells in humans and rats carry the major histocompatibility complex class II (MHC-II) molecule and the enzyme cathepsin E. This enzyme is a protease present in APCs and believed to be involved in the degradation of ingested proteins for antigen presentation. The presence of these molecules in M cells of humans and rats suggests an ability to digest and present antigens to the lymphoid cells with which they are in close contact (Finzi et al., 1993). Rabbit M cells are able to secrete interleukin-1 (IL-1) in response to bacterial

lipopolysaccharide (LPS) *in vitro*, indicating that these cells, in addition to transportation of antigens, may also send signals that activate the immune response (Pappo and Mahlman, 1993).

1.3.1.3.3 - Markers for M Cells

A major disadvantage of using only morphological criteria to identify M cells is that their morphology is extremely variable among species and among different tissues in the same species. Additionally, in the intestine of pigs, cells with morphology not compatible with M cells have been demonstrated to take up yeasts (Gebert et al., 1994). Thus, numerous studies have attempted to identify specific markers for these cells, but unfortunately, there is still no universal M cell marker that can be used in the different tissues and species (Gebert and Pabst, 1999).

The first method used to detect M cells was histochemistry using alkaline phosphatase as a negative marker. Enterocytes have high levels of digestive enzymes in the apical membrane, one of them being alkaline phosphatase, but in M cells this enzyme is markedly less active. Reduced phosphatase activity has been used in several species for M cell identification; however, it is not reliable in rabbits because of large variation in the levels of this enzyme in enterocytes (Jepson et al., 1993a). Additionally, goblet cells are also negative for alkaline phosphatase (Gebert et al., 1996; Gebert, 1997b).

Since structure and function are distinct in M cells and enterocytes, and cellular shape and function are related to the cytoskeleton, intermediate filaments, which form part of cytoskeleton, have been investigated in M cells. The epithelial nature of M cells is confirmed by the presence of cytokeratins (CKs), the intermediate filaments that are found in epithelial cells (Gebert et al., 1996). Rabbit M cells, in addition to CKs, also possess the intermediate filament vimentin which is considered an M cell marker in this species. In rabbits, vimentin is present not only in M cells of PP and the appendix but also in M cells of the palatine tonsils and BALT, however, vimentin is not present in M cells of humans, mice, rats, pigs, cat and guinea pig (Jepson et al., 1992; Gebert et al., 1994; Gebert et al., 1995). Vimentin is the intermediate filament found in the cytoplasm of mesenchymal cells, such as macrophages, fibroblasts, muscle and endothelial cells, and also in atypical epithelial cells such as from tumours and cell cultures. The general

function of vimentin as an intermediate filament, as well as its function in the M cell of rabbits, is unknown. It is believed that it might be related to cellular shape and biomechanical properties (Jepson et al., 1992; Gebert et al., 1995).

Cells of simple epithelium normally express CK-8, 18 and 19. In the PP of pigs, the amount of CK-18 in M cells is higher than in the adjacent enterocytes, whereas CK-19 is lower in M cells and higher in enterocytes. CK-18 is probably associated with M cell function: a ring of fibrils, positively stained for this marker, was observed to encircle particles that were taken up by M cells. CK-18 is considered a marker for porcine intestinal M cells, but is not applicable to other species, since this pattern of CK expression was not observed in cats, guinea-pigs, rabbits and rats (Gebert et al., 1994). A disadvantage of CK-18 as a marker for M cells is that other intestinal cells such as goblet and enteroendocrine cells also express high levels of this protein (Gebert, 1997b).

Various carbohydrate binding proteins called lectins interact with different types of glycoconjugates (glycolipids or glycoproteins) and are extensively used to recognize specific determinants on the cell surface. Lectins occur widely in nature and are found on the cell surfaces in animals, vegetables and microorganisms. Many of the receptors for microorganisms that are present on the outer membrane of the host cells are glycoconjugates that bind specifically to the microorganism lectin (Sharon, 1996). Surface glycoconjugates on M cells have been largely investigated for the presence of a specific pattern that could be correlated with the selective binding of pathogens. In mice, the fucose-specific lectin, *Ulex europaeus* agglutinin type-I (UEA-I), is a marker for M cells of the small intestine, while in the large intestine galactose-specific lectins specifically recognize M cells (Clark et al., 1994; Giannasca et al., 1994). In rabbits, the lectin peanut-agglutinin (PNA) is a marker for M cells in PP and the appendix (Jepson et al., 1993a), and fucose and N-acetylgalactosamine binding lectins specifically react with the M cells of the cecal lymphoid tissue (Gebert and Hach, 1993). No specific lectin-binding pattern was observed in M cells of cats, rats, guinea-pigs (Gebert et al., 1996) and people (Kato and Owen, 1999).

Different sub-patterns of glycoconjugates have been observed in the intestinal M cells of mice and rabbits, indicating that there are different subpopulations of these cells.

In the intestine, the epithelium on the villi and on the follicles of the PP originates from the different intestinal crypts that are situated around those villi and follicles. The individual crypt gives rise to only one distinct subpopulation of M cells in the FAE, i.e. all the M cells arising from one crypt have homogeneous surface properties. This finding was suggested as a way of expanding the ability of M cells to recognize different pathogens and antigens. Supporting this hypothesis is the observation that *Salmonella typhimurium* adheres in a non-uniform manner to the M cell in mice (Giannasca et al., 1994; Gebert and Posselt, 1997).

Monoclonal antibodies specific for rabbit M cells were developed from M cell-enriched fractions of PP and appendix (Pappo, 1989). The epitopes recognized by these antibodies are unknown and none of these antibodies reacts with M cells of other species (Gebert, 1997b).

1.3.1.3.4 – M Cell Origin

The epithelial nature of the M cell is evident from the presence of desmosomes and tight junctions and from the expression of cytokeratins. M cells, in the same way as enterocytes, are derived from stem cells of the intestinal crypts; however, morphological features of the M cells are evident only on the epithelium overlying the lymphoid follicles. Therefore, there are two opposing theories on the origin of M cells: 1) the development of M cells is predetermined at the point of origin in the crypt; 2) M cells are derived from mature enterocytes in the FAE. Evidence exists to support both.

Studies in mice (Giannasca et al., 1994) and rabbits (Jepson et al., 1993a; Gebert and Posselt, 1997) demonstrated that cells in the intestinal crypts neighbouring the follicle do not have morphological features of M cells. Some of these immature cells, however, have the same lectin-binding pattern as mature M cells (Jepson et al., 1993a; Giannasca et al., 1994; Gebert and Posselt, 1997). In rabbits, these immature cells also react with the M cell marker vimentin (Jepson et al., 1993a) and the M cell antibody (Pappo, 1989), supporting the theory that some of the undifferentiated crypt cells are predetermined to be M cells.

On the other hand, the restriction of M cells to MALT suggests that these cells differentiate in response to cells or secreted factors from these lymphoid tissues (Neutra,

1998). Several pieces of evidence support this hypothesis. M cells in the PP of rabbits increase in number after bacterial inoculation, seen as early as 30 minutes after challenge (Borghesi et al., 1996). This argues against the theory of M cell determination in the crypts, since a cell requires more than 24h to migrate from the crypt to the FAE (Kato and Owen, 1999). Internalized bacteria, infiltration of lymphoid cells and release of soluble factors from lymphoid cells have all been suggested as being responsible for M cell differentiation (Borghesi et al., 1996). Additional evidence supporting the concept that bacteria and/or lymphoid cells influence M cell differentiation was obtained from one study using severe combined immunodeficient (SCID) mice which lack PP. In this study SCID mice were divided in 2 groups: one inoculated with lymphocytes derived from BALB/c mice that were infected with *Salmonella typhimurium*, and the other with lymphocytes of BALB/c mice that were uninfected. SCID mice from both groups developed PP with the presence of M cells in the FAE. The number of M cells was higher in mice that received *S. typhimurium* primed lymphocytes and was proportional to the number of lymphocytes inoculated. Therefore the development of M cells was likely in response to inoculated lymphocytes and/or their soluble factors, i.e. cytokines (Savidge and Smith, 1995).

Further important evidence in favour of the development of M cells from mature enterocytes came from studies using a coculture of lymphocytes from PP and an enterocyte Caco-2 cell line. In two days the differentiated enterocytes transformed into cells with morphology and functional capability of M cells. The digestive enzyme sucrase-isomaltase, which is normally present in differentiated enterocytes, decreased in the Caco-2 cells cocultured with lymphocytes. Villin, a protein associated with the brush border of enterocytes, was diffusely distributed throughout the cytoplasm, the pattern normally seen in M cells (Kerneis et al., 1997). These same authors were able to convert enterocytes into M cells in mice, by the inoculation of PP lymphocytes in areas of the intestine that normally lack these lymphoid tissues. By 9 days post-inoculation PP-like structures with M cells developed at the inoculation site, and these M cells possessed the murine M cell marker UEA-I (Kerneis et al., 1997).

The differentiation pathway of the intestinal M cells of rabbits was evaluated using two different lectins that label the different M cell subpopulations in this species. It was noticed that there are cells in the intestinal crypts that bear the lectin pattern of M cells on the outer membrane, suggesting that they are immature M cells. However, although this lectin pattern is present on the cell surface of the crypt epithelial cells that are migrating to the adjacent FAE and adjacent villi M cells develop not on the villi, but only in the FAE. These findings lead to a unifying theory on the origin of M cells: M cell origin is predetermined in the crypt, but maturation into M cells can only occur under the influence of the microenvironment of the lymphoid follicle (Gebert and Posselt, 1997).

1.3.2 – Tonsils of the Upper Aerodigestive Tract

1.3.2.1- Anatomy of the Tonsils in Humans and Domestic Mammals

The tonsils are organized mucosal lymphoid tissues located in the pharynx, close to the entrances of the upper aerodigestive tract. In humans these pharyngeal lymphoid tissues are arranged as a ring, called Waldeyer's ring, which comprises the nasopharyngeal tonsil (adenoid) in the roof of the pharynx, the paired tubal tonsils at the pharyngeal openings of the Eustachian tubes, the paired palatine tonsils in the oropharynx, and the lingual tonsil in the posterior third of the tongue (Perry and Whyte, 1998).

In domestic animals, while the pharyngeal lymphoid tissues are also organized as a ring, the components vary depending on the species. The nasopharyngeal tonsil is present in all domestic mammals. In carnivores the nasopharyngeal tonsil is flat, without crypts, and is located in the roof of the nasopharynx, between the openings of the auditory tubes. In horses the nasopharyngeal tonsil has crypts and is situated similarly to carnivores. The nasopharyngeal tonsil of ruminants and pigs is raised with a rugose surface and is located at the caudal end of the pharyngeal septum. While crypts are present in the nasopharyngeal tonsil of pigs, ruminants lack them (Nickel et al., 1979).

The tubal tonsils are absent in carnivores, but present in the other domestic mammals. Crypts are present in the tubal tonsils of pigs, but absent in ruminants. In

horses the tubal tonsils do not form a distinct organ, but consist of small non-organized accumulations of lymphocytes and isolated lymphoid nodules (Nickel et al., 1979).

The palatine tonsils of carnivores are located on the lateral wall of the oropharynx, within the tonsillar fossa, a fold that covers each palatine tonsil. The palatine tonsil of carnivores lacks crypts. In ruminants the palatine tonsils are located on the dorso-lateral wall of the oropharynx within a fossula, and have crypts. While in small ruminants the palatine tonsils are located superficially in the fossulae, in cattle they are situated more deeply, within the musculature and connective tissue of the pharyngeal dorso-lateral wall. The palatine tonsils of horses are located on the oropharynx, lateral to the glosso-epiglotic fold, and extending caudally to the base of the epiglottis and they have crypts (Nickel et al., 1979). In pigs, the palatine tonsils, also called the tonsils of the soft palate, are located on each side of the medial pharyngeal septum of the ventral surface of the soft palate; they are large, flat, extending to the free border of the soft palate, and the surface is pitted with many small openings that correspond to the entrance of the crypts (Belz and Heath, 1996).

The lingual tonsils are located in the mucosa of the root of the tongue. In carnivores the lingual tonsils comprise non-organized lymphocytic infiltrates and isolated lymphoid nodules. In pigs, ruminants and horses the lingual tonsils consist of isolated lymphoid follicles associated with a crypt. In pigs they are few and can also be found within the lingual papillae, while in ruminants and horses they are numerous (Nickel et al., 1979).

1.3.2.2 – Histology of the Palatine Tonsil

The palatine tonsils comprise of an organized lymphoid tissue overlain by an epithelium, as an organ of MALT, and are partially encapsulated by connective tissue. The lymphoid tissue contains primary and mostly secondary follicles which consist mainly of B lymphocytes and surrounded by a perifollicular or extrafollicular area populated mainly by T lymphocytes, but also by macrophages and dendritic cells. In the perifollicular area, HEV and lymphatic vessels are found (Belz, 1998a; Belz, 1998b; Bernstein et al., 1999). The oral epithelium covering the palatine tonsil is stratified, squamous, and nonkeratinized and generally invaginates deeply into the lymphoid tissue

to form crypts. Crypts vary in number depending on the species, and are absent in carnivores. While in rabbits the palatine tonsil is monocryptic (Olah et al., 1972), in humans there are between 10 to 20 crypts per tonsil (Perry and Whyte, 1998), and pigs have between 160 to 190 crypts (Belz and Heath, 1996). Tonsillar crypts ramify and can connect to each other. The presence of crypts greatly increases the epithelial surface of the tonsil. It is estimated that while the epithelium covering the oral surface of the human palatine tonsil is on average 45 cm^2, the total area of the crypt epithelium is 295 cm^2 (Perry, 1994; Perry and Whyte, 1998). Crypts overlie one or more lymphoid follicles.

The oral superficial epithelium of the palatine tonsil is similar to the oropharyngeal epithelium elsewhere and is underlaid by a dense band of connective tissue, the lamina propria (Ham and Cormarck, 1979b). The epithelium lining the crypts is highly infiltrated by lymphoid cells and is in close contact with the lymphoid tissue underneath, separated from it only by the basement membrane which is often discontinuous. In these areas the epithelial cells are distorted due to non-epithelial cell infiltration, creating a lattice appearance giving rise to the name "reticulated epithelium". Reticulation varies both in extent and in number of infiltrating cells so that either epithelial or non-epithelial cells may predominate. Cells that are found in these reticulated areas are small lymphocytes, lymphoblasts, plasma cells, macrophages, dendritic cells, and less often polymorphonuclear leukocytes and mast cells. Epithelial cells in reticulated areas contain less glycogen than cells from non-reticulated areas, as demonstrated by periodic acid-Schiff (PAS) staining (Yamanaka et al., 1983; Perry, 1994; Belz and Heath, 1996; Perry and Whyte, 1998).

1.3.2.3 – Ultrastructure of the Crypt Epithelium of the Palatine Tonsil

Scanning electron microscopy (SEM) is a very useful tool to evaluate the superficial appearance of cells. The luminal surface of the crypt epithelium is mostly smooth, with projecting dome-shaped areas that correspond to the lymphoid follicles underneath. The epithelial cells lining the crypt are flat and polygonal, like squames, and their surface has fine microplications (Howie, 1980; Perry et al., 1988; Perry, 1994; Belz and Heath, 1995; Belz and Heath, 1996). Occasionally, cells with a different

superficial morphology are recessed between these squame-like cells in the reticulated areas of the epithelium, and are covered with either small or long irregular microvilli, or microfolds. These cells have been described in humans, dogs and pigs, and interpreted to be M cells (Howie, 1980; Belz and Heath, 1995; Belz and Heath, 1996). Such cells were not identified in other investigations on the crypt epithelium of humans (Perry et al., 1988; Perry, 1994). In rabbits, most of the crypt epithelium is reticulated, and round, raised cells that are rich in microvilli cover 30% to 50% of surface of this epithelium (Olah and Everett, 1975; Gebert, 1995). Occasionally, the surface of the crypt epithelium is disrupted, exposing non-epithelial cells (Perry et al., 1988; Belz and Heath, 1995). In cross-section the reticulated areas of the crypt epithelium consist of epithelial cells and their thin cytoplasmic processes, forming a network of wide interconnecting spaces that are filled with lymphoid cells (Howie, 1980).

Transmission electron microscopy (TEM) allows the evaluation of structural details of the intracellular environment, i.e. organelles and cytoskeleton, and relationships among cells. The crypt epithelium is stratified, as in humans (Perry, 1994) and pigs (Belz and Heath, 1996), and typical for a stratified epithelium (Ham and Cormarck, 1979b), comprises of basal, intermediate and superficial cell layers. The cells of the basal layer are connected to the basal lamina through hemidesmosomes, and to each other by desmosomes. The basal cells are cuboidal or cylindrical, have a large nucleus, are rich in free ribosomes, and have minimal RER and Golgi apparatus. Tonofilaments form intracytoplasmic bundles that are mainly arranged parallel to the long axis of the cell (Perry, 1994; Belz and Heath, 1996). All these features are found in a typical stratified epithelium (Farbman, 1988). In the crypts however, the basal lamina is frequently disrupted by non-epithelial cells infiltrating into the epithelium. The epithelial cells of the intermediate layer may be polygonal, elongated, star-shaped or irregular in shape, varying according to the degree of reticulation. Their cytoplasm is rich in tonofilaments bundles, ribosomes and membrane-bound vesicles. Little Golgi apparatus is seen in intermediate cells (Perry, 1994). In the superficial layer different morphological types of epithelial cells are observed. Some cells are flattened (squamous) and arranged in several layers, as a typical stratified squamous epithelium.

The cytoplasm of these squamous cells is usually rich in filaments that are aligned parallel to the surface, and the nucleus of the cells of the uppermost layers is pyknotic. Cytoplasmic electron density varies from light to dark in different cells. In other areas, there is only one layer of superficial epithelial cells overlying non-epithelial cells. These superficial epithelial cells bulge into the lumen, have an intact nucleus, are rich in organelles (RER, Golgi, and free ribosomes) and have numerous vesicles that are located mainly in the apical cytoplasm. The apical cytoplasmic membrane has short microvilli (Perry, 1994; Belz and Heath, 1996). The ultrastructure of the palatine tonsil crypt epithelium has been compared to the skin of foetus (periderm) in which the superficial epithelial cells also are rich in organelles and vesicles. The foetal periderm is normally exposed to a hydrophilic environment and acts not only as a passive protective layer, but also in absorptive and secretory functions (Perry, 1994).

The presence of M cells in the palatine tonsil is a subject of controversy, as mentioned earlier. Except for rabbits, these cells are considered difficult to detect and are identified by the close contact with non-epithelial cells forming thin cytoplasmic processes around them, the presence of irregular microvilli or microfolds on the apical cytoplasmic membrane, cytoplasm rich in organelles (mitochondria and Golgi apparatus) and vesicles located mainly at the apical portion of the cytoplasm, and by a scarcity of filaments. M cells are connected to other epithelial cells by desmosomes (Olah and Everett, 1975; Howie, 1980; Belz and Heath, 1995; Gebert, 1995; Belz and Heath, 1996). These features described for M cells are very similar to some crypt epithelial cells described by Perry, who did not identify M cells in the palatine tonsils of humans. Instead, this author compared the crypt epithelium with the fetal periderm, as described above (Perry, 1994). Hence, Perry probably was describing the same type of cell as described by other authors as M cells (Olah and Everett, 1975; Howie, 1980; Belz and Heath, 1995; Belz and Heath, 1996).

Since the structure of M cells is known to differ not only among different species, but also among different tissues within the same species, morphological identification alone is not considered sufficient for the detection of these cells. The use of specific markers and functional studies in association with morphology are necessary

(Gebert and Pabst, 1999), however, except for rabbits, such markers are not available, and only in this species have studies associating morphology and function of the crypt epithelium of the palatine tonsil been performed (Gebert, 1995).

1.3.2.4 – Markers for the Specialized Epithelium of the Crypt Palatine Tonsils

While the epithelial cells of the superficial epithelium of the palatine tonsils have a pattern of progressive differentiation from basal cuboidal cells to superficial squamous cells, similar to the epidermis (Ham and Cormarck, 1979a), the epithelial cells of the reticulated areas of palatine tonsil crypt epithelium usually lack this pattern. In epithelia in general, the expression of CKs changes according to the type of epithelium and to the stage of cell differentiation. While cells of simple epithelia express CK- 8 and -18, in stratified squamous epithelia, such as oral mucosa, these CKs are present only in basal cells (Moll et al., 1982; Sato et al., 1990). Hence, CK expression by the crypt epithelium has been investigated to look for any specific pattern in this epithelium. In the palatine tonsils of humans, the epithelium of the surface has the same CK pattern as the oral epithelium, i.e. CK- 8 and -18 are restricted to the basal layer. In contrast, the reticulated areas of the crypt epithelium have the same pattern of CK expression as the cells of the basal layer, suggesting that the infiltration of lymphocytes induces a change in the CK expression in the epithelial cells and consequently a change in cell shape (Sato et al., 1988; Sato et al., 1990; Wilson et al., 1998). It was speculated that these cells might be M cells, but some of the features of M cells, such as cytoplasm rich in vesicles, were lacking and the cells were located not only at the luminal surface, but also in the deeper layers of the epithelium (Sato et al., 1990). Similarly, in the palatine tonsils of pigs, CK-14 which is present only in the basal cells of the oral surface of the palatine tonsils, is also expressed by suprabasal cells of the reticulated areas of the crypt; CK-13 on the other hand, present in the cells of suprabasal layers of the stratified epithelium, is absent in the reticulated areas; and CK-18, considered a marker for porcine intestinal M cells (Gebert, 1994), is not present in the crypt epithelium of the palatine tonsils of pigs (D. M. Middleton, unpublished results).

In rabbits, antibodies to vimentin label epithelial cells in the reticulated areas of the crypt epithelium. These vimentin positive cells have been interpreted to be M cells,

comparable to the gut M cells, and comprise between 30% and 75% of the epithelial cells that reach the crypt luminal surface (Gebert et al., 1995). Using an ultrastructural immunohistochemistry (immunogold) technique, it was confirmed that the vimentin positive cells in the palatine tonsils of rabbits are morphologically compatible with M cells. Additionally, these cells with M cell morphology were shown by electron microscopy to transport horseradish peroxidase from the crypt lumen to the subepithelial lymphoid tissue (Gebert, 1995).

The lectin-binding pattern of the crypt epithelium of the palatine tonsils was investigated in humans (Wilson et al., 1998) and in rabbits (Gebert, 1996; Gebert, 1997a). In humans, the glycoconjugate pattern on the crypt epithelium is different from that seen in the oral surface (Wilson et al., 1998). In rabbits, cells with elongated microvilli, numerous cytoplasmic vesicles and close contact with lymphocytes, all ultrastructural features consistent with M cells, were selectively labelled with fucose and N-acetylgalactosamine binding lectins, while the other squamous epithelial cells were labelled with galactose binding lectins (Gebert, 1996). The fucose labelling was restricted to the apical membrane, pocket membrane and vesicles of the putative M cells (Gebert, 1997a). Additionally, these cells were positive when double stained for UEA-1 (fucose binding lectin) and vimentin. It was concluded, therefore, that similar to tissues of GALT, M cells do exist in the palatine tonsils of rabbits (Gebert, 1996).

Although there are epithelial cells in the reticulated areas of the crypt epithelium with a distinct morphology, different cytoskeleton and membrane markers, only in rabbits has the existence of M cells been confirmed by combined morphological and functional studies (Gebert, 1995). Therefore, whether a distinct cell type, similar to the intestinal M cell that performs antigen uptake and transcytosis, is present in the palatine tonsils of other species is not clear and only studies that associate morphology and function will answer this question (Gebert and Pabst, 1999).

1.3.2.5 – Function of the Tonsils of the Upper Aerodigestive Tract

There is ample supporting evidence that the palatine tonsils, together with the other components of the Waldeyer's ring, have a role in the defence of the upper aerodigestive tract similar to that of GALT in the lower digestive tract. In the nasal

mucosa, the salivary and the lacrimal glands of humans there is a predominance of B lymphocytes with the phenotype IgD^+ IgM^- $CD38^+$, which is unique to the tonsils, suggesting that those tissues are seeded with lymphocytes originating from the tonsils. When human tonsillar B lymphocytes are inoculated into SCID mice there is a preferential migration of these cells to the lungs, whereas there is none to the intestine (Nadal et al., 1991; Bernstein et al., 1999; Brandtzaeg et al., 1999b).

The incubation *in vitro* of human tonsillar B lymphocytes with either specific antigens or mitogens results in the production of specific antibody against diphtheria toxoid, *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus*, and the lipopolysaccharide of *Escherichia coli* (Bernstein et al., 1999). The palatine tonsils likely have an important role in controlling Epstein-Barr virus (EBV) infection in the oropharynx of humans, since the specific cytotoxic activity against EBV is significantly higher in tonsillar than in peripheral blood lymphocytes (Hirao et al., 1996). An epidemiological association between paralytic poliomyelitis and combined tonsillectomy and adenoidectomy was observed in the 1940's. The IgA titers against poliovirus in the nasopharyngeal fluid of children immunized with poliovaccine and subjected to combined tonsillectomy and adenoidectomy was 4 fold lower than in children with intact tonsils, suggesting that these lymphoid organs have an important role in the defence against pathogens that colonize the naso- and oro-pharynx (Ogra, 1971).

As mentioned earlier in section 1.3.1.2, a regionalization of the immune responses of MALT exists. One factor responsible for this regionalization is the variation in adhesion molecules on lymphocytes and on venular endothelium of the different components of MALT and effector sites of the immune responses. The upper aerodigestive tract seems to form a partially distinct compartment of MALT, i.e., immune responses induced in the tonsils are effective mostly in the nasal, bronchial, salivary and lacrimal glands. Results of studies support a dichotomy between the upper aerodigestive tract and the gut with regard to homing of lymphocytes into MALT. The adhesion molecule MAdCAM-1 is present in inductive and effector sites of GALT, but only a very small amount of this molecule is present in human tonsils (Brandtzaeg et al., 1999b). Additionally, MAdCAM-1 is absent in the lungs, salivary and mammary glands

and uterus of humans, and also the murine uterus (Briskin et al., 1997; Perry et al., 1998). Further supporting this dichotomy theory is the fact that in humans, mice and sheep, the leukointegrin $\alpha_4\beta_7$, which is a homing receptor for GALT, is not an important homing receptor for lymphocytes into the respiratory tract (Wagner et al., 1996; Abitorabi et al., 1996). The leukointegrin $\alpha_4\beta_1$ is a possible homing molecule involved in the migration of NALT lymphocytes. This molecule seems to be involved in the migration of primed mucosal B lymphocytes from upper aerodigestive tract to secretory tissues outside the gut. The ligand for $\alpha_4\beta_1$ is the vascular cell adhesion molecule-1 (VCAM-1), which is expressed on the venular endothelium of human bronchial and nasal mucosae and also in the murine female genital tract (Perry et al., 1998; Brandtzaeg et al., 1999b). Experimental studies in mice have shown that $\alpha_4\beta_1$ and VCAM-1 are involved in the homing of lymphocytes to the genital tract (Perry et al., 1998), suggesting that lymphocytes primed in NALT can seed distant tissues. The urogenital tract seems to have a homing mechanism similar to the upper aerodigestive tract. Evidence supports the notion that primed immune cells from NALT migrate to the urogenital tract. High levels of IgG and IgA are found in the cervicovaginal secretions of mice immunized intranasally against several different types of pathogens, including herpes simplex and HIV (Brandtzaeg, 1997). Similar findings were documented in monkeys immunized intranasally with *Streptococcus mutans* (Russel et al., 1996). Vaccine experiments in humans demonstrated that immunization via the nasal cavity and the palatine tonsils has the potential to result in seeding of distant sites with vaccine-specific antibody-secreting cells, since such cells were detected in the peripheral blood in addition to palatine tonsils, adenoids and nasal secretions. No vaccine response was present in the intestinal cell suspensions, however, and oral and parenteral vaccination induced only a poor response in the palatine tonsils (Quiding-Jarbrink et al., 1995).

Collectively, these findings demonstrate that the tonsils have a regionalized function not only in the defence of the upper aerodigestive tract, but also at distant sites such as the urogenital tract, and have the potential to seed remote tissues with memory and effector lymphocytes. Also, these lymphoid tissues function independently of GALT and have the advantage of exposure to intact antigens, without the destructive

action of digestive enzymes. These are valuable features to be explored in developing mucosal vaccines.

1.3.2.6 - Palatine Tonsils as a Portal of Entry and Carrier Site for Pathogens

The palatine tonsils have a strategic position, close to the entrance of both the respiratory and alimentary tracts, sites of constant exposure to pathogenic and non-pathogenic microorganisms. Thus, they are a common portal of entry and site of multiplication and persistence of microorganisms in both humans and domestic animals.

1.3.2.6.1 - Bacterial Pathogens

The palatine tonsils are a site of persistence of several important bacterial pathogens of pigs, e.g., *S. suis* (Staats et al., 1997), *Actinobacillus pleuropneumoniae*, a pathogen with worldwide distribution and the cause of pleuropneumonia in pigs (Chiers et al., 1999; Taylor, 1999a), *Mycoplasma hyosynoviae,* which causes arthritis in growing-finishing pigs (Hagedorn-Olsen et al., 1999), and *Yersinia enterocolitica*, usually an inapparent infection in pigs, but one that can be associated with fever and enteritis (Taylor, 1999b). *Salmonella typhimurium,* a very important cause of enterocolitis in pigs has been demonstrated experimentally to enter the host through the tonsillar epithelium (Fedorka-Cray et al., 1995) and is known to persist in the palatine tonsils (Wood et al., 1989). *S. typhimurium* and *Y. enterocolitica* are also important causes of food-born infection in humans and pork is a major source of this infection (Thibodeau et al., 1999; Schwartz, 1999).

The tonsils may play a significant role in the pathogenesis of *Pasteurella multocida* type D infection. This bacterium is considered the primary agent of atrophic rhinitis of pigs, a widespread infectious disease characterized by atrophy of the nasal turbinate bones and deformity of the snout (de Jong, 1999). It is believed that the tonsils act as a source of bacteria to colonize the turbinates or as a site for secretion of the toxin responsible for turbinate atrophy (Ackerman et al., 1991).

An important agent of pneumonia in cattle and sheep and of septicaemia in lambs, *Mannheimia* (formerly *Pasteurella*) *haemolytica*, is frequently isolated from the palatine tonsils (Al Sultan and Aitken, 1985; Shoo et al., 1990).

The most common agents of human acute bacterial tonsillitis, Group A beta-haemolytic streptococci, which can also cause fatal sepsis, are often carried in the tonsils of clinically healthy individuals (Stevens et al., 1989; Stenfors and Raisanen, 1991).

1.3.2.6.2 - Viral Pathogens

There are many examples of viruses that replicate and persist in the palatine tonsils. The epithelium of the palatine tonsils is the primary site of replication of hog cholera (classical swine fever) virus, the agent of a highly contagious and usually fatal disease of pigs characterized by disseminated hemorrhages and encephalitis (Oirschot, 1999). The agent of Aujeszky's disease (pseudorabies), a herpesvirus causing encephalitis, abortion and pneumonia in pigs, replicates initially in the palatine tonsils (Narita et al., 1984). This tissue is also a site of latent infection for herpesvirus, which is an important feature for diagnostic and research purposes, as the trigeminal ganglia, sites of latency of this virus, can only be examined after the death of the animal (Cheung, 1995).

Several important feline viruses replicate in the palatine tonsils: the feline leukemia virus (Rojko & Kociba, 1991), the herpesvirus causing feline rhinotracheitis (Gaskell and Povey, 1979), the coronavirus causing feline infectious peritonitis (Stoddart et al., 1988), and the calicivirus that causes oro-respiratory infection. In carrier animals the palatine tonsils are site of persistent infection with feline calicivirus (Wardley and Povey, 1977; Dick et al., 1989).

The palatine tonsils play an important role in the infection caused by bovine viral diarrhoea (BVD) virus. This is a major pathogen of cattle and responsible for several manifestations such as diarrhoea, abortions, congenital defects, and immunosuppression that may facilitate the action of other pathogenic microorganisms in the respiratory and gastrointestinal tracts. BVD virus and viral antigens are found in the crypt epithelium, lymphoid follicles and macrophages of the palatine tonsils of both chronic and acutely infected animals. In the tonsillar crypt epithelium, areas of degeneration and necrosis are seen in association with BVD virus (Ohmann, 1983; Marshall et al., 1996). Together with the nasal mucosa, the tonsils are considered the primary site of BVD virus

replication. High viral titres are reached in the tonsils before the virus spreads throughout the host (Bruschke et al., 1998).

The palatine tonsils play a role in the pathogenesis of many important viral diseases of dogs. Parvovirus, a common cause of an often fatal enteritis in dogs, replicates initially in the palatine tonsil, retropharyngeal and mesenteric lymph nodes (Meunier et al., 1985). Canine adenovirus-1, the agent of infectious canine hepatitis, multiplies in the palatine tonsils, causing tonsillitis, before spreading to other tissues (Kelly, 1993). A similar pathogenesis occurs with the agent of canine distemper, which localizes and replicates in the palatine tonsils and bronchial lymph nodes before disseminating to other tissues (Appel, 1969; Dungworth, 1993).

The tonsils are sites of replication of the HIV-1. A large number of syncytial cells, which originate from dendritic cells and contain HIV-1, are detected in the lymphoepithelium of the palatine and nasopharyngeal tonsils of patients with chronic HIV-1 infection (Frankel et al., 1997). Measles virus is found in the tonsils of individuals who die from subacute sclerosing panencephalitis which is a fatal disease of children resulting from persistent infection with this virus (Brown et al., 1989).

1.3.2.6.3 - Non-conventional Pathogens

The tonsils are important organs for the diagnosis of new variant Creutzfeldt-Jakob disease in human beings. This neurological disease appeared in the mid 1990's in the United Kingdom and was linked to dietary exposure to the agent of bovine spongiform encephalopathy (BSE). Initially, confirmation of the diagnosis was possible only by brain biopsy or at necropsy. Later, it was shown that the prion protein associated with the disease can be detected in lymphoid follicles of the tonsils (Hill et al., 1997). The same is true for the disease scrapie, a spongiform encephalopathy of sheep. Due to their accessible location and high concentration of the scrapie-associated form of the prion protein, the palatine tonsils are the most appropriate lymphoid tissue for biopsy in the diagnosis of scrapie in live sheep (van Keulen et al., 1996). Previously, the diagnosis of this disease was performed only through brain biopsy or after death.

All these examples of animal and human diseases illustrate the importance of the tonsils in the pathogenesis of infectious diseases. This is not unexpected, since the

majority of pathogens invade the host through the oro-respiratory route and the tonsils are major lymphoid tissues located at the entrance of this route.

1.4 - Lymphocyte Subsets and their Roles in the Immune Response

1.4.1 - Lymphocyte Subsets and Functions

B and T lymphocytes originate from the bone marrow and play a central role in the immune responses of vertebrates. B lymphocytes were so named because it was identified that in birds they mature in the bursa (B) of Fabricius. In humans and mice these cells mature in the bone marrow, but in pigs (Lunney and Butler,1998) and sheep (Griebel et al., 1992), B cells maturate in the Peyer's patches . T lymphocytes mature in the thymus, hence the name 'T' cells. The unique feature of B and T lymphocytes is their ability to recognize an antigen specifically and to mount a response specific to that antigen. Additionally, these cells are able to proliferate to expand this specific response, and also to develop memory, which gives a more rapid and efficient response on second exposure to the same antigen. Antigen specificity is dictated by the presence of membrane bound receptors. There are many different clones of lymphocytes in one individual, each with a different antigen specific receptor, thus creating a large repertoire of lymphocytes able to discriminate between 10^9 to 10^{11} distinct antigen determinants (Abbas et al., 2000e).

B and T lymphocytes differ in their antigen receptors and in their functions. When a B cell receptor (membrane bound immunoglobulin) binds to its specific antigen determinant or epitope, the cell undergoes activation and differentiates into an immunoglobulin or antibody-secreting cell, i.e., a plasma cell. In this way the immunity provided by B lymphocytes can be transferred via the serum, and hence the term 'humoral immunity'. Antibodies can have a direct protective action by interfering with the binding of a microorganism to the host tissues or by neutralizing a bacterial toxin. More often, antibodies act in concert with cells of the immune system. The antibodies IgG and IgM, for example, are able to initiate the classical pathway of complement, leading to the release of complement fragments such as C3b, an opsonin for which there are receptors on macrophages and neutrophils that enhance phagocytosis. Other

complement factors that are released, C3a and C5a, are chemotactic factors for neutrophils. Antibodies also act as opsonins: microorganisms that are coated with antibodies, bind efficiently to antibody receptors on phagocytic cells, enhancing the phagocytic process. Antibodies can mediate antibody-dependent cellular cytotoxicity, such as in the elimination of helminths by eosinophils mediated by IgE. B cell activation is mostly dependent on the cooperation of T lymphocytes, and immunoglobulin class switching, antibody affinity improvement, and memory B lymphocytes can develop only with the help of T lymphocytes (Delves and Roitt, 2000b; Abbas et al., 2000d).

T lymphocytes are involved in the induction of a cellular immune response that can be transferred only by cells. In T lymphocytes the antigen specific receptor is known as the T cell receptor (TCR) and is not secreted by the cell, as in B lymphocytes. While B lymphocytes can recognize antigen in its native form, T lymphocytes can recognize antigens only after they have been processed and presented by APC. T lymphocytes recognize small linear peptide fragments of protein antigens that are presented by APC in association with major histocompatibility complex molecules (MHC). Upon the interaction of an antigen peptide and MHC with the TCR on the T cell surface, the CD3 molecule which is linked to TCR to form the TCR complex, sends a biochemical signal that leads to the activation of the T cell. The CD3 molecule is used as a marker of T lymphocytes, because it differentiates T lymphocytes from other lymphocytes (Abbas et al., 2000a).

There are two classes of T cell, based on differences in the peptide chains of the TCR, namely $\alpha\beta$ TCR and $\gamma\delta$ TCR. Cells bearing the $\alpha\beta$ TCR are usually the predominant type of T lymphocytes. T lymphocytes have accessory molecules that participate in antigen recognition and cell activation processes. Two important accessory molecules are CD4 and CD8. Mature T lymphocytes bearing $\alpha\beta$ TCR express either CD4 or CD8, but usually not both. These molecules, whose main function is signal transduction, are also called co-receptors because they operate together with TCR and MHC at the time of antigen recognition. The presence of CD4 or CD8 on an $\alpha\beta$ T cell is used as a marker of the lymphocyte phenotype and function. CD4 positive cells, also known as T helper cells, recognize antigens in association with the MHC-II

molecule which is present on professional APCs, namely dendritic cells (DCs), activated macrophages and B lymphocytes. Antigens presented in association with MHC-II are derived mostly from the extracellular environment, for example from bacteria. Once activated by its cognate antigen, a CD4 naïve T cell responds by secreting IL-2, resulting in autocrine stimulation, proliferation and clonal expansion. These activated CD4 cells are able to initiate the effector phase of the immune response, where different sets of cytokines are responsible for distinct forms of response, influencing and improving the function of other cells of the immune system, like B lymphocytes, CD8 cells and inflammatory cells (Delves and Roitt, 2000a & b; Abbas et al., 2000a).

CD8 positive T lymphocytes, also known as cytotoxic T lymphocytes, recognize peptides in association with the MHC-I molecule which is present on all nucleated cells. MHC-I presents antigen peptides derived from the cytosol, e.g., viral peptides, to CD8 cells. For activation of the CD8 cell, other signals are necessary in addition to recognition of a MHC-I-associated foreign peptide. These signals, called second signals, are believed to be provided by APC or helper T lymphocytes. Professional APCs can also present antigens in association with MHC-I, and while presenting antigens to CD8 cells, send a second signal via costimulatory molecules. This second signal induces CD8 cells to proliferate and differentiate into cytotoxic cells. Another possible second signal can be provided by a CD4 helper cell, which interacts via MHC-II with the same APC as the CD8 cell, or a CD4 cell that instead interacts with an APC in the vicinity of the CD8 cell, and at the same time secretes IL-2 that stimulates clonal proliferation and differentiation of CD8 cells. Once activated, CD8 cells can kill cells, such as virus-infected cells or tumour cells, which present foreign antigens in association with MHC-I. This killing is antigen specific, dependent on the close contact of both cells, and is mediated by cytotoxic granules (perforin and granzymes) and by the interaction of the apoptosis-inducing receptor (Fas) ligand on the plasmalemma of the CD8 cell with Fas on the cytoplasmic membrane of the target cell. Perforin is a pore-forming protein that is released into the target cell and induces cell death by osmotic defects. Granzymes penetrate the target cell mainly through the pores formed by perforin and induce apoptosis. Finally, the interaction of Fas with its ligand, also leads to apoptosis (Abbas

et al., 2000c). Similar to CD4 cells, CD8 cells are also able to secrete cytokines that mediate the immune response (Vukmanovic et al., 2000).

The second subpopulation of T lymphocytes based on TCR receptor differences comprises cells that express the γδ TCR. These cells recognize antigens via TCR and require CD3 for signal transduction, as do the αβ T lymphocytes; however, they lack CD4 and usually also CD8 molecules. Unlike CD4 and CD8 cells, γδ T lymphocytes do not require protein-processing pathways for antigen recognition and are not MHC-restricted. The functions of γδ T lymphocytes and what these cells recognize are unclear. Both peptides and non-peptides are able to stimulate γδ T lymphocytes. Many antigens are recognized by these cells, such as mycobacterial protein and non-protein extracts, tetanus toxoid, heat shock proteins, LPS, and listeriolysin O. It has been suggested that γδ T lymphocytes recognize antigens common to microorganisms and stressed cells. They increase significantly in the peripheral blood of humans during a variety of infectious diseases such as tuberculosis, salmonellosis, leishmaniasis, toxoplasmosis and AIDS (Bukowski et al., 1999). γδ T lymphocytes can modulate the response of both T and B lymphocytes by the production of cytokines. They are present in the early phase of infections, preceding CD4 and CD8 cell activation, and appear to modulate the outcome of the immune response. They are also present in the later phases of infection and are believed to downregulate the αβ T cell response. An illustration of this is the observation that γδ T lymphocytes from patients with arthritis due to Lyme disease are capable of inducing apoptosis in αβ T lymphocytes from the same host (King et al., 1999; Born et al., 1999).

γδ T lymphocytes appear to participate in the inflammation and repair, regardless of the etiology of the injury. This is well illustrated by knockout mice deficient in γδ T lymphocytes that were submitted to a microbial (*Nocardia asteroides*) and a chemical (ozone) pulmonary insult; in both situations there was development of severe necrotic lung lesions that lacked an inflammatory response. In contrast, control mice that were exposed to the same insults, had mild lesions characterized by infiltration of neutrophils and by repair (King et al., 1999). γδ T lymphocytes also appear to participate in

epidermal healing through the secretion of keratinocyte growth factor (King et al., 1999; Born et al., 1999).

γδ T lymphocytes have been largely associated with mucosal immunity since they are found in higher numbers in epithelial surfaces than in other lymphoid tissues, supporting the theory that these cells have a role as a first line of defence. In the intestinal epithelium of mice and chickens, more than 50% of intraepithelial lymphocytes are γδ T lymphocytes and most of the murine intraepidermal T lymphocytes express γδ receptor (Bucy et al., 1988; Raulet, 1989; Beard et al., 2000; Abbas et al., 2000a). In humans approximately 30% of the intestinal intraepithelial lymphocytes are γδ T cells (Deusch et al., 1991). In cattle, sheep and pigs, unlike other species, γδ cells represent a large proportion of the peripheral blood T lymphocytes but the significance of this finding is unknown (Hein and Mackay, 1991; Davis et al., 1998). In young animals of these species, γδ T lymphocytes are the predominant T cell subpopulation in the peripheral blood and it has been suggested that they have an important role in immunity until the maturation of the αβ subsets by antigenic experience takes place (Hein and Mackay, 1991; Yang and Parkhouse, 1996).

Another subpopulation of lymphocytes is natural killer (NK) cells. NK cells are mostly related to innate immunity and are activated before a specific response takes place, but they also cooperate in the acquired response. Unlike T and B lymphocytes, these cells do not have a specific receptor for antigen recognition. They recognize and kill infected cells and tumour cells. The signal to kill these cells is reduction of MHC-I expression, i.e., MHC-I is an inhibitory signal for NK cells. Since all nucleated cells express MHC-I, they are not killed in normal conditions, but virus-infected cells and tumour cells may lose this molecule and hence be killed by NK cells. NK cells also have receptors for Fc of IgG, and by this means they can kill IgG-coated target cells by antibody-dependent cellular cytotoxicity. The method of NK killing is similar to that of CD8 cytotoxic T lymphocytes, i.e., by perforin and granzymes. NK cells can also secrete interferon gamma (IFN-γ), a cytokine that activates macrophages (Delves and Roitt, 2000a; Abbas et al., 2000g).

1.4.2 - The Role of the Lymphocyte Subsets in the Pathogenesis of Infectious Diseases

Understanding the function of different lymphocyte subsets and their participation in the various infectious diseases is extremely important. An appropriate type of immune response involving the right types of cells and cytokines is responsible for protection and recovery, while the activation of the wrong type of response, or an exuberant appropriate type of response, may be associated with failure of protection, disease and even death. The knowledge obtained from the mechanisms of protective immunity in infectious diseases can be translated into effective treatments and vaccines.

In mice and humans, immune responses may be polarized into Th1 (T helper 1) and Th2 (T helper 2), depending on the pattern of cytokines produced. This polarization is mediated mainly by CD4 lymphocytes upon antigen recognition. Th1 response is characterized by a substantial production of IFN-γ, tumour necrosis factor beta (TNF-β) and IL-2, while Th2 response is via the production of IL-4, IL-5, IL-6, IL-10 and IL-13. Th1 response is effective against intracellular microbes, such as viruses and mycobacteria, against tumour cells, and in graft rejection. In this type of response there is activation of macrophages, NK cells and cytotoxic CD8 cells. An increase in IgG2a production is also a feature of Th1 response. This antibody promotes opsonization and activation of the classic pathway of complement, both mechanisms that improve elimination of intracellular microbes. Th2 response is induced mainly against extracellular pathogens. In this response there is an increase in IgG1, for example, which neutralizes extracellular microbes. There is also attraction and activation of eosinophils that are important in the response against helminths. Th1 and Th2 responses are antagonistic to each other so that cytokines, such as IFN-γ, produced by Th1 cells inhibit the Th2 response, while IL-10 and IL-13 produced by Th2 cells inhibit the Th1 response. In this way there is a cross-regulation of the immune response, defining the predominance of one type of response and limiting the pathological effects of exaggerated immune and inflammatory responses. This polarization is more evident in chronic processes. CD8 cells can also participate in the modulation of the immune response by cytokine production, which influences the CD4 response. Hence, Tc1 (T

cytotoxic 1) cells produce IFN-γ that inhibit the Th2 response, and Tc2 (T cytotoxic 2) cells produce IL-4 that has an inhibitory effect on Th1 response and activates Th2 response (Romagnani, 1997; Vukmanovic et al., 2000; Abbas et al., 2000b).

In many infectious diseases the Th1/Th2 polarization of the immune response is evident. One classical example is the disease caused by *Mycobacterium leprae* (*M. leprae*) in humans. Patients with the tuberculoid form of leprosy, which is characterized by granulomas, have less tissue destruction and a paucity of bacteria which are confined to the granulomas. These patients develop a Th1 type of response. In contrast, individuals with the lepromatous form of the disease have extremely severe lesions and high numbers of bacteria. In these individuals, a Th2 type of response is produced, which accounts for the inability to control the disease. Since *M. leprae* is an intracellular pathogen, a Th1 response is more effective in controlling the infection (Abbas et al., 2000f). Other examples illustrate the polarization of the immune responses: mice susceptible to intracellular parasite *Leishmania major* develop a Th2 response, while the resistant ones develop a Th1 response. In contrast, in helminthic infestations, mice resistant to the extracellular parasite *Trichuris muris* develop a Th2 response, while a Th1 response is observed in susceptible mice (Mosmann and Sad, 1996).

Many studies involving the identification of lymphocyte subsets and cytokine production have been performed lately to investigate the pathogenesis of infectious diseases in domestic animals, e.g. Aujeszky's disease (Bouma et al., 1997), PRRS (Kawashima et al., 1999) and hog cholera in pigs (Summerfield et al., 2001), Johne's disease (Beard et al., 2000; Stabel, 2000) and tuberculosis (Waters et al., 2000) in ruminants, and distemper in dogs (Wünschmann et al., 1999; Wunschmann et al., 2000). *Mycobacterium paratuberculosis* infection (Johne's disease) in ruminants is characterized by a Th1 type of response in the subclinical stage of infection. This response wanes and is replaced by Th2 type which is associated with clinical disease (Stabel, 2000). In pigs Th1/Th2 polarization of the immune response is observed in Aujeszky's disease virus infection and protection from this disease correlates with a Th1 response. Vaccination with inactivated Aujeszky's virus induces a Th2 response, a non-protective type of response; however, if the pigs receive recombinant IL-12 at the time of

the vaccination, there is enhancement of the Th1 response which is protective (Zuckermann, 2000).

1.4.3 – Lymphocyte Subsets and Functions in Pigs

The anatomic and physiologic similarities of pigs and humans have led to intense research efforts to develop the pig as a model for human studies and as a potential donor for xenotransplantation (Kaiser, 2002). For these reasons, and also because of the importance of the swine industry in the world, many studies have been focused on the porcine immune system. Monoclonal antibodies against porcine lymphocyte subpopulations have been generated by many laboratories around the world, and three international workshops have been held to standardize the monoclonal antibodies reactive with porcine leukocyte differentiation antigens and to define new clusters of antibodies (Lunney et al., 1994; Saalmuller, 1998; Haverson et al., 2001). Many monoclonal antibodies against porcine CD molecules are now available, leading to enhanced understanding of the porcine immune system.

In general, pigs have the same subsets of lymphocytes and respective functions as the other species, i.e. B lymphocytes, αβ and γδ T lymphocytes and NK cells. αβ T lymphocytes are also divided in CD4+ and CD8+, with helper and cytotoxic functions, respectively (Lunney and Pescovitz, 1987; Zuckermann, 1999). However, some features are unique to the porcine immune system, e.g., a significant proportion of porcine T lymphocytes co-express CD4 and CD8 molecules. CD4 and CD8 are generally mutually exclusive in mature lymphocytes; however, pigs, chickens and monkeys have a high proportion of CD4/CD8 double positive (DP) cells (Zuckermann, 1999). These DP T lymphocytes can vary from 8 to 64% of the resting peripheral-blood T lymphocytes in adult pigs (Saalmuller et al., 1987) and they are also present in lymphoid tissues (Zuckermann and Gaskins, 1996). In humans, DP T lymphocytes are rare in peripheral blood and comprise around only 3% in healthy individuals (Blue et al., 1985). Higher proportions (15%) however, are associated with disease states such as mononucleosis, auto-immune diseases and neoplastic processes (Ortolani et al., 1993).

The function of DP cells in pigs has not been specifically determined. First, it was though that they were immature T lymphocytes that emigrated prematurely from the

thymus. However, the absence of CD1 antigen, a marker for immature thymic cells, makes it unlikely (Saalmuller et al., 1989; Pescovitz et al., 1990; Pescovitz et al., 1994). Evidence now suggests that in this species these DP T lymphocytes are memory cells. This subpopulation increases with age both in blood and lymphoid tissues. DP cells comprise less than 2% of the peripheral blood lymphocytes in 1-week-old pigs, 7% at 7 months of old, 15% between 18 and 21 months, and continue to increase to reach 24% at 32 months of age (Zuckermann and Husmann, 1996). DP cells are prominent in inflammatory sites, such as in pneumonic lung of pigs infected with *Mycoplasma hyopneumoniae*, in the cerebrospinal fluid of pigs with herpesvirus encephalitis, and among the cells infiltrating porcine kidney allografts (Zuckermann, 1999). These cells respond in a secondary fashion to viral and helminthic antigens (Dillender and Lunney, 1993; Pescovitz et al., 1994; Zuckermann and Husmann, 1996); and 75% of DP porcine cells express high levels of a homologue to human β1 integrin (CD29) molecule which is expressed in high levels in human memory T lymphocytes (Zuckermann and Husmann, 1996). Antigenic stimulation seems to play a role in the generation of DP cells as they are abundant in lymphoid tissues that have a high antigenic load such as tonsils and PP, but are scarce in peripheral lymph nodes that receive less exposure to antigens. Supporting this theory further is the observation that viral and bacterial infections in pigs correlate with an increase in the proportion of DP cells in the peripheral blood (Zuckermann, 1999). DP cells in pigs are different from those observed in humans; in pigs DP cells are small, resting lymphocytes (Saalmuller et al., 1987), while in humans these cells are lymphoblastic, activated cells (Blue et al., 1985).

The reason for simultaneous expression of CD4 and CD8 molecules on this subset of T lymphocytes in pigs is not clear. DP cells are MHC-II restricted, and it is believed that these cells originate from single CD4+ cells (Zuckermann, 1999). Zuckermann (1999) suggested that the co-expression of these molecules seems to enhance the avidity of the TCR for its target, facilitating the activation of a memory DP cell in conditions where the antigen level is low or there is a scarcity of APCs, such as at sites outside the lymphoid tissues .

Another unique feature of the porcine immune system is the high number of γδ T lymphocytes. These form the dominant subpopulation of T lymphocytes in the peripheral blood of young pigs. The NK cell population ($CD3^-$, $CD2^+$, $CD8^{low}$) is also very high in the peripheral blood of young pigs (Yang and Parkhouse, 1996). They comprise 28% of the peripheral blood lymphocytes (PBL) of 4 week-old pigs, while only 5% of the PBL in 1-year-old pigs are NK cells. This profile is not seen in humans, where the proportion of NK cells in umbilical cord blood (19%) is not very different from the proportion found in the blood of adults (15%). Hence, it seems that prior to the maturation of the αβ TCR subsets, young pigs depend on the innate immunity, represented by NK cells, in association with γδ T lymphocytes (Yang and Parkhouse, 1996).

1.4.4 – Distribution of Lymphocyte Subsets in the Blood and Lymphoid Tissues of Pigs

Since the advent of monoclonal antibodies which identify porcine lymphocyte subpopulations, numerous studies have been done to evaluate the distribution of the various lymphocyte subsets in the peripheral blood and lymphoid tissues of pigs. These studies involve mainly the use of fluorescence activated cell sorter technology (FACS) and less often immunohistology.

1.4.4.1 – Fluorescence Activated Cell Sorter (FACS) Studies

The results of FACS studies vary not only according to animal age but also among different laboratories. This is not unexpected since the antigenic load for the animals will vary according to the housing conditions. Animals bred and reared in isolation in research facilities certainly have different lymphocyte subset proportions than those in commercial establishments. Even among commercial farms, differences may be expected due to the variety of environmental conditions (Solano-Aguilar et al., 2001). The intraepithelial lymphocytes of the small intestine, for example, are influenced by environmental conditions. In germ-free pigs, there is no increase in intraepithelial lymphocytes between 1 and 2 months of age, while in conventionally reared and specific

pathogen free (SPF) pigs of the same age this population of lymphocytes increases, especially in the conventionally reared animals (Rothkötter et al., 1999).

In 4-week-old pigs, 32% of the PBL are B lymphocytes, 12% are αβ T lymphocytes, 24% are γδ T lymphocytes and 32% are non-T, non-B lymphocytes. The αβ T subset is divided in 4 subpopulations, all of which are positive for the CD2 marker, a molecule found on 90% of mature T lymphocytes, on 50% to 70% of thymocytes, and on NK cells. The four subsets comprise single positive (SP) $CD4^+CD8^-$ (2.1%), DP $CD4^+CD8^{low}$ (2.2%), SP $CD4^-CD8^{low}$ (3.7%), and SP $CD4^-CD8^{high}$ (4%). γδ T lymphocytes consist of 3 subsets: $CD2^-CD4^-CD8^-$ (21%), $CD2^+CD4^-CD8^{low}$ (2.2%) and $CD2^+CD4^-CD8^-$ (1%) cells. The non-T, non-B lymphocytes are also divided into 3 subsets: $CD2^+CD8^{low}$ (28%), which are the NK cells, $CD2^+CD8^-$ (2.3%) and $CD2^-CD8^-$ (2%) cells. Low expression of CD8 molecule in CD4+ cells and in γδ T lymphocytes is interpreted as a sign of previous cell activation (Yang and Parkhouse, 1996) . These subset percentages are different in older animals. Pigs of 8 months have higher proportions of αβ T lymphocytes (30%), of which 7% are SP CD4, 18% are CD8 SP and 5% are DP T lymphocytes (Yang and Parkhouse, 1996). Studies performed by others demonstrated higher proportions of these αβ lymphocyte subsets: 17% of the lymphocytes were SP CD4, 30% CD8 SP, and 12% DP cells, in pigs of a similar age (6-7-month-old) (Zuckermann and Gaskins, 1996). The proportion of γδ T lymphocytes in the peripheral blood declines with age: 67% of the T lymphocytes in 4-week-old pigs, 55% in 8-month-old, and 48% in pigs at 16 months. A decrease in proportion of the non-T, non-B lymphocytes in the peripheral blood is also observed, from 32% in 4-week-old piglets to 11% in 16 month-old pigs (Yang and Parkhouse, 1996).

The proportions of lymphocyte subsets in lymphoid tissues are different from the peripheral blood. In the spleen of 4-week-old pigs, 23% are αβ T lymphocytes, which contrasts with 12% in the peripheral blood. Approximately 70% of the splenic αβ T subset is CD4 positive (including SP and DP), which is twice that found in the peripheral blood. The subpopulation of non-T, non-B lymphocytes in this organ is much smaller, close to half of that found in the blood. The proportion of γδ T lymphocytes in the spleen (26%) is similar to that found in the peripheral blood (24%); however, while in

the spleen they are mostly $CD2^+$, in the peripheral blood the majority of γδ T lymphocytes are $CD2^-$ (Yang and Parkhouse, 1996).

The lymph nodes of 4-week-old pigs have a higher proportion of αβ T lymphocytes (+/-55%) than peripheral blood and spleen, of which approximately 70% are CD4 positive. The proportions of γδ T lymphocytes and non-T non-B lymphocytes in the lymph nodes are very small, 4% to 6% and 6% to 8%, respectively (Yang and Parkhouse, 1996). The proportions of lymphocyte subsets in the lymph nodes of 4-week-old pigs (Yang and Parkhouse, 1996) are not very different from those in 6-7 months old pigs (Zuckermann and Gaskins, 1996). While in the younger group the DP cell proportion varies from 8.7% to 9.9%, in older pigs it is between 10% and 13%. Single positive CD4 cells account for 26% to 33% in 4-week-old pigs and 25% to 27% in 6-month-old pigs.

The proportions of lymphocyte subsets in MALT differ from those in the lymph nodes. The palatine tonsils of 4-week-old pigs have a high proportion of B (57%) and non-T, non-B lymphocytes (18%), as compared to approximately 33% of B lymphocytes and 7% of non-T, non-B in lymph nodes. In contrast, smaller percentages of αβ T (23%) and γδ T (3%) cells are found (Yang and Parkhouse, 1996). Of all the lymphoid tissues examined to date, the palatine tonsils have the highest proportion of CD4/CD8 DP T lymphocytes, comprising 62% of the CD4 positive lymphocytes, compared to 35% in the lymph nodes. This higher proportion of DP cells is consistent with the position of the tonsils at a site of a constant antigenic exposure and with the fact that DP cells are memory or activated cells (Zuckermann and Gaskins, 1996). The proportion of DP cells in the tonsils increases dramatically with age. Three-day-old pigs lack DP cells, 8% of the tonsillar lymphocytes of 4-week-old pigs are DP, and at 6 months this amount increases to 18% (Yang and Parkhouse, 1996; Zuckermann and Gaskins, 1996). These findings give even more support for the theory that DP cells are antigen experienced or memory cells and lead to speculation that the tonsils are the sites of generation of DP T lymphocytes (Zuckermann and Husmann, 1996).

In the small intestine the proportions of lymphocyte subsets differ from those in the palatine tonsils, and within different areas of the intestine the lymphocyte subsets

also vary. The ileal PP has a higher proportion of B lymphocytes (63%) than the jejunal PP (46%). In the ileal PP, the interfollicular area contains only a very small number of T lymphocytes (Binns and Licence, 1985; Zuckermann and Gaskins, 1996), only 4% of the T lymphocytes are DP, and the proportions of single CD4 and CD8 are 7% and 8%, respectively. In contrast, in the jejunal PP 10% of the lymphocytes are DP, 12% are SP CD4, and 28% are SP CD8 (Zuckermann and Gaskins, 1996). Although the intestine is not a classical lymphoid tissue, it is an effector site of the GALT, as described earlier. The intraepithelial lymphocytes from the jejunum and ileum are mostly T lymphocytes (90%), consisting largely of CD8 cells (>90%) (Zuckermann and Gaskins, 1996).

1.4.4.2 – Immunohistological Studies

While there are numerous descriptions on the proportions of lymphocyte subsets in lymphoid tissues of pigs, very few studies have addressed their morphological distribution. In the spleen B lymphocytes are present in the lymphoid follicles, and T lymphocytes are present mostly around the central splenic arteries in the periarteriolar lymphoid sheath. Very few T lymphocytes are found in the splenic lymphoid follicles, and those are mainly CD4 cells. In the periarteriolar lymphoid sheath CD4 cells outnumber CD8 cells, but in the red pulp CD8 cells predominate. The major immunoglobulin containing B cell in the spleen is IgM (Jonjic et al., 1987; Bianchi et al., 1992).

The morphological distribution of lymphocyte subsets in lymph nodes is similar to that in the spleen. The lymphoid follicles are composed mainly of B lymphocytes, with few CD4 cells and rare CD8 cells. In the perifollicular area CD4 cells predominate over CD8 cells, but CD8 cells predominate in the medulla. IgA containing cells predominate in the mesenteric lymph nodes, but in the peripheral lymph nodes, it is the IgG containing cells (Bianchi et al., 1992).

The same pattern of morphological distribution of lymphocyte subsets is observed for the palatine tonsils i.e., T lymphocytes, mainly CD4, in the perifollicular areas and B lymphocytes with scattered CD4 cells and very few CD8 cells in the follicles. The B lymphocytes of the lymphoid follicles are mainly IgG and IgM positive cells. These cells are also present in the perifollicular area, but are concentrated adjacent

to the crypt epithelium and infiltrated in the crypt epithelium. An intense reaction for these immunoglobulins is seen in the lumen of the crypts. IgA positive cells are not detected in the tonsillar lymphoid follicles and in the crypt epithelium the number of IgA positive cells is lower than IgM and IgG cells (Ramos et al., 1992).

In the intestinal tract, lymphocytes are distributed in 3 main regions: within the PP, scattered through the lamina propria and within the epithelium. In the PP, the pattern of distribution of cells is similar to the other lymphoid tissues. IgM, IgG and IgA containing cells are present in the PP, but IgA cells predominate. The epithelium overlying the PP has a predominance of B lymphocytes while CD8 cells, which are the major intraepithelial lymphocyte in areas outside the PP, are lacking (Bianchi et al., 1992).

A compartmentalization of lymphocytes is seen in the lamina propria of the small intestine. B lymphocytes are concentrated in the area subjacent to the crypts and they are mostly immunoglobulin containing cells, with a predominance of IgA and IgM over IgG (Bianchi et al., 1992; Vega-Lopez et al., 1993). IgA is present in the lumen of the crypts and in the basal portion of the cytoplasm of the crypt epithelial cells, indicating secretion of IgA at this site (Lackovic et al., 1999). T lymphocytes are found mainly in the area subjacent to the villi, with a predominance of CD4 positive cells. These CD4 cells form a sheet of cells in close association with MHC-II positive cells with the morphology of dendritic cells, suggesting that antigen presentation takes place at this site (Bianchi et al., 1992; Vega-Lopez et al., 1993).

Within the intraepithelial T cell subpopulations of the small intestine, CD8 cells form the largest subset (Bianchi et al., 1992). CD4/CD8 double negative T lymphocytes are also found in high number in this epithelium and were interpreted as γδ T lymphocytes (Vega-Lopez et al., 1993). In a later study, however, no γδ T lymphocytes were observed in the epithelium of the small intestine of pigs (Rothkötter et al., 1999). The failure to detect γδ T lymphocytes in this latter study could be explained by the fact that the antibody used to detect these cells, MAC320, marks only $CD2^-$ γδ T lymphocytes (Davis et al., 1998). As stated earlier in section 1.4.4, γδ T lymphocytes may or not express CD2 molecule. It is still possible therefore, that $CD2^+$ γδ T

lymphocytes are present in the intestinal epithelium and that they may indeed be the CD4/CD8 double negative T lymphocytes observed in the previous study of the intestinal epithelium of pigs (Vega-Lopez et al., 1993), which were in fact $CD2^+$.

1.5 - Summary

This literature review has demonstrated the importance of *S. suis* infection in swine production, how disseminated it is in the world, and how difficult it is to eliminate this infection from herds. Despite the numerous studies on virulence factors, the pathogenesis of this disease is still unclear, and no fully effective vaccine is available. The importance of the palatine tonsils as a portal of entry and site of persistence for *S. suis* has also been illustrated. The persistence of *S. suis* infection in herds is believed to be due to the presence of carrier animals that bear this pathogen in the tonsils. Therefore, considering the importance of the palatine tonsils in *S. suis* infection, there is a clear need to better understand the function of this organ and the initial steps in colonization of the palatine tonsils by this pathogen. As an organ of MALT, the tonsils share many of the features of these tissues and seem to have an important role in the defence of the upper respiratory and digestive tracts, and also in the defence of other distant tissues. However, much remains to be determined about the function of the palatine tonsils and their role in infectious diseases.

Over the last 15 years monoclonal antibodies that identify specific subpopulations of lymphocytes have been developed and characterized for the porcine species, leading to an increase in the knowledge of the porcine lymphoid tissues and pathogenesis of infectious diseases in this species.

This review forms the background of this study on the crypt epithelium of the palatine tonsils of pigs. The next chapter addresses the hypotheses and objectives that are the foundations of this work.

2 – Research Hypotheses and Objectives

This research concentrated on the palatine tonsils because they form the largest mucosa-associated lymphoid tissue in the oro-naso-pharynx of pigs, and are common sites of *S. suis* isolation. In the palatine tonsils, the barrier zone between the external environment and the underlying lymphoid tissue is formed by the crypt epithelium. Hence, the crypt epithelium, together with its intraepithelial leukocytes, is the first zone of interaction with pathogens and antigens and is a potential site for the initiation of immune responses in the palatine tonsils. In this study, structure, function and role of the crypt epithelium in the pathogenesis of *S. suis* infection are investigated.

2.1 - Hypotheses

1. The crypt epithelium of the palatine tonsils of pigs is infiltrated by a variety of lymphocyte subpopulations.
2. The leukocyte subpopulations of the crypt epithelium of the palatine tonsils of pigs change in response to bacterial infection.
3. The crypt epithelium of the palatine tonsils of pigs is specialized in the same way as the lymphoepithelium of other MALT, and has epithelial cells and/or M cells that are able to take up pathogens and antigens from the lumen.

2.2 – Objectives

1. Characterize the lymphocyte subpopulations in the tonsillar crypt epithelium of healthy pigs.
2. Characterize the leukocyte subpopulations in the tonsillar crypt epithelium of pigs challenged with *S. suis* serotype 2.
3. Assess *S. suis* serotype 2 transport across the crypt epithelium.

4. Perform a detailed study of the morphology of this epithelium and investigate if M cells are present in the palatine tonsils of pigs.

The characterization of the lymphocyte subsets in the crypt epithelium was performed in market weight 6-month-old healthy pigs (Chapter 3), and in 3-week-old pigs experimentally infected with *S. suis* serotype 2 (Chapter 4), using monoclonal antibodies against leukocyte markers and an immunohistochemistry technique. The transport of *S. suis* serotype 2 across the crypt epithelium (Chapter 5) and the morphology of this epithelium (Chapter 6) were evaluated by transmission electron microscopy.

3 - The Lymphocyte Subsets in the Crypt Epithelium of the Palatine Tonsils of Pigs

3.1 - Introduction

The palatine tonsils, secondary lymphoid organs strategically located in the oropharynx, are part of the MALT, a specialized compartment of the immune system that works as a first line of defence at the interface between external and internal environments. They play an important role in the defence of the upper respiratory and alimentary tracts, and even of distant sites such as mammary glands and urogenital tract (Brandtzaeg and Halstensen, 1992, Brandtzaeg et al., 1999). They are a well-known portal of entry and site of multiplication and persistence for pathogens in humans and domestic animals (Appel, 1969, Williams et al., 1973, Fekadu et al., 1983, Perry and Whyte, 1998). The tonsillar epithelium forms deep branching crypts, markedly increasing the surface area between the external environment and lymphoid tissue. The crypt epithelium is a modified type of stratified squamous epithelium which is variably highly infiltrated by non-epithelial cells, mainly lymphocytes, giving it a reticular appearance (Brandtzaeg, 1984). This lymphoepithelium forms the primary barrier of the tonsil and can sample and translocate antigens to the underlying lymphoid tissue (Perry and Whyte, 1998). In pigs the palatine tonsils are oval, paired structures in the soft palate, with 160 to 190 crypts, and the crypt epithelium, as in other species, is extensively and highly infiltrated by lymphocytes (Belz and Heath, 1996). Experimental transport of carbon particles and bacteria across this epithelium to the underlying lymphoid tissue has been demonstrated (Williams and Rowland, 1972, Williams et al., 1973). The palatine tonsils of pigs are important portals of entry and carrier sites for several species of microorganisms (Williams et al., 1973, Narita et al., 1984, Wood et al., 1989, Fedorka-Cray et al., 1995, Arends et al., 1997).

In the last 15 years, a large panel of monoclonal antibodies reacting with specific cell subpopulations of the porcine immune system has been developed and characterized in the First, Second and Third International Workshops on Swine Leukocyte Differentiation Antigens (Lunney et al., 1994, Saalmuller, 1998, Haverson et al., 2001). Since then, numerous studies have characterized the leukocyte subpopulations in the blood and lymphoid tissue of pigs (Jonjic et al., 1987, Bianchi et al., 1992, Vega-Lopez et al., 1993, Yang and Parkhouse, 1996, Zuckermann and Gaskins, 1996, Zuckermann and Husmann, 1996, Lackovic et al., 1999). Although some of these studies included the palatine tonsil (Jonjic et al., 1987, Yang and Parkhouse, 1996, Zuckermann and Gaskins, 1996, Zuckermann and Husmann, 1996), the tonsillar crypt epithelium has not been investigated specifically. Except for one study (Jonjic et al., 1987), all of these used flow cytometric analysis and consequently did not describe the specific morphologic distribution of cell subpopulations.

Given the primary barrier function of the tonsillar crypt epithelium and its capability of sampling and translocating antigens, a detailed knowledge of the lymphocyte subpopulations in this site is essential to understand the initial steps in the pathogenesis of infectious diseases and to develop efficient methods of mucosal vaccination. Since there are no reports on the lymphocyte subpopulations in the porcine tonsillar crypt epithelium, the specific subsets in healthy 6-month-old market pigs are characterized using monoclonal antibodies against CD3, CD4, CD8, γδ TCR, and λ-immunoglobulin light-chain in an ABC technique.

3.2 - Material and methods

3.2.1 - Animals and samples

Palatine tonsils from 10 healthy market pigs, approximately 6-month-old and derived from the same minimum-disease herd, were obtained from a commercial slaughterhouse. A tissue sample from the caudal half of the left tonsil from each pig was collected. The tissues were kept on ice for 2 hours until reaching the laboratory where they were embedded with Tissue-Tek O.C.T. compound (Miles), snap frozen in liquid nitrogen and stored at -70 C.

3.2.2 - Monoclonal antibodies

The following antibodies against porcine lymphocyte markers were used: anti-CD3 and anti-immunoglobulin light-chain (Ig-Lc) donated by Dr K. Haverson, Bristol, UK; anti-CD4 and anti-CD8α (American Type Culture Collection, Manassas, USA); and anti-CD8β and anti-γδ TCR (Veterinary Medical Research and Development, Pullman, USA). The Ig-Lc used is now known to recognize only a subset of the λ-light chain positive B lymphocytes (Sinkora et al, 2001). Anti-CD8α antibody binds to both $CD8^{high}$ and $CD8^{low}$ expressing lymphocytes, corresponding to the classic cytotoxic T lymphocytes and the CD4/CD8 double positive (DP) cells, respectively. Anti-CD8β antibody binds only to the $CD8^{high}$, the cytotoxic T lymphocytes (Zuckermann et al., 1998). As negative substitution controls, isotype matched irrelevant antibodies were used, namely mouse IgG1, IgG2a and IgG2b (DAKO). Antibody specificities and concentrations are given in Table 3.1.

3.2.3 - Immunohistochemistry

Serial 6 μm frozen sections were cut from each block of palatine tonsils, air-dried for a minimum of 5 minutes, pre-fixed in cold acetone (4°C) for 10 seconds and stored at -70°C. Prior to staining, the slides were fixed in cold acetone (4°C) for 5 minutes. Slides to be stained for CD8α and β were fixed instead in 100% cold ethanol (4°C) for 8 minutes. The staining technique used was a modification of an ABC technique (Griebel et al., 1992). All steps were carried out at room temperature, unless otherwise indicated, with three 5-minute washes in automation buffer (Biomeda Corp.) between each step. Non-specific binding of antibodies to tissue sections was inhibited by incubating slides for 20 minutes in a solution of 5% horse serum in phosphate-buffered saline (PBS). This same solution was used as diluent for the primary and secondary antibodies. Non-specific tissue binding of avidin and biotin was blocked (Avidin/Biotin Blocking Kit, Vector Laboratories). The sections were incubated with the primary antibody solution overnight at 4°C and on the following day with biotinylated horse anti-mouse IgG (secondary antibody) at 1/400 for 30 minutes (Vector). The secondary antibody solution contained 10% normal porcine serum to inhibit any cross-reactivity with host tissue

immunoglobulins. Endogenous peroxidase was blocked by incubation for 20 minutes in a solution of 0.1% sodium azide and 0.03% H_2O_2 in PBS. After incubation for 60 minutes in ABC solution (Vectastain ABC, Vector), the peroxidase was developed for 5 minutes in a solution of 1 mg/ml of 3,3 diaminobenzidine tetra-hydrochloride (Sigma) and 0.1% H_2O_2 in PBS. Sections were counterstained for 30 seconds in Gill's hematoxylin solution (Sigma), washed, dehydrated through graded concentrations of ethanol, cleared in xylol and mounted. Prior to secondary fixation, the slides for Ig-Lc staining were soaked in PBS for 7 hours at 4°C to reduce staining of intercellular immunoglobulin.

3.2.4 - Image Analysis and Statistics

For each animal, 10 fields of crypt epithelium were captured from a light microscope using the image analysis software Northern Eclipse (Empix Imaging, Inc.) and monochrome CCD camera. This procedure was carried out for each antibody and repeated for the corresponding areas from control slides. For each antibody, an average of 1 mm^2 of crypt epithelium was captured per animal. The image was enhanced by blue filter #47 and camera controls so that positive staining appeared black and background staining white to gray. The area of crypt epithelium was measured and stained cells were counted in each field. For each test antibody, the number of non-specifically stained cells was subtracted from the total. For CD8 staining, adjacent serial sections were used and the fields captured from slides stained with anti-CD8α antibody were matched with the corresponding field of slides stained with anti-CD8β antibody. From the 10 fields, a mean of the number of positive cells per mm^2 of crypt epithelium was calculated for each antibody in every animal; then a mean value for each antibody was calculated from 10 animals. For each of the six antibodies, the data obtained from individual animals and the combined data from all ten were displayed in separate box plots.

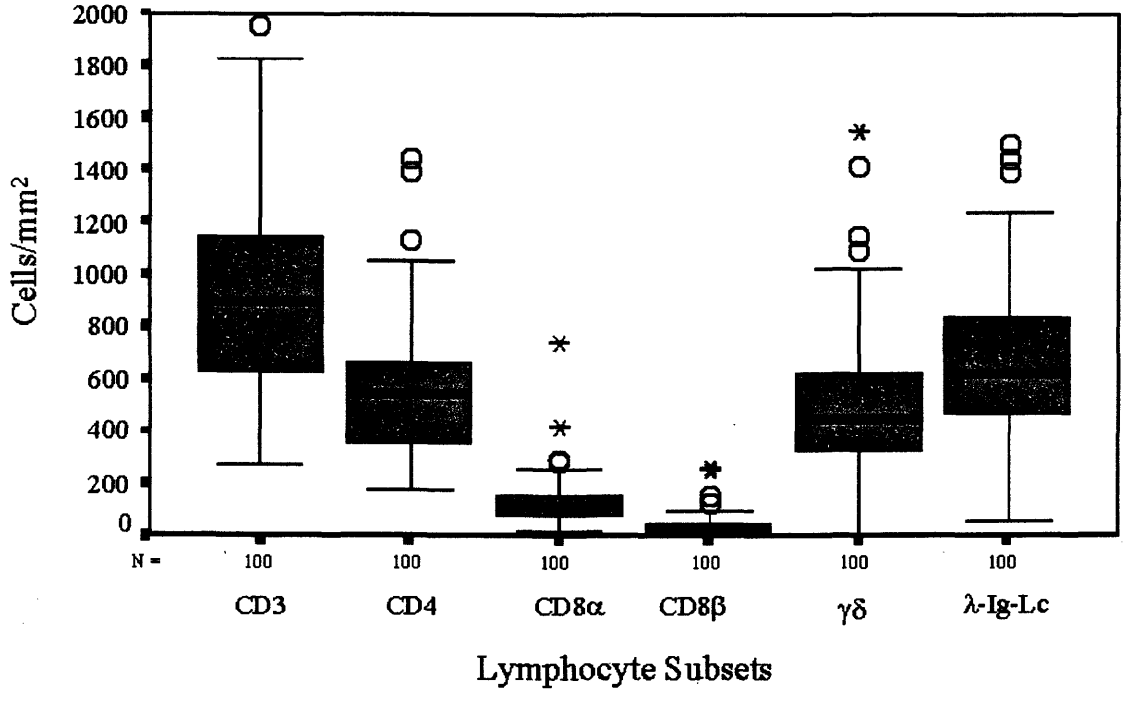
Table 3.1 - Monoclonal antibodies: specificities and concentrations

Antibody	Ig Isotype	Specificity	Concentration (μg/ml)
FY1H2	IgG1	CD3	1.2
74-12-4[a]	IgG2b	CD4	0.62
76-2-11[b]	IgG2a	CD8α	3.82
PG164A	IgG2a	CD8β	10
PGBL22A	IgG1	γδ TCR	2
K139.3E1	IgG2a	Ig-Lc[c]	0.08
X 0931[d]	IgG1	control[d]	1 (CD3)[e] 2 (γδ TCR)
X 0943[d]	IgG2a	control[d]	2 (CD8 α & β) 0.1(Ig-lc)
X 0944[d]	IgG2b	control[d]	0.58 (CD4)

[a] ATCC HB-147.
[b] ATCC HB-143.
[c] λ-Immunoglobulin light-chain
[d] Mouse negative control (DAKO) with specificity against *Aspergillus niger* glucose oxidase which is not present in mammalian tissues.
[e] Corresponding antibodies for which each control was used are given in parenthesis.

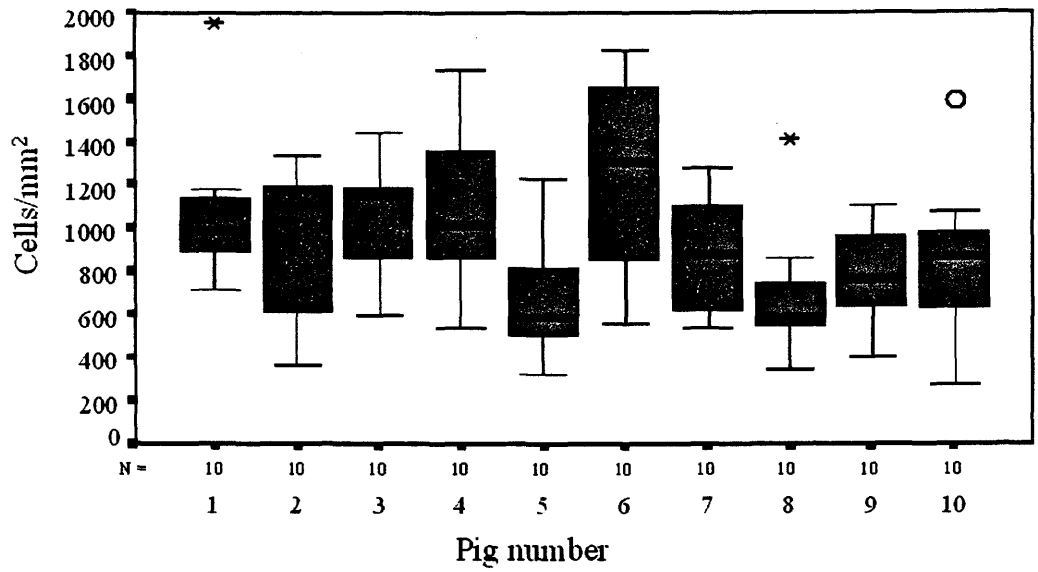
3.3 - Results

In this study, anti-CD3 and anti-λ-Ig-Lc were used to define the major lymphocyte populations, namely T and B lymphocytes, respectively. Anti-CD4, anti-CD8 and anti-γδ TCR were used to determine the relative proportions of the T cell subpopulations. For each lymphocyte subset, the number of cells infiltrated in the tonsillar crypt epithelium was variable among animals and within individual animals (Figs. 3.1 to 3.7). For example, the coefficient of variation (CV = 100 x standard deviation/mean) of B lymphocytes for 10 animals was 26%, and the CV of B lymphocytes for pig 1 was 41%.



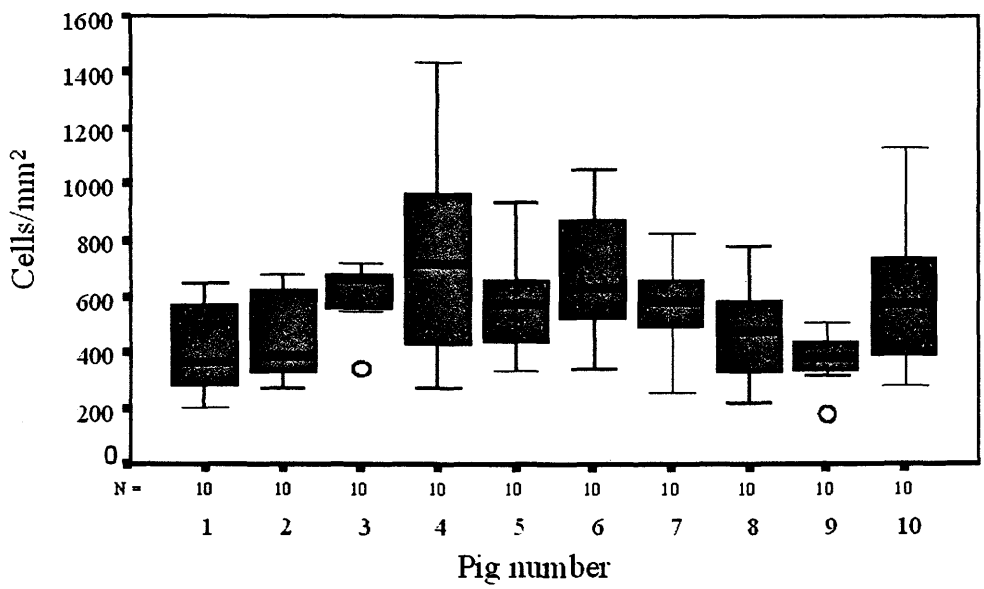
Means: CD3 = 920, CD4 = 552, CD8α = 128, CD8β = 37, γδ = 517, λ-Ig-Lc = 654

Fig. 3.1 – Distribution of lymphocyte subsets in the tonsillar crypt epithelium of 6-month-old pigs. Box plot of the data obtained from 10 fields in each of 10 animals. The box contains 50% of the values. Vertical extension lines represent 95% of the values. The horizontal line within the box indicates the median, (o) outliers, and (*) far outliers.



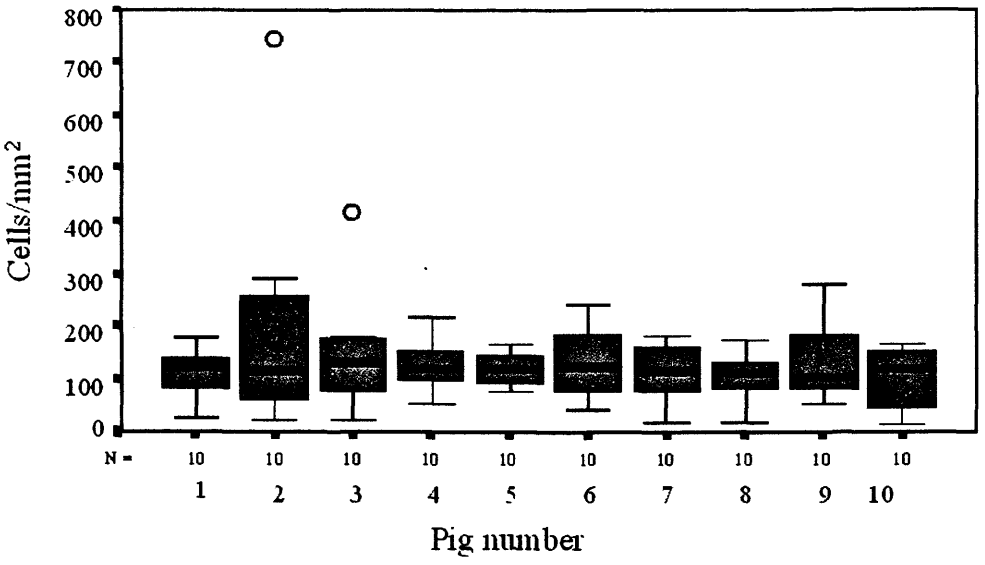
Means: 1 = 1066, 2 = 952, 3 = 1044, 4 = 1069, 5 = 647, 6 = 1240, 7 = 876, 8 = 681, 9 = 779, 10 = 850

Fig. 3.2 – CD3 positive lymphocytes in the crypt epithelium of the palatine tonsils. Data obtained from 10 fields in each of 10 six months old pigs (box plot, see Fig. 3.1).



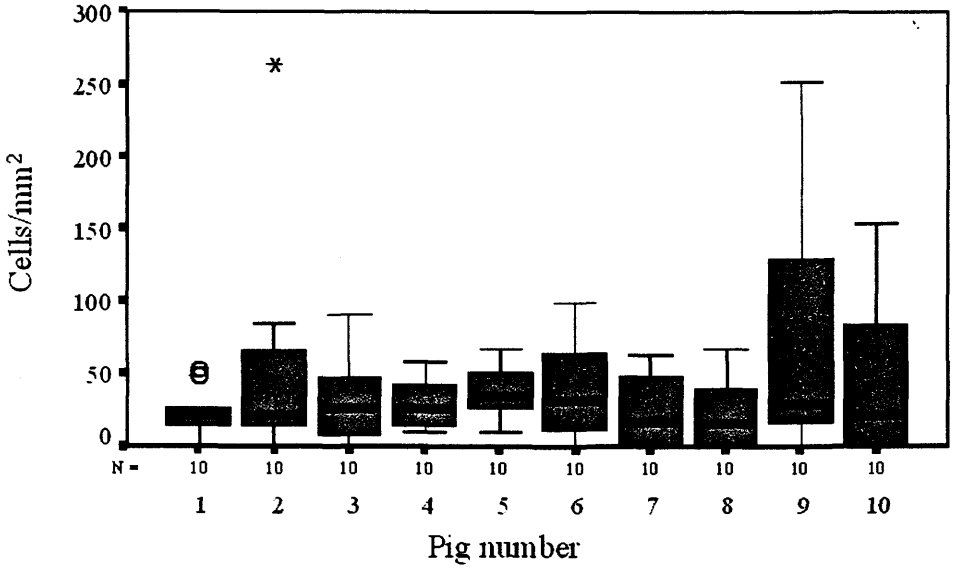
Means: 1 = 412, 2 = 441, 3 = 612, 4 = 791, 5 = 568, 6 = 675, 7 = 554, 8 = 465, 9 = 379, 10 = 621

Fig. 3.3 – CD4 positive lymphocytes in the crypt epithelium of the palatine tonsils. Data obtained from 10 fields in each of 10 six months old pigs (box plot, see Fig. 3.1).



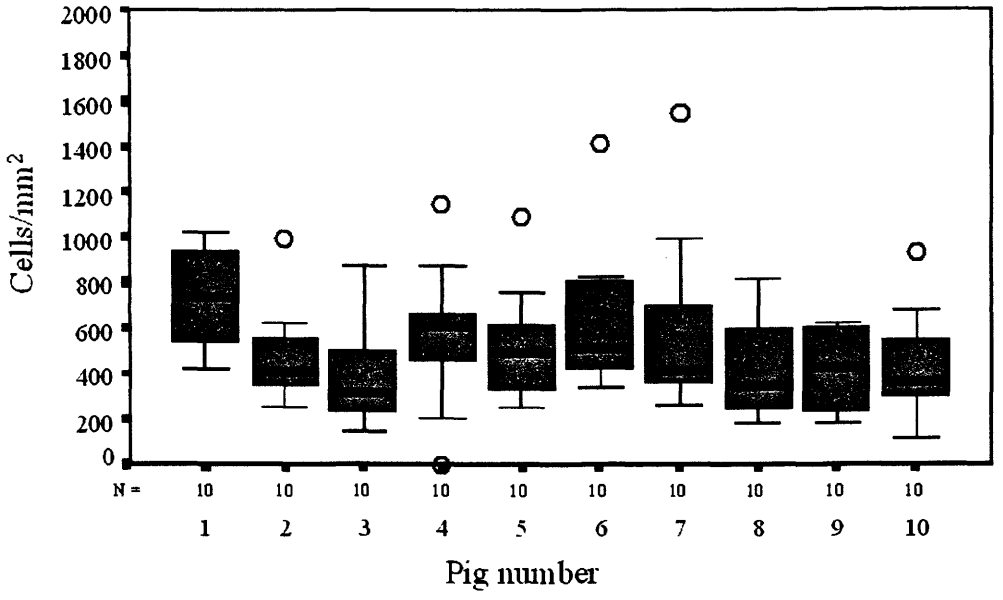
Means: 1 = 111, 2 = 194, 3 = 144, 4 = 121, 5 = 120, 6 = 133, 7 = 112, 8 = 108, 9 = 136, 10 = 103

Fig. 3.4 – CD8 α positive lymphocytes in the crypt epithelium of the palatine tonsils. Data obtained from 10 fields in each of 10 six month-old pigs (box plot, see Fig. 3.1).



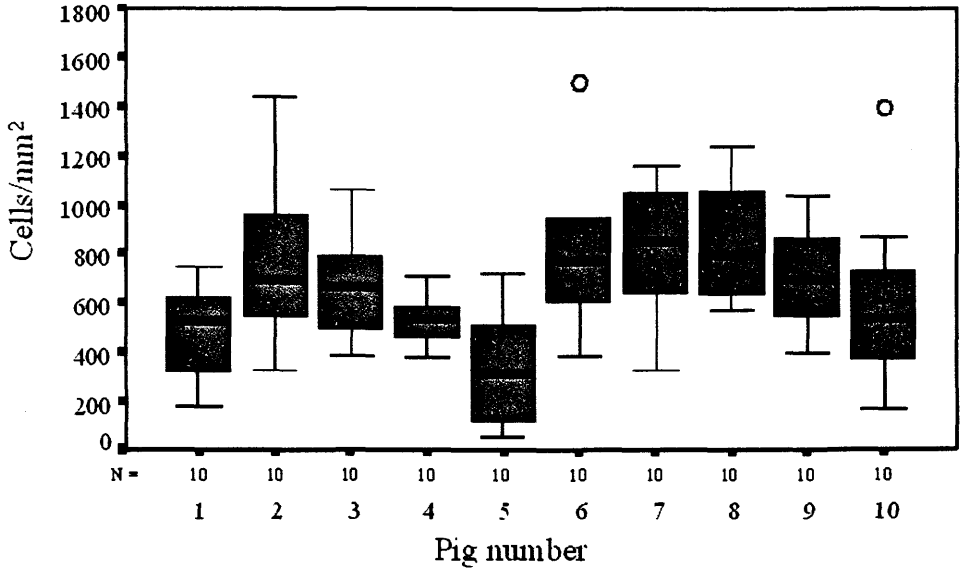
Means: 1 = 23, 2 = 52, 3 = 33, 4 = 48, 5 = 38, 6 = 40, 7 = 23, 8 = 23, 9 = 67, 10 = 42

Fig. 3.5 – CD8 β positive lymphocytes in the crypt epithelium of the palatine tonsils. Data obtained from 10 fields in each of 10 six month-old pigs (box plot, see Fig. 3.1)



Means: 1 = 724, 2 = 468, 3 = 382, 4 = 567, 5 = 534, 6 = 635, 7 = 591,
8 = 412, 9 = 419, 10 = 438

Fig. 3.6 - γδ positive lymphocytes in the crypt epithelium of the palatine tonsils. Data obtained from 10 fields in each of 10 six month-old pigs (box plot, see Fig. 3.1).



Means: 1 = 481, 2 = 749, 3 = 685, 4 = 523, 5 = 329, 6 = 805, 7 = 826,
8 = 854, 9 = 696, 10 = 550

Fig. 3.7 - λ-Ig-Lc positive lymphocytes in the crypt epithelium of the palatine tonsils. Data obtained from 10 fields in each of 10 6-month-old pigs (box plot, see Fig. 3.1).

There were more CD3 cells than λ-Ig-Lc positive cells in the crypt epithelium and both subpopulations were diffusely distributed in this site (Fig. 3.8). CD3 cells were also diffusely and uniformly distributed throughout the interfollicular area, with a moderate number in the follicles.

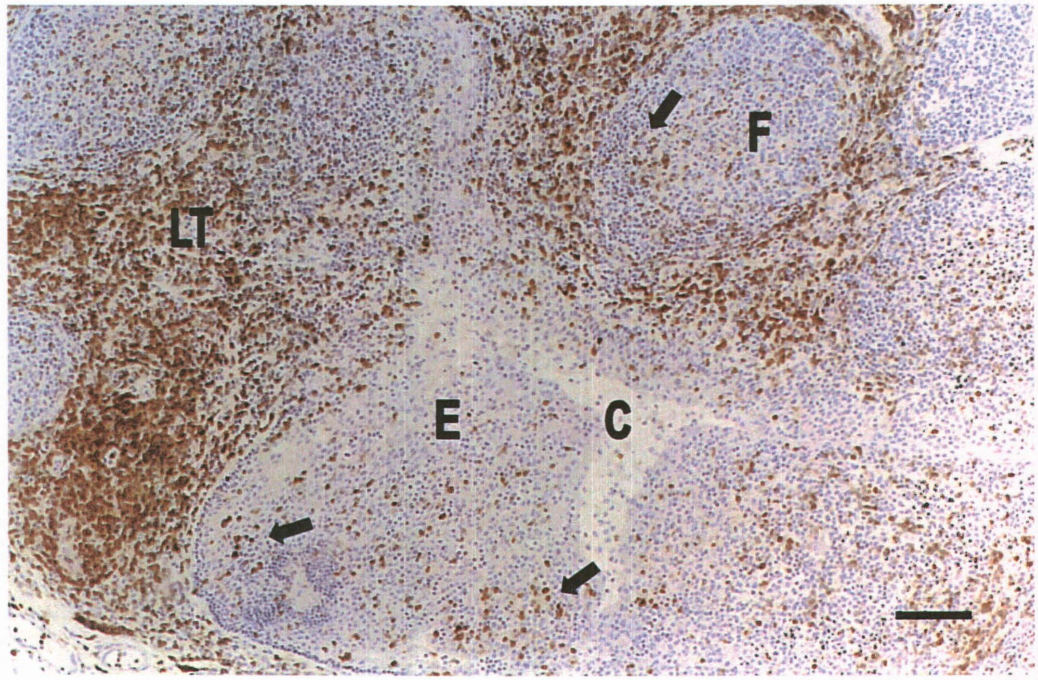


Fig. 3.8 – Section of palatine tonsil stained with ABC for CD3 (pan-T cell marker). There are many CD3 positive cells (arrows) in the crypt epithelium. In the subepithelial lymphoid tissue, the CD3 cells are widely and uniformly distributed throughout the interfollicular area, and many positive cells are also present in follicles (arrow).
C – crypt lumen, E - crypt epithelium, LT – lymphoid tissue, F – lymphoid follicle.
Bar = 100μm.

In contrast, λ-Ig-Lc positive cells were mainly in the crypt epithelium and subjacent to it (Fig. 3.9), with few cells in the follicular mantle zone (3-10 cells per follicle) and in the interfollicular area (10-20 cells at 20x magnification field). Strong intercellular positive staining was present centrally in the germinal centres. Most of λ-Ig-Lc positive cells had abundant cytoplasm and an eccentric nucleus. Occasionally,

individual or small groups of crypt epithelial cells were positive for λ-Ig-Lc and intense amorphous positive staining was frequently observed in the lumen of the crypts.

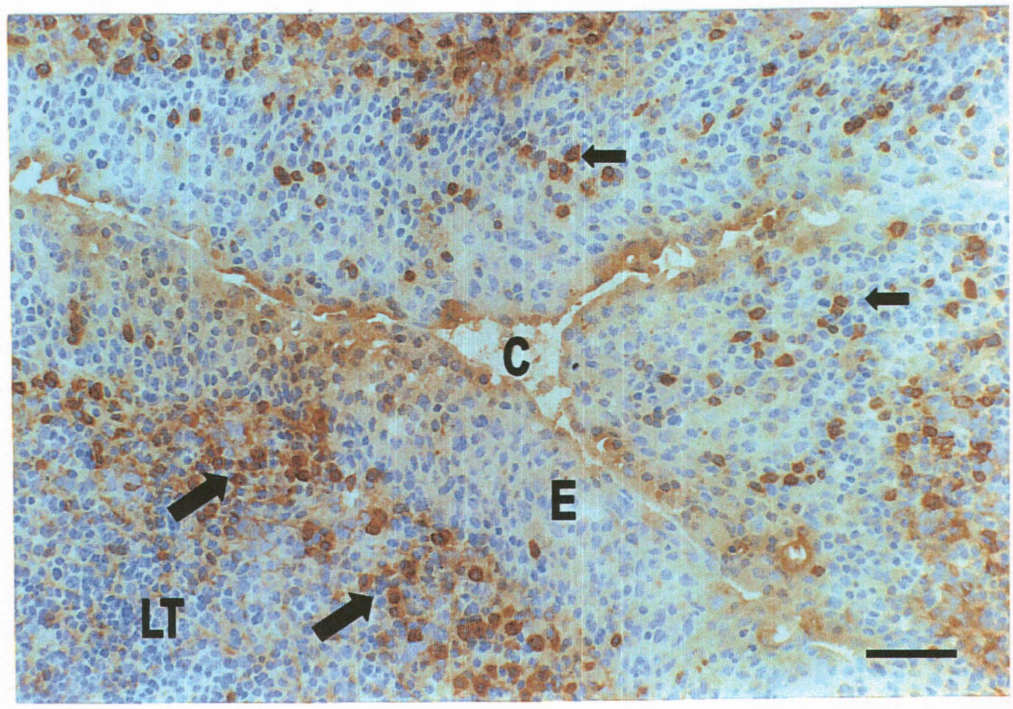


Fig. 3.9 – Section of palatine tonsil stained with ABC for λ-immunoglobulin light-chain (B lymphocytes). Many positive cells (small arrows) are present in the crypt epithelium and in the immediately subjacent lymphoid tissue (arrows).
C- crypt, E - crypt epithelium, LT – lymphoid tissue. Bar = 60μm.

Within the T cell population, the helper T CD4 cells (Fig. 3.10) and the γδ cells (Fig. 3.11) formed the largest subsets; both were extensively distributed throughout the crypt epithelium. CD4 cells were widely and homogeneously distributed in the perifollicular area, with many cells (20 to more than 50) in the follicles. In contrast, in lymphoid tissue γδ cells had a patchy pattern of distribution, were less numerous, usually concentrated subjacent to the crypt epithelium (Fig. 3.11) and rarely present in the follicles. The number of γδ cells in the epithelium was variable among animals (CV = 22%) and from one field to another within each animal, e.g., the CV was 32% in pig 1.

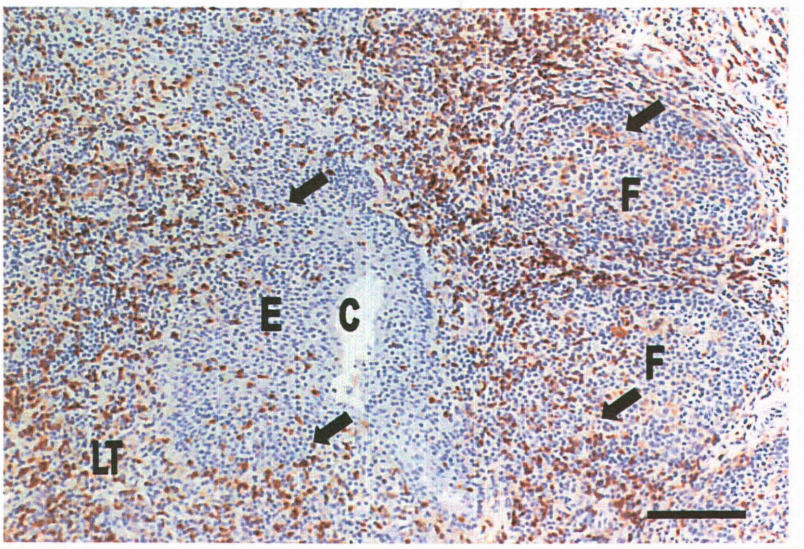


Fig. 3.10 - Section of palatine tonsil stained with ABC for CD4 (T helper lymphocytes). The crypt epithelium is highly infiltrated by CD4 cells (arrows). CD4 cells are widely distributed in the interfollicular area and many positive cells (arrows) can also be seen in the lymphoid follicles. C – crypt lumen, E - crypt epithelium, LT - lymphoid tissue, F- lymphoid follicle. Bar = 150μm.

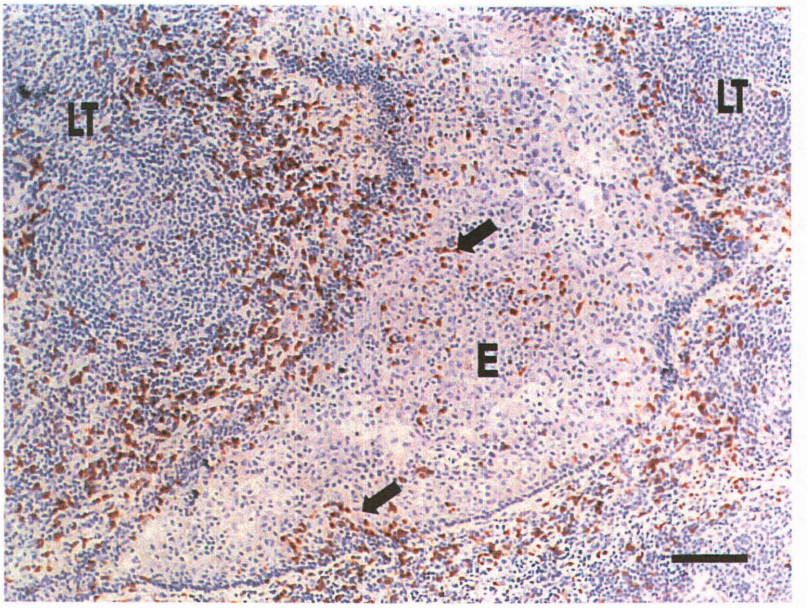
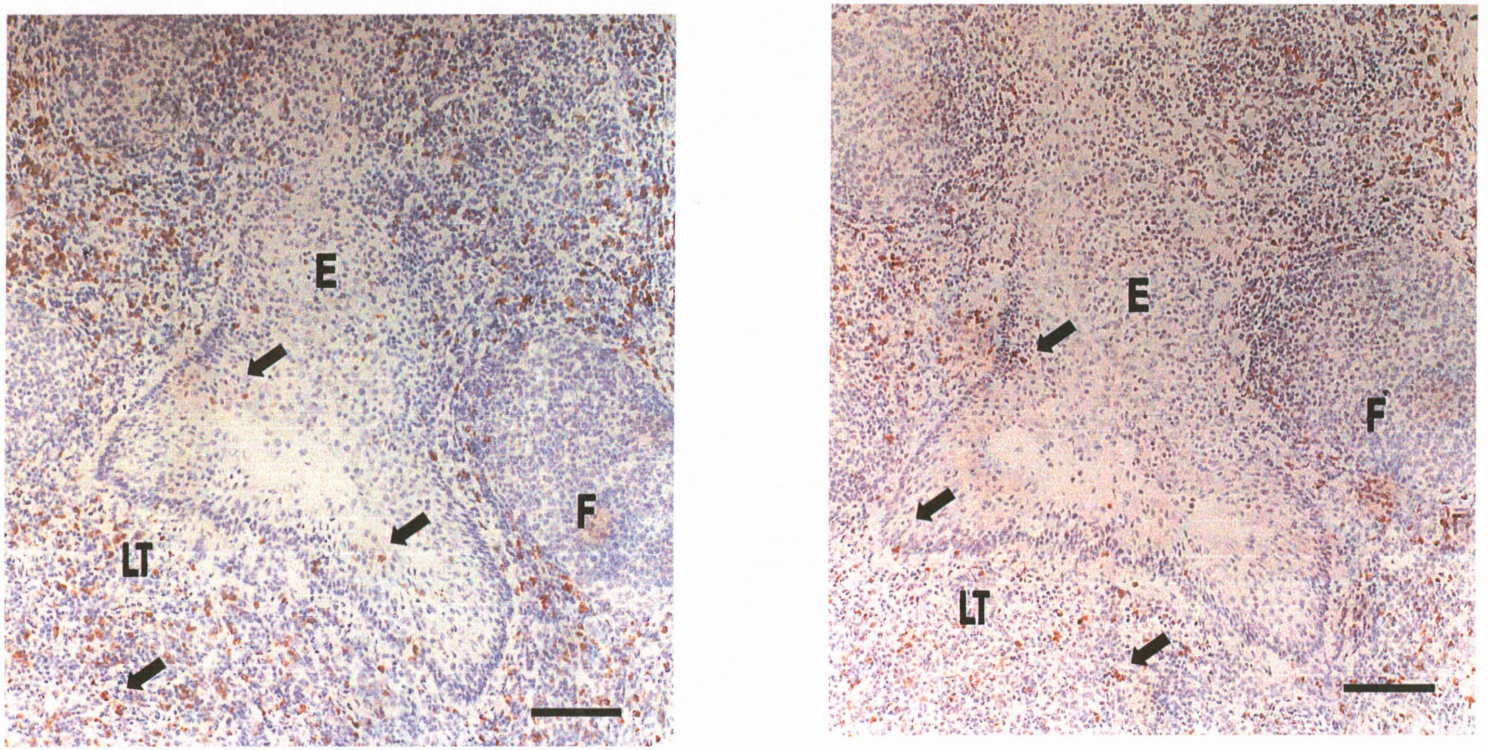


Fig. 3.11 - Section of palatine tonsil stained with ABC for γδ T lymphocytes. γδ T lymphocytes (arrows) are concentrated in the crypt epithelium and in the immediately subjacent lymphoid tissue. E – crypt epithelium, LT – lymphoid tissue. Bar = 100μm.

Both the total CD8 cells (CD8α) and the cytotoxic CD8 cells (CD8β) were distributed sparsely in the crypt epithelium (Fig. 3.12a & b). The distribution of CD8α and CD8β in the perifollicular area was very uneven and the numbers extremely variable among animals. Only a very few CD8α and CD8β cells were present in the follicles.



a b

Fig. 3.12 – Serial adjacent sections stained with ABC for CD8α (total CD8 cells) and CD8β (cytotoxic CD8 cells). **a** - CD8α T lymphocytes: very few positive cells are present in the crypt epithelium (arrows), and they are low in number in the lymphoid tissue (arrows) especially in the follicles. **b** - CD8β T lymphocytes: positive cells (arrows) are even fewer than CD8α T lymphocytes.
E – crypt epithelium, LT- lymphoid tissue, F – lymphoid follicle. Bars =100μm.

3.4 - Discussion

A diversity of lymphocyte subsets was identified in the crypt epithelium, and marked variation within individual animals and among animals was also observed. This high level of variation among animals could be influenced by factors such as health status, environment or age. In this study all animals were apparently healthy, came from the same herd on the same day and were approximately the same age; therefore, we would not expect these factors to underlie this variation. Pigs from different pens might have been subjected to different environments, including exposure to different penmates. Another potential influencing factor could be the sample size. Perhaps ten animals and ten fields per animal were inadequate, given the unexpected individual variation obtained. Finally, the large variation in the intra-epithelial lymphocyte subsets in different crypts within the same animal could contribute to the differences noticed among animals. A variable degree of lymphocyte infiltration throughout the crypt epithelium of the same individual is in accordance with findings in humans (Perry, 1994) and pigs (Belz and Heath, 1996) and probably reflects heterogeneous antigenic stimulation from one crypt to another (Perry, 1994).

The sum of the individual T cell subpopulations examined (CD4, CD8 and γδ) exceeded the number of the total T cell population (CD3). A number of factors may have contributed to this. Within each animal, the fields analyzed for the different antibody markers did not necessarily correspond to each other, except for the CD8 staining. Another factor is that double-positive CD4/CD8 (DP) cells present would be included in both CD4 and CD8 compartments. The method used did not allow the simultaneous identification of both markers. Based on the findings of the CD8 staining, however, a maximum of 17% of the CD4 cells could co-express CD8. As CD8α antibody reacts with both cytotoxic ($CD8^{high}$) and DP cells ($CD8^{low}$), and CD8β antibody binds only the classic cytotoxic T lymphocytes, the difference between these two subpopulations represents the $CD8^{low}$. Other subsets that may also express CD8 at low densities are αβ single CD8, γδ CD8 and natural killer cells (Yang and Parkhouse, 1996, Zuckermann et al., 1998). While CD4 and CD8 molecules are generally considered to be mutually exclusive in mature lymphocytes, pigs, chickens and monkeys can have a

high proportion of DP cells (Zuckermann, 1999). DP cells can vary from 8 to 64% of the resting peripheral T lymphocytes in adult pigs (Saalmuller et al., 1987). In humans, DP cells are rare in peripheral blood, comprising around 3% in healthy individuals (Blue et al., 1985); higher proportions (15%), however, have been seen, associated mainly with disease (Ortolani et al., 1993). DP cells are also detected in porcine lymphoid tissues such as lymph nodes, Peyer's patches and tonsils, with the greatest proportion, 62% of the CD4 lymphocytes, in the tonsil, compared to 35% in lymph nodes (Zuckermann and Gaskins, 1996). While the function of DP cells in pigs has not been specifically determined, evidence suggests that they are memory cells (Dillender and Lunney, 1993, Pescovitz et al., 1994, Zuckermann and Husmann, 1996, Summerfield et al., 1996).

If the calculated memory cell population (DP cells) is 17% or less of the intraepithelial CD4 cells for this 6-month-old age group, then the remainder (83%) represents the CD4 subset of naïve or recently activated cells. This contrasts with the fact that in flow cytometric analyses, DP cells formed a large proportion (60%) of the CD4 cells in preparations from whole tonsils of 6-month-old pigs (Zuckermann and Gaskins, 1996). It also contradicts evidence that most lymphocytes in the crypt epithelium of the palatine tonsils of humans are positive for memory markers (Ruco et al., 1995), and that at epithelial surfaces memory cells are expected to dominate (Mackay, 1993). To confirm however if there is indeed a higher proportion of naïve cells in the crypt epithelium of the palatine tonsils of pigs, the use of antibodies against activation markers, such as CD45RA and CD45RC, which are present in naïve lymphocytes (Zuckermann et al., 2001), will be necessary.

The findings of this study indicate that approximately half of the T cell population in the crypt epithelium belongs to the γδ subset. The functions of γδ T lymphocytes and what they recognize are still unclear. This particular subset lacks CD4 and usually CD8. It does not require protein-processing pathways for antigen recognition and is not MHC-restricted, unlike CD4 and CD8 cells. Evidence suggests that γδ T lymphocytes recognize antigens common to microorganisms and stressed cells, that their response is rapid and precedes CD4 and CD8 cells activation, and that by producing the appropriate cytokines, they have an immunoregulatory role in the early

phase of infection (Boismenu and Havran, 1997). These cells participate in the maintenance of the epithelial integrity by secreting growth factors and recruiting inflammatory cells (King et al., 1999). Although they comprise only 4% of the human peripheral blood T cell population, they increase significantly during a variety of infectious diseases (Bukowski et al., 1999). They are found in higher proportions on epithelial surfaces. In mice, γδ T lymphocytes predominate in the epithelia such as the intestine and skin (Raulet, 1989), and in humans approximately 30% of the intestinal intraepithelial lymphocytes are γδ T lymphocytes (Deusch et al., 1991). Unlike other species, γδ T lymphocytes represent a large subpopulation of peripheral blood T lymphocytes in cattle, sheep and pigs (Davis et al., 1998). In young pigs, they are the dominant T cell subpopulation in the peripheral blood and they may have an important role in immunity prior to maturation of the αβ TCR subsets. In flow cytometric studies on tonsils of 4-week-old pigs, only 2.5% of the lymphocytes were γδ T lymphocytes (Yang and Parkhouse, 1996), but no studies have documented their presence in the crypt epithelium. In human tonsils, γδ T lymphocytes are more numerous in chronic infections and are especially concentrated in the crypt epithelium (Graeme-Cook et al., 1993, Yamanaka et al., 1996, Olofsson et al., 1998).

In this study, B lymphocytes were identified using an antibody to immunoglobulin light-chain (Ig-Lc), which detects cytoplasmic, membrane bound and free immunoglobulin, without specificity for immunoglobulin class (Denham et al., 1998). The number of B lymphocytes detected was most likely an underestimation, since the antibody used to detect these cells was recently described to be specific for the λ chain of the immunoglobulin light chain (Sinkora et al., 2001). In pigs, B lymphocytes express approximately the same proportions of the two light chains, κ and λ (Sinkora et al., 2001); hence the number of these cells in the crypt epithelium could be twice, or more, the value obtained in this study. The morphology of the majority of λ-Ig-Lc positive cells in our study was consistent with that of plasma cells. The strong amorphous positive staining in the crypt lumen was likely free immunoglobulin, similar to the pattern described in tonsils of pigs stained with anti-IgG antibody (Ramos et al., 1992). The source of these luminal immunoglobulins could be the crypt epithelium B

cells or the saliva. Despite the absence of secretory component (SC) in human tonsillar epithelial cells (Brandtzaeg et al., 1978, Tang et al., 1995), immunoglobulins are transferred to the lumen by intercellular diffusion (Brandtzaeg et al., 1978). Although there are no studies showing the presence of SC in the porcine tonsillar epithelium, the positive staining of few epithelial cells for Ig-lc could indicate active immunoglobulin transport. The strong intercellular positive staining in the germinal centres probably represents immune complexes (Bianchi et al., 1992).

In a study of the intra-epithelial lymphocyte subsets in the small intestine of pigs, there was a predominance of CD4-CD8- and CD8+ over CD4+ cells (Vega-Lopez et al., 1993), the double-negative subset probably representing mainly γδ T lymphocytes. In the present study, using an antibody against γδ TCR, these cells were found in high number also in the tonsillar crypt epithelium. In contrast, the proportions of CD4+ and CD8+ in the tonsillar crypt epithelium were reversed, the CD4+ cells being numerous and CD8+ cells few, possibly reflecting exposure to a different microenvironment. In pigs, while CD8+ cells are known to predominate in the intestinal epithelium, B lymphocytes predominate in the epithelium overlying the PP, and no CD8+ cells are observed at these sites (Bianchi et al., 1992). These findings seem to reflect the different functions of these epithelia in the intestine. The crypt epithelium, being part of MALT, likely has a function comparable to that of the epithelium overlying the PP. In the intestine of humans, the high number of intraepithelial CD8+ cells (50% to 80% of the intraepithelial lymphocytes) has been associated with oral tolerance (Kelsall and Strober, 1999); maybe this is also the case in pigs, and could explain the higher proportion of CD8+ cells in the intestinal epithelium in contrast to the tonsillar crypt epithelium. The findings of this study are in agreement with those on human tonsillar crypt epithelium where a predominance of CD4+ cells over CD8+ cells and a high concentration of γδ T lymphocytes were noticed (Graeme-Cook et al., 1993, Ruco et al., 1995, Tang et al., 1995).

The intraepithelial presence of both T lymphocytes and B lymphocytes indicates that the tonsillar crypt lymphoepithelium is capable of participating in both cellular and humoral immune responses. CD4 cells predominate over CD8 cells. γδ T lymphocytes

represent a large proportion of the T lymphocytes, which could indicate that these cells in pigs, as reported for mice, have an important role in mucosal defence.

4 - Changes in the Leukocyte Subpopulations of the Palatine Tonsillar Crypt Epithelium of Pigs in Response to *Streptococcus suis* serotype 2 Infection

4.1 - Introduction

The palatine tonsils are part of MALT, a specialized compartment of the immune system distributed at the mucosal surfaces lining the gastrointestinal, respiratory and genitourinary tracts of mammals and birds (Gebert and Pabst, 1999). In pigs the palatine tonsils are large paired structures located strategically within the soft palate near the entrance of both respiratory and alimentary tracts (Belz and Heath, 1996). The importance of tonsils in the pathogenesis of infectious diseases is illustrated by the many pathogens utilizing them either as a portal of entry, or site of replication or persistence; e.g. *Actinobacillus pleuropneumoniae* and *Mycoplasma hyosynoviae* in pigs (Chiers et al., 1999; Hagedorn-Olsen et al., 1999), *Mannhemia* (*Pasteurella*) *haemolytica* in lambs and cattle (Al Sultan and Aitken, 1985; Shoo et al., 1990), herpes viruses in pigs and cattle (Narita et al., 1984; Schuh et al., 1992), poliovirus, Epstein-Barr virus and HIV, and *Neisseria meningitidis* in humans (Ogra, 1971; Hirao et al., 1996; Frankel et al., 1997; Bevanger et al., 1998). The tonsillar lymphoid tissue is overlain by mucosal epithelium and lacks afferent lymphatics, features specific to MALT. In pigs particularly, the surface area of the palatine tonsil is markedly increased by deep epithelial invaginations within the lymphoid tissue forming numerous branching crypts. The crypt epithelium is extensively infiltrated by leukocytes, giving it a reticulated appearance (Perry, 1994). This lymphoepithelium forms the first line of defence of the palatine tonsil and can sample and translocate antigens from the lumen to the underlying lymphoid tissue (Williams and Rowland, 1972; Williams et al., 1973; Perry and Whyte, 1998).

In Chapter 3, specific lymphocyte subsets in the crypt epithelium of the palatine tonsils of healthy market-weight pigs, approximately 6-month-old, are characterized. Here the changes that occur in this epithelium, in response to *Streptococcus suis* (*S. suis*) serotype 2 infection, are investigated. *S. suis* serotype 2 is a common porcine pathogen, distributed worldwide, and frequently causes major economic loss due to meningitis, arthritis, pneumonia, and septicaemia, mostly in animals between 3 and 16 weeks old (Staats et al., 1997). Although many virulence factors are described for *S. suis*, the pathogenesis of this disease is still not clarified and no efficient vaccine has been developed (Staats et al., 1997; Gottschalk and Segura, 2000). The palatine tonsils are a portal of entry (Williams et al., 1973) and an important carrier site of this pathogen (Davies and Ossowicz, 1991). Understanding the changes that occur in the first barrier of the tonsil, i.e. the crypt epithelium, in response to *S. suis* is essential for clarifying the initial steps in the pathogenesis of this disease and for the future development of efficient methods of mucosal vaccination.

The purpose of this chapter is to describe the early changes that occur in the leukocyte subpopulations in the crypt lymphoepithelium of the palatine tonsils of 3-week-old piglets in response to an experimental infection with *S. suis* serotype 2, using monoclonal antibodies against a variety of leukocyte markers in an ABC technique.

4.2 – Material and Methods

4.2.1 - Construction of *Streptococcus suis* SX 426

A strain of *S. suis* serotype 2 was constructed at the Veterinary Infectious Disease Organization (VIDO), with genetic markers to enable its identification and to facilitate re-isolation following experimental infection. The *sagp* gene, encoding an acidic glycoprotein of *S. suis*, was isolated from a genomic library and mutated by the insertion of a DNA fragment encoding a variant of the jellyfish *Aequorea victoria gfp* gene, and the *aph*A-3 gene conferring resistance to kanamycin (Suarez et al., 1997). The mutated form of *sagp* was cloned into a temperature-sensitive shuttle vector pEU904 encoding resistance to streptomycin (a gift from Dr J. R. Scott, Emory University, Atlanta, GA). This plasmid was named pJPC214 and transformed into *S. suis* serotype 2 by

electroporation, followed by selection for the clones in which a double crossover took place by varying the incubation temperatures and examining the resistance to antibiotics (Perez-Casal et al., 1993). After incubating at 37°C on THY-agar containing 100 μg/ml of kanamycin, the colonies were screened for sensitivity to spectinomycin and fluorescence under a long-wave (360nm) ultra-violet lamp. All the colonies were sensitive to spectinomycin and did not show fluorescence, indicating loss of the plasmid containing this marker (data not shown). The constructed strain was named *S. suis* SX 426. Southern blots, carried out with genomic DNA of SX 426 to map the location and integrity of the inserted plasmid DNA, indicated that the pJPC214 DNA was integrated at the 5' end of the *sagp* gene of *S. suis* serotype 2 SX 332 by a single cross-over event. A portion of the *gfp* together with the spectinomycin genes was deleted in the *S. suis* strain (data not shown). The recombination at the 5' end of the *sagp* gene resulted in a strain still capable of expressing SAGP, as confirmed by western blot analyses of concentrated culture supernatants with specific anti-SAGP rabbit serum (data not shown).

The *S. suis* strain SX 426 was derived from the virulent strain SX 332 of *S. suis* serotype 2. SX 426 was positive for suilysin, and on sodium dodecyl sulfate-polyacrylamide gel electrophoresis presented 136 kDa and 110 kDa bands, corresponding to MRP and EF, respectively.

4.2.2 - Animals and samples

Sixteen 3-week-old healthy piglets derived from the same minimum-disease herd, free of *S. suis* serotype 2, were used in this study. Eight pigs were challenged with *S. suis*. The challenge was accomplished by spraying, using a nasal actuator dispenser 232 GI (Valois of America, Greenwich, CT), 1 ml of Todd-Hewitt broth containing 10^8 CFU/ml of *S. suis* serotype 2 strain SX426 on the surface of the palatine tonsils. Eight piglets were sprayed with the same volume (1 ml) of sterile broth and used as controls. These procedures were done under halothane anaesthesia, in accordance with the principles of Canadian Council of Animal Care. The University of Saskatchewan Campus Committee on Animal Care reviewed and approved the experimental protocols. Blood samples were collected, using heparin as anti-coagulant, on the day prior to the

challenge and at 18h, 24h, 48h and 72h post-challenge. Two pigs from each group were euthanised at the following intervals after challenge: 18h, 24h, 48h and 72h. One control pig was eliminated from the experiment due to unrelated disease. From each pig, a tissue sample from the caudal half of the left palatine tonsil was collected, embedded in Tissue-Tek OCT compound (Miles), snap frozen in liquid nitrogen and stored at – 70 ° C until use. Samples of palatine tonsils were also fixed in 10% neutral buffered formalin. The cranial portion of the left palatine tonsil, the mandibular, retropharyngeal and pulmonary hilar lymph nodes, the spleen, lung, cerebellum and hock joint were collected for bacteriology.

4.2.3 - Bacteriology

A 10% suspension in 145 mM NaCl of each of the different tissues collected at the necropsy was prepared by maceration in a stomacher. The tissue suspensions were then cultured on tryptic soy agar with 5% sheep blood (PML Microbiologicals, Wilsonville, OR) and selective media containing Todd-Hewitt broth (Oxoid, Hampshire, England), purified agar (15g/L; Oxoid) and kanamycin (0.1g/L; Sigma). Blood samples were cultured similarly. Representative kanamycin resistant and α- haemolytic colonies were selected for additional evaluation of biochemical reactions (API 20 Strep, Bio Mérieux, Inc., Hazelwood, MO).

4.2.4 - Monoclonal Antibodies

The following monoclonal Abs against porcine leukocyte markers were used: anti-CD3 and anti-λ-immunoglobulin light-chain (λ-Ig-Lc) donated by Dr K. Haverson, Bristol, UK; anti-CD4, anti-CD8α and anti-myeloid (SWC3) cells (American Type Culture Collection, Manassas, USA), and anti-γδ TCR (Veterinary Medical Research and Development (VMRD), Pullman, USA). Anti-CD8-α Ab binds to both $CD8^{high}$ and $CD8^{low}$ expressing lymphocytes, corresponding to the classic cytotoxic T lymphocytes and the CD4/CD8 double positive (DP) cells, respectively (Zuckermann et al., 1998). Anti- CD8β, an antibody that reacts only with single positive CD8 cells, was not used in this experiment due to very weak staining leading to equivocal results. For the detection of cells expressing the major histocompatibility complex class II (MHC-II), a pool of

monoclonal Abs against bovine MHC-II was used: TH16A, H42A, TH22A, TH81A and TH14B (VMRD, Pullman, USA). These monoclonal Abs cross-react with porcine MHC-II (Davis et al., 1987; Tanimoto and Ohtsuki, 1996). As negative substitution controls, isotype-matched irrelevant Abs were used, namely mouse IgG1, IgG2a and IgG2b (DAKO). For the detection of *S. suis* in the tonsils, a rabbit polyclonal Ab against *S. suis* serotype 2, donated by Dr M. Gottschalk, Univ. of Montreal, St. Hyacinthe, Can., was used. Monoclonal antibody concentrations and specificities are given in Table 4.1.

Table 4.1 - Monoclonal antibodies: specificities and concentrations

Antibody	**Ig Isotype**	**Specificity**	**Concentration (μg/ml)**
FY1H2	IgG1	CD3	1.2
74-12-4[a]	IgG2b	CD4	0.62
76-2-11[b]	IgG2a	CD8α	1.91
PGBL22A	IgG1	γδ TCR	2
K139.3E1	IgG2a	Ig-Lc[c]	0.08
74-22-15	IgG1	Myeloid (SWC3)	0.8
MHC-II[d]	IgG2a	MHC-II	1
X 0931[e]	IgG1	Control	1 (CD3 & myeloid)[f], 2 (γδ TCR)
X 0943[e]	IgG2a	Control	2 (CD8 α),1 (MHC-II), 0.1 (Ig-Lc)
X 0944[e]	IgG2b	Control	0.58 (CD4)

[a] ATCC HB-147.
[b] ATCC HB-143.
[c] Immunoglobulin λ-light-chain subset.
[d] Pool anti-bovine MHC-II antibodies: TH16A, H42A, TH22A, TH81A and TH14B.
[e]Mouse negative control (DAKO) with specificity against *Aspergillus niger* glucose oxidase which is not present in mammalian tissues.
[f]Corresponding antibodies for which each control was used are given in parenthesis.

4.2.5 - Immunohistochemistry

The same ABC technique as described in Chapter 3 was used in this experiment. For each Ab used, adjacent sections were stained for *S. suis* serotype 2 using rabbit anti-*S. suis* serotype 2 Ab and the same ABC technique, except that biotinylated goat anti-rabbit Ab was used as secondary Ab, and goat serum replaced the horse serum. For MHC-II, 4 μm sections from the formalin-fixed, routinely processed, paraffin-embedded tonsillar blocks were used instead of frozen, as a better definition of the positive cells was achieved, which facilitated cell counting.

4.2.6- Image analysis and statistics

For each infected animal, fields (at 20x magnification) of palatine tonsillar crypt epithelium positive for *S. suis* were matched with fields in adjacent sections stained with test (i.e., specific leukocyte marker) and control Abs, and were captured from a light microscope using image analysis software Northern Eclipse (Empix Imaging) and a digital DVC camera. This procedure was repeated for each test Ab: anti-CD3, CD4, CD8, γδ TCR, λ-Ig-Lc, myeloid, MHC-II, and respective control Abs. For control animals the same procedures were carried out; however, crypts with any weak cross-reactivity for *S. suis* were excluded from analysis, as rabbit anti-*S. suis* serotype 2 Ab was previously demonstrated to cross-react slightly with other streptococci of porcine tonsillar flora (Middleton, Gottschalk and Willson, unpublished data). The number of fields captured was determined by the number of available crypts that fulfilled the above criteria, and therefore differed for each leukocyte marker and for each time frame. The number of fields ranged from 4-19 and 0-23 for control and infected animals, respectively. In one instance, for the λ-Ig-Lc marker, at 48h post-infection, *S. suis* positive crypts were identified in sections from only one infected animal. Table 4.2 summarizes the number of fields analyzed for each animal. At the time of analysis, the observer was blinded to the treatment (infected or control) administered. The area of crypt epithelium was measured and positive cells were counted. For each field, the number of non-specifically stained cells in slides stained with control Ab was subtracted from the number of positive cells in the matching field from slides stained with the test

Ab. For CD8 and γδ TCR, the count was performed manually due to the weak staining obtained with these Abs. For CD3, CD4, λ-Ig-Lc, and MHC-II the count was automated with a minimal adjustment of the threshold for each image. For the myeloid marker, due to confluence and intensity of staining which obscured cellular detail, the results for positive staining are given as percentage of analysed area (method adapted from Josefsen and Landsverk, 1997).

Analysis of variance, based on a split-plot design, was performed in order to include both the variations within individual pigs and among the different pigs. First, a split-plot test, designed as cells/mm^2 (or percentage of positive area) by interval after challenge (hour), was performed with the data from control animals to evaluate if, among control pigs over the time frames, there was any significant difference that could have resulted from an effect of the sterile broth. Since none was detected, in order to encompass the high degree of expected variation in the number of cells in the crypt epithelium, the data from control pigs (7 animals) were pooled and compared with those of infected pigs of each time frame (2 animals). A split-plot test, designed as cells/mm^2, or percentage of positive area, by treatment, was used to evaluate the differences in the number of positive cells, or percentage of positive area, for each leukocyte subset at each time frame, in the crypt epithelium of *S. suis* serotype 2 infected and control pigs. The split-plot tests were done using a general linear model procedure from SPSS (Statistical Package for the Social Sciences) version 10 (Chicago, Illinois).

Table 4.2 – *Streptococcus suis* serotype 2 experimental infection in 3-week-old pigs. Number of fields captured for each animal for the different leukocyte markers.

Hours post-infection	Pig	CD3	CD4	CD8	γδ TCR	λ-Ig-Lc	MHC-II	Myeloid
18	1 (control)	4	4	4	9	8	7	8
	2 (control)	10	9	9	10	10	10	10
	3 (infected)[a]	3	3	3	4	1	6	3
	5 (infected)	7	8	8	10	9	2	10
24	8 (control)	9	8	8	10	10	10	10
	9 (control)	8	7	7	8	6	10	7
	10 (infected)	7	8	8	6	5	7	2
	11 (infected)	5	3	3	8	5	1	5
48	15 (control)	10	10	10	10	10	10	10
	16 (infected)	3	4	4	2	0	2	3
	17 (infected)	5	7	7	10	13	12	8
72	20 (control)	10	10	10	10	12	5	10
	21 (control)	16	14	14	18	18	19	18
	22 (infected)	7	8	8	6	7	12	7
	23 (infected)	20	19	19	17	23	12	18

[a] In infected animals all fields correspond to *S. suis* infected crypts.

4.3 - Results

The bacteriological results of infected and control animals are summarized in Table 4.3.

Table 4.3 – *Streptococcus suis* serotype 2 experimental infection in 3-week-old pigs. *Post-mortem* isolation of *S. suis* serotype 2 (CFU/g) from tissues of infected piglets.

Hours post-infection	Pig	Lung	Pulmonary hilar lymph node	Spleen
18	1 (control)	–[a]	–	–
	2 (control)	–	–	–
	3 (infected)	–	–	–
	5 (infected)	–	–	–
24	8 (control)	–	–	–
	9 (control)	–	–	–
	10 (infected)	–	–	–
	11 (infected)	–	$1.33x10^2$	–
48	15 (control)	–	–	–
	16 (infected)	–	$2.67x10^3$	–
	17 (infected)	–	–	$1.33x10^2$
72	20 (control)	–	–	–
	21 (control)	–	–	–
	22 (infected)	–	$4.00x10^2$	–
	23 (infected)	$1.55x10^4$	–	–

[a] *S. suis* serotype 2 not recovered. Threshold of detection is approximately 1×10^2 CFU/g.

Kanamycin resistant, α-haemolytic bacteria were isolated from the tonsils of most control and infected piglets; however, the morphology and the API test results were not consistent with *S. suis*. The challenge strain of *S. suis* was isolated from internal organs of challenged pigs at 24 (1 of 2 pigs), 48 (2 of 2 pigs) and 72 (2 of 2 pigs) hours after challenge, but not at 18h. *S. suis* was isolated from the blood of pig 23 at 72h post-

infection. All remaining infected pigs (16, 17, 22 and 23), but not controls, developed respiratory signs, such as snuffling and sneezing 36h post-infection. Piglet 16 had fever (41.4 °C) at 48h post-infection, and pig 23 was febrile (42.1°C) and shivering at 72h post-infection. Other pigs did not show clinical signs of disease.

Using ABC technique, it was demonstrated that *S. suis* was irregularly distributed throughout the tonsillar crypts of infected pigs and that organisms were mostly concentrated within the crypt lumina and epithelium, with scant positive reaction observed in the subjacent lymphoid tissue (Fig. 4.1). Weak staining, observed occasionally in crypt lumina and epithelium with the *S. suis* Ab, was interpreted as cross-reaction.

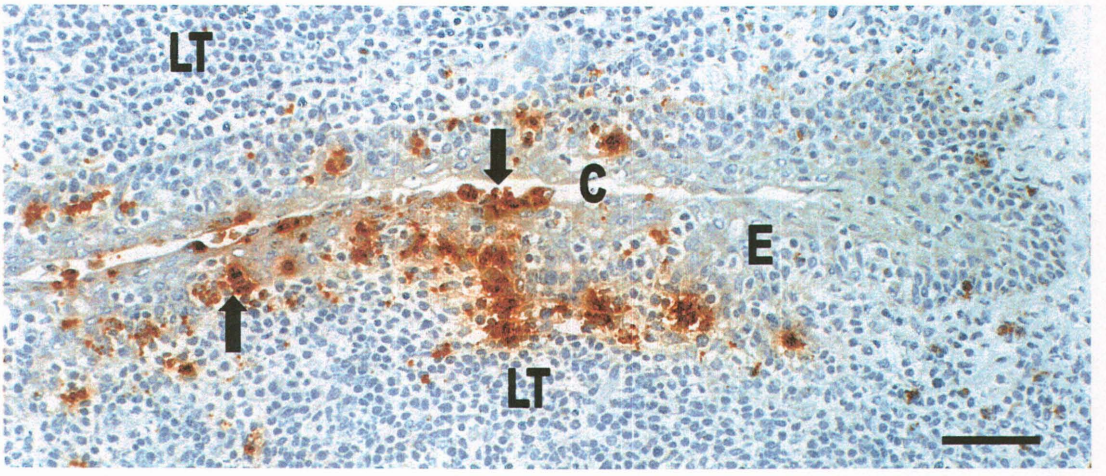
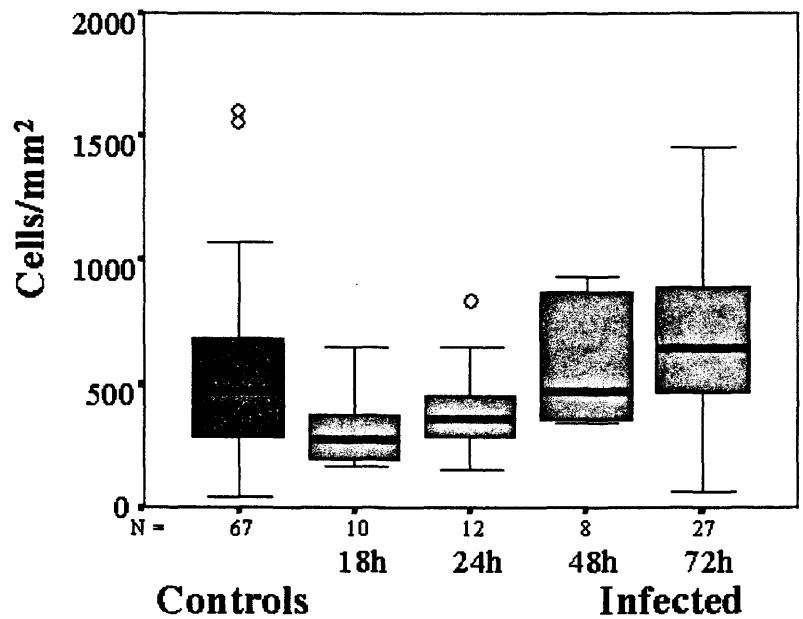


Figure 4.1 - Section of palatine tonsil of a 3-week-old pig experimentally infected with *Streptococcus suis* serotype 2. Bacteria are concentrated in the crypt epithelium and lumen (arrows). C - crypt lumen, E - crypt epithelium, LT - lymphoid tissue. Pig 23, 72h post-infection. Tissue stained for *S. suis* serotype 2 in an ABC technique. Bar = 60μm

In this study, anti-CD3 and anti-λ-Ig-Lc Abs were used to define the T and B lymphocyte populations, and anti-CD4, anti-CD8α and anti-γδ TCR Abs to determine the relative proportions of T cell subpopulations. Anti-myeloid Ab served as a marker of neutrophils and macrophages, and anti-MHC-II Ab of APC. For each leukocyte subset,

the degree of infiltration in the tonsillar crypt epithelium was highly variable among and within individual animals. Numbers of cells of each leukocyte subset in the epithelium of infected and control animals, along with respective degrees of significance, are presented in Figs. 4.2 to 4.7, and the percentage of area of epithelium analyzed that was positive for myeloid marker is presented in Fig. 4.8.

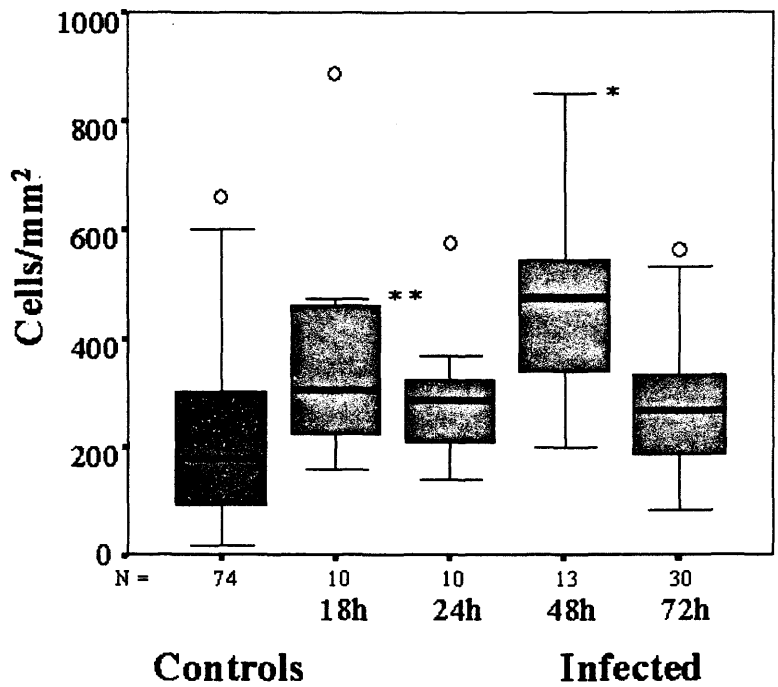


Means: controls = 528; infected: 18h = 317, 24h = 397, 48h = 585, 72h = 705

Fig. 4.2 – Distribution of CD3 positive cells in the palatine tonsillar crypt epithelium of 3-week-old pigs infected with *Streptococcus suis* serotype 2 and non-infected controls (pink and green boxes, respectively). Box plot of the data obtained from 2 infected animals for each time frame after infection, and from 7 control pigs. 'N' represents the total number of crypts analyzed. The box contains 50% of the values. Vertical extension lines represent 95% of the values. The horizontal line within the box indicates the median, (o) outliers. $^{*}p < 0.05$, $^{**}p < 0.01$.

The staining patterns of the CD3, CD4, CD8α, γδ TCR and λ-Ig-Lc subsets were similar to those described previously in the palatine tonsils of market weight pigs

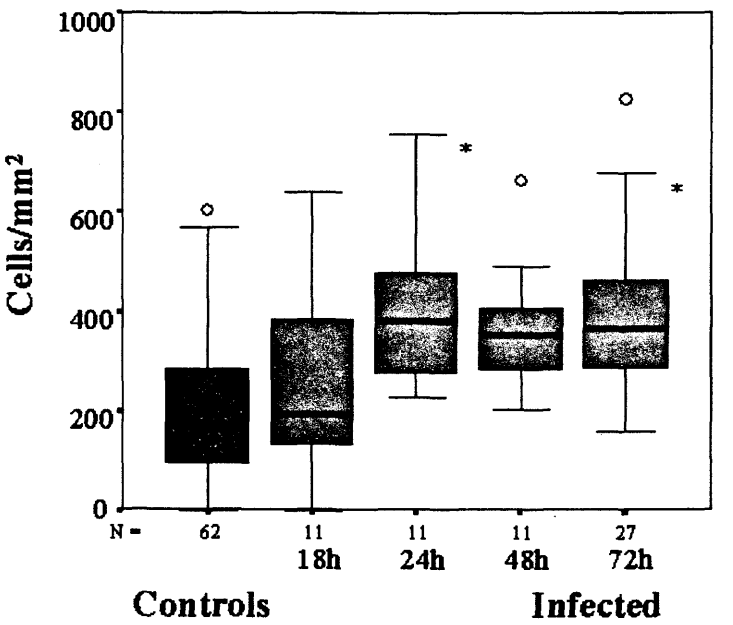
(Chapter 3), although the number in each subset was substantially lower. CD3 (T) cells outnumbered λ-Ig-Lc (B) positive cells in the epithelium of both infected and control animals, except at 18h post-infection, where the reverse was true (Figs. 4.2 & 4.3). The number of λ-Ig-Lc positive cells in the crypt epithelium was higher in infected than in control animals at all time frames, with a significant difference at 18 and 48h (Fig. 4.3).



Means: controls = 214; infected: 18h = 370, 24h = 294, 48h = 464, 72h = 277

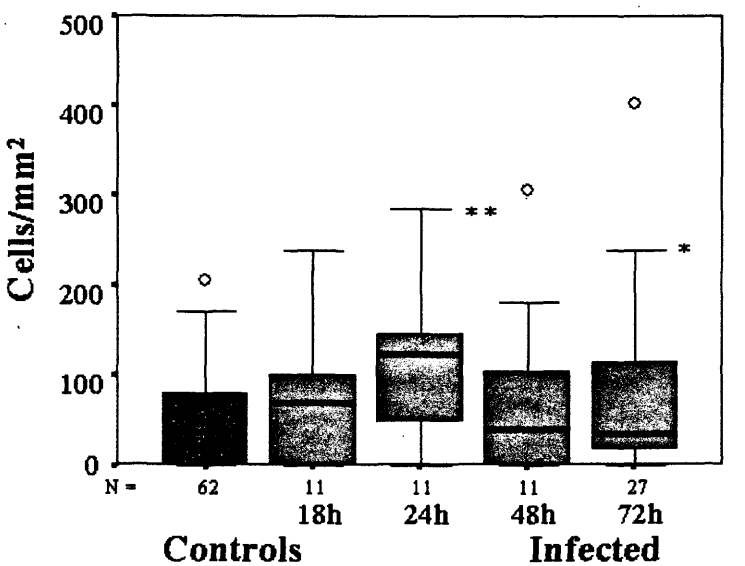
Fig. 4.3 – Distribution of λ-immunoglobulin light-chain positive cells (B cell subset) in the palatine tonsillar crypt epithelium of 3-week-old pigs infected with *Streptococcus suis* serotype 2 and non-infected controls (box plot, see Fig.4.2).

Within the T cell subpopulations, CD4 (T helper) cells formed the largest subset in infected and control animals, γδ cells the next, and the CD8 (T cytotoxic) cell subset the smallest. Both CD4 and CD8 cells were increased in infected animals, and a significant difference was detected at 24h and 72h post-infection (Fig. 4.4 & 4.5).



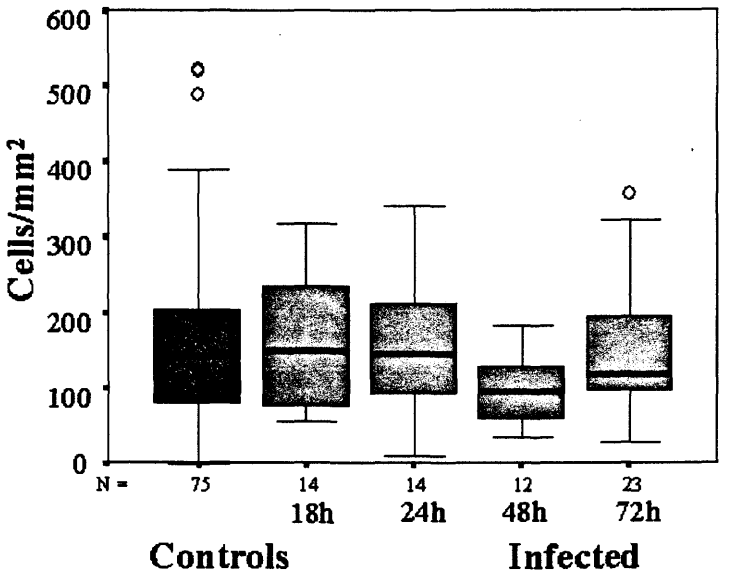
Means: controls = 208; infected: 18h = 251, 24h = 408, 48h = 367, 72h = 393

Fig. 4.4 – Distribution of CD4 positive cells in the palatine tonsillar crypt epithelium of 3-week-old pigs infected with *Streptococcus suis* serotype 2 and non-infected controls (box plot, see Fig.4.2).



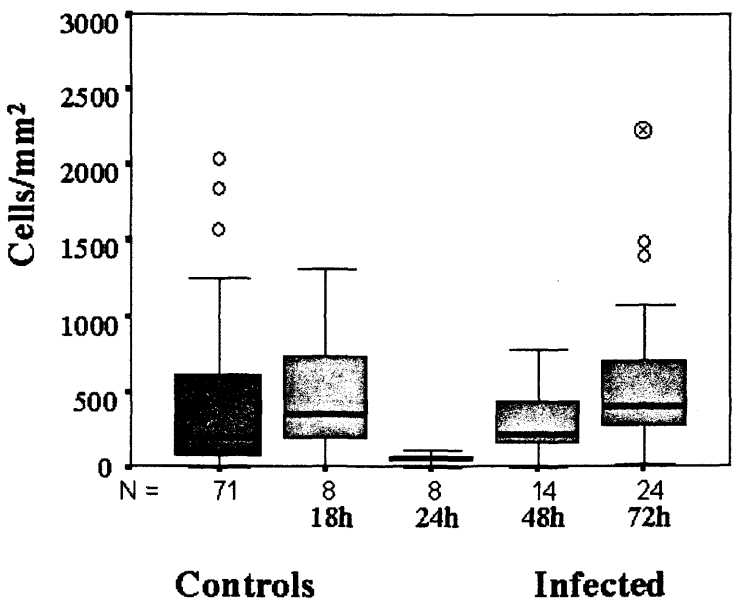
Means: controls = 48; infected: 18h = 69, 24h = 112, 48h = 72, 72h = 80

Fig. 4.5 - Distribution of CD8α positive cells in the palatine tonsillar crypt epithelium of 3-week-old pigs infected with *Streptococcus suis* serotype 2 and non-infected controls (box plot, see Fig.4.2).



Means: controls = 156; infected: 18h = 165, 24h = 155, 48h = 99, 72h = 144

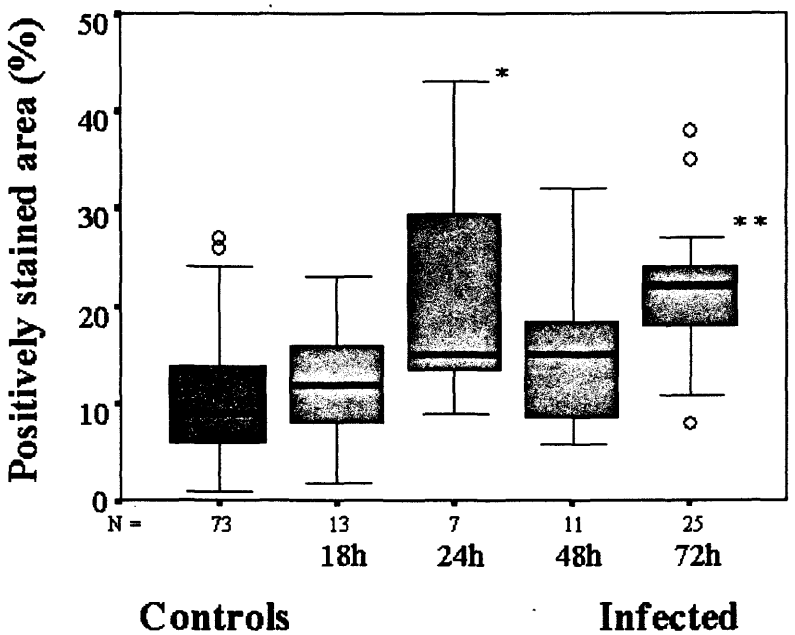
Fig. 4.6 - Distribution of γδ TCR positive cells in the palatine tonsillar crypt epithelium of 3-week-old pigs infected with *Streptococcus suis* serotype 2 and non-infected controls (box plot, see Fig.4.2).



Means: controls = 381; infected: 18h = 484, 24h = 59, 48h = 269, 72h = 603

Fig. 4.7 - Distribution of MHC-II positive cells in the palatine tonsillar crypt epithelium of 3-week-old pigs infected with *Streptococcus suis* serotype 2 and non-infected controls (box plot, see Fig.4.2). (⊗) far outliers

The area of crypt epithelium positive for myeloid marker was larger in the crypts infected with *S. suis*, and was significantly different from controls at 24h and 72h post-infection (Fig. 4.8).



Means: controls = 11%; infected: 18h = 13%, 24h = 22%, 48h = 16%, 72h = 21%

Fig. 4.8 - Percentage of analyzed area of palatine tonsillar crypt epithelium of 3-week-old pigs infected with *Streptococcus suis* serotype 2 and non-infected controls, with positive staining for porcine myeloid (SWC3) marker (box plot, see Fig.4.2).

In addition to neutrophils (Fig. 4.9) and macrophages (Fig. 4.10), the anti-myeloid (SWC3) Ab stained large foci within the crypt epithelium, frequently in highly reticulated areas (Fig. 4.11).

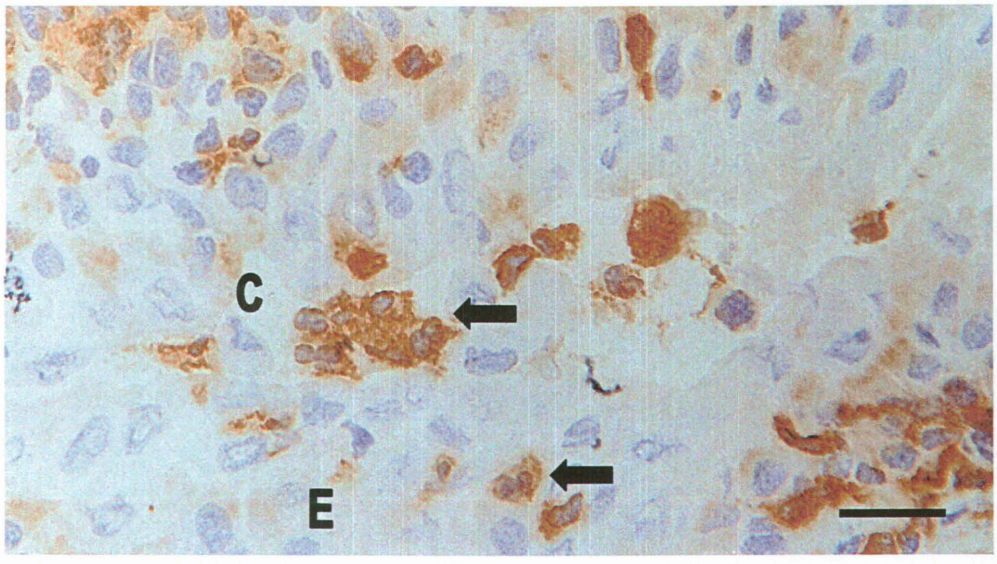


Fig. 4.9 - Section of palatine tonsil of a 3-week-old pig experimentally infected with *Streptococcus suis* serotype 2. Positive staining neutrophils can be observed in the crypt lumen and epithelium (arrows). C- crypt lumen, E - crypt epithelium. Pig 23, 72h post-infection. Tissue stained for myeloid cells in an ABC technique. Bar = 20μm.

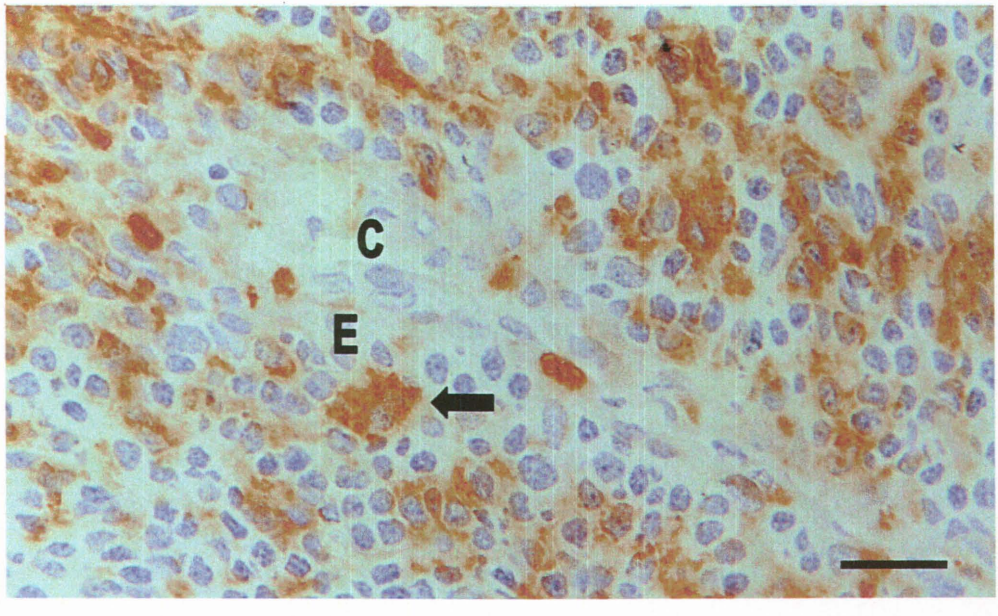


Fig. 4.10 - Section of palatine tonsil of a 3-week-old pig experimentally infected with *Streptococcus suis* serotype 2. An isolated positive staining macrophage can be observed in the crypt epithelium (arrow). The other cells have a confluent positive staining, making it difficult to distinguish between macrophages and epithelial cells. C - crypt lumen, E - crypt epithelium. Pig 23, 72h post-infection. Tissue stained for myeloid cells in an ABC technique. Bar = 25μm.

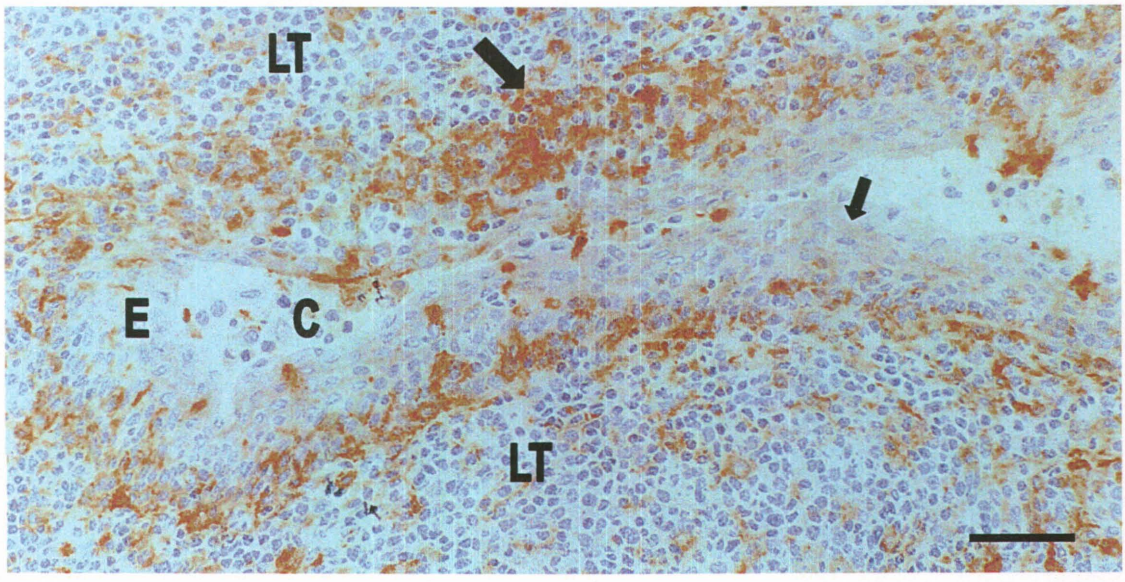


Fig. 4.11 - Section of palatine tonsil of a 3-week-old pig experimentally infected with *Streptococcus suis* serotype 2. The highly reticulated area of the crypt epithelium has extensive positive staining (large arrow); in contrast, non-reticulated areas have no positive staining (small arrow). C - crypt lumen, E - crypt epithelium, LT - lymphoid tissue. Pig 23, 72h post-infection. Tissue stained for myeloid cells in an ABC technique. Bar = 50μ.

No significant difference in the number of CD3, γδ T cell receptor and MHC-II positive cells was noticed in the crypts of infected animals compared to controls (Figs. 4.2, 4.6 & 4.7). MHC-II positive cells were mononuclear, with either scant or abundant cytoplasm, considered to correspond to lymphocytes and macrophages, respectively (Fig. 4.12). Single cells or small clusters of cells with long cytoplasmic projections and dendritic-like processes, and positive for MHC-II marker, were frequently observed (Fig. 4.13). It was difficult to determine for a few of the positive cells if they were epithelial or mononuclear cells (Fig. 4.14).

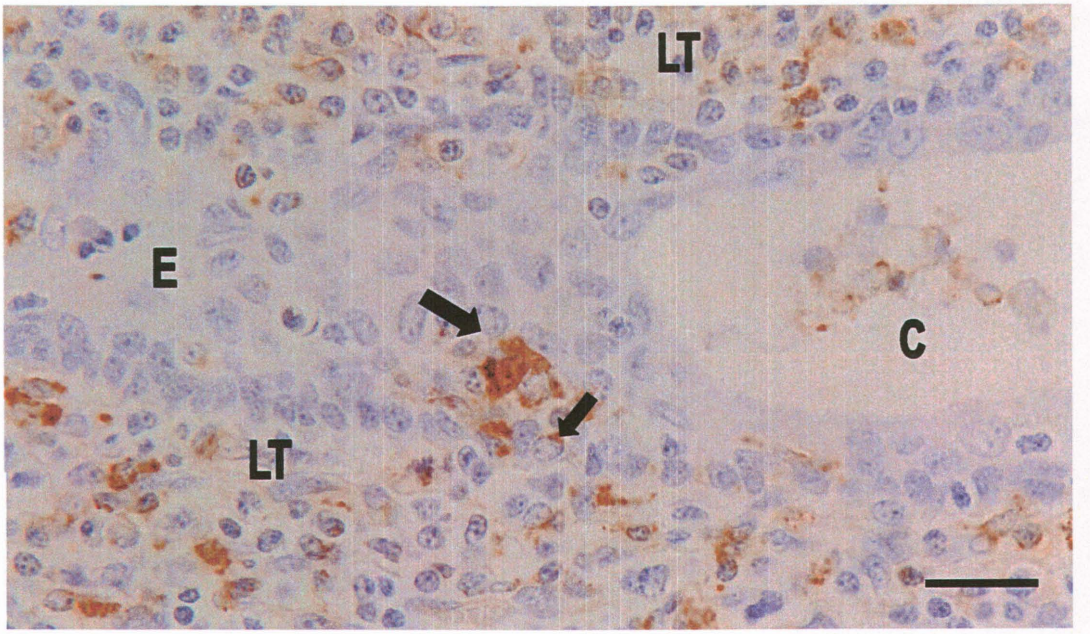


Fig. 4.12 - Section of palatine tonsil of a 3-week-old pig experimentally infected with *Streptococcus suis* serotype 2. A positively staining cell for MHC-II with the morphology compatible with a macrophage (large arrow) is present in the crypt epithelium. Another cell, with morphology compatible with a lymphocyte, i.e., with only a small rim of cytoplasm around the nucleus, is positively stained (small arrow). C - crypt lumen, E - crypt epithelium, LT - lymphoid tissue. Pig 23, 72h post-infection. Tissue stained for MHC-II in an ABC technique. Bar = 25μm.

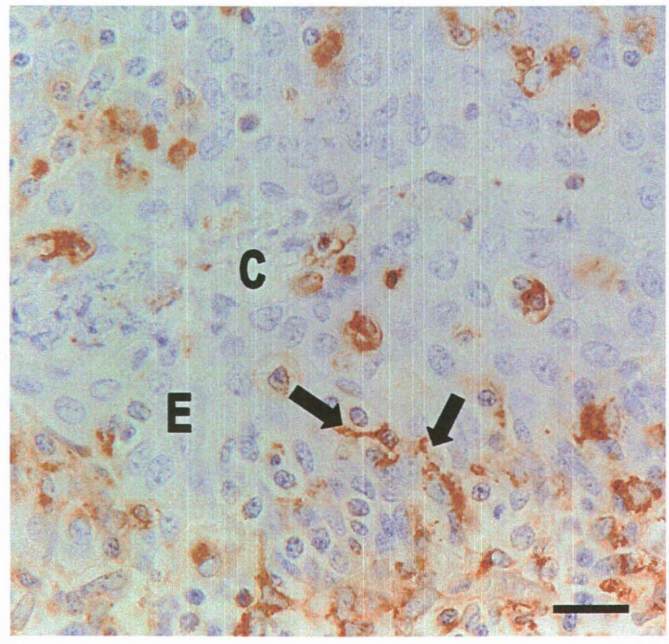


Fig. 4.13 - Section of palatine tonsil of a 3-week-old pig experimentally infected with *Streptococcus suis* serotype 2. Dendritic-like processes (arrows) staining positive for MHC-II can be seen in the crypt epithelium. C - crypt lumen, E - crypt epithelium, LT - lymphoid tissue. Pig 23, 72h post-infection. Tissue stained for MHC-II in an ABC technique. Bar = 20μm.

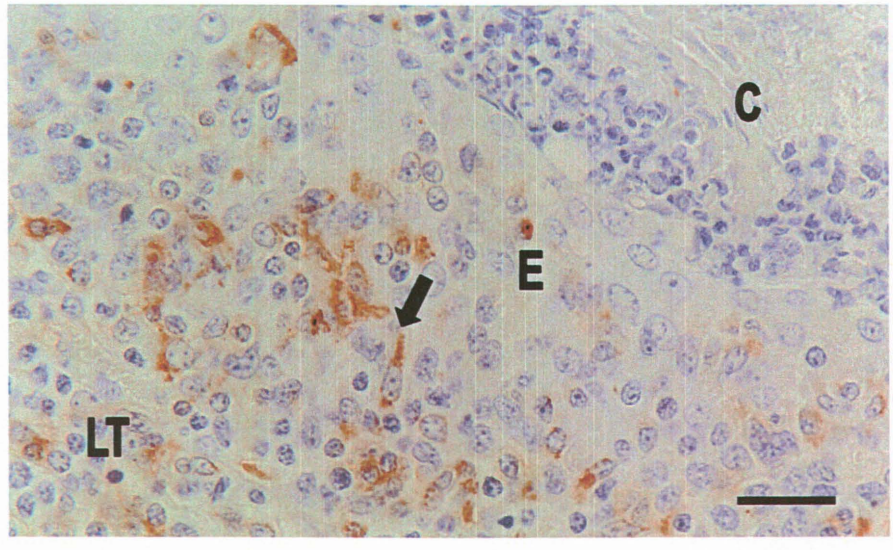


Fig. 4.14 - Section of palatine tonsil of a 3-week-old pig experimentally infected with *Streptococcus suis* serotype 2. Several cells in the crypt epithelium are positively stained for MHC-II. One of these cells (arrow) has morphology suggestive of an epithelial cell. C –crypt lumen, E - crypt epithelium, LT - lymphoid tissue. Pig 22, 72h post-infection. Tissue stained for MHC-II in an ABC technique. Bar = 25μm.

4.4 - Discussion

This study was performed to identify the alterations occurring in leukocyte subpopulations within the crypt epithelium of the palatine tonsils of piglets during acute infection with *S. suis* serotype 2 SX426. Challenged piglets developed clinical signs of infection 36h post-infection. Additionally, *S. suis* serotype 2 was recovered from internal tissues in one animal at 24h post-infection and the remaining challenged animals 48h and 72h post-infection, confirming that *S. suis* did disseminate from the tonsils and/or oro-pharyngeal area to other tissues. *S. suis* was demonstrated in the palatine tonsils of all infected piglets by IHC, despite the fact that it was not re-isolated by culture. The failure to recover *S. suis* serotype 2 was possibly because the portion of tonsil cultured was small, the *S. suis* distribution non-uniform, and other bacteria normally present in the tonsils grew more rapidly to obscure any *S. suis* colonies.

The number of CD3, CD4, CD8, γδ and λ-Ig-Lc positive lymphocytes in the 3-week-old animals in this study was substantially lower than that in 6-month-old pigs described in Chapter 3. This lower number of lymphocytes is potentially of importance in the susceptibility of younger animals to infectious diseases.

The substantial variation in the number of cells, or percentage of positive area, in each leukocyte subpopulation of the crypt epithelium in infected as well as in control animals, and within individual animals, is consistent with observations made previously in Chapter 3. For this reason, since no significant differences were detected among control pigs, the data from control pigs were pooled when compared with infected pigs, in order to encompass and mitigate this expected high variation in the number of cells in the crypt epithelium, as observed in Chapter 3. Factors such as environment, age, and health status, were unlikely to play a role, since these animals came from the same source and were the same age. The variations in specific leukocyte subpopulations among crypts of the same animal are likely a reflection of the heterogeneous antigenic stimulation from one site to another in the palatine tonsil (Perry, 1994). The non-uniform distribution of *S. suis* observed in the crypts of infected animals is probably similar for other bacteria of the tonsillar flora, and demonstrates that there is indeed a

variation in antigenic stimulation among the crypts. Consequently, each crypt unit likely represents a unique microenvironment.

The number of T lymphocytes in the crypt epithelium outnumbered that of the B lymphocytes, except for the infected animals of 18h post-infection. The Ab used to detect B lymphocytes has recently been found to recognize only a subset of porcine λ-light chain positive B lymphocytes; also λ and κ light chains in serum immunoglobulins and peripheral B lymphocytes are expressed in similar proportions in pigs (Sinkora et al., 2001). Hence, the number of B lymphocytes in the crypt epithelium determined may be an underestimation and could be more than double the number obtained here.

No change in the number of γδ T lymphocytes was noticed in the crypts infected with *S. suis*, suggesting that no apparent role exists for these cells in the early response against *S. suis*. This observation was unexpected since these cells are believed to play an important role in the early phase of infection (Boismenu and Havran, 1997). Without increasing in numbers, however, these cells could have participated in early pathogen recognition and secreted cytokines to trigger the recruitment of other lymphocyte subsets in which an increase was observed. Additionally, γδ T lymphocytes did not constitute the dominant T cell subset in the crypt epithelium of the palatine tonsils as described for the peripheral blood T cell subpopulation of 4 week-old pigs. γδ T lymphocytes are believed to have an important role in the immune response of young pigs prior to the maturation of the other subsets (Yang and Parkhouse, 1996).

Both B lymphocytes and CD4 (T helper) lymphocytes were increased in number in the crypt epithelium of infected animals, indicating early recruitment and suggesting a potential for the development of humoral and cellular immune responses against *S. suis* in the tonsils. Suilysin, a virulence factor of *S. suis*, which was present in the strain used in this experiment, can trigger the production of IL-6 by porcine monocytes and alveolar macrophages (Lun et al., 2001). Therefore, the early increase in the B cell population could perhaps have resulted from the production of IL-6, which is a growth factor for differentiated B lymphocytes (Abbas et al., 2000b).

Studies on the immune response of pigs against *S. suis* have concentrated largely on the systemic humoral response (Holt et al., 1988; Blouin et al., 1994; del Campo

Sepúlveda et al., 1996; Staats et al., 1997). A cellular response is likely required for an efficient clearance of these bacteria, since *S. suis* is able to persist inside phagocytes (Brazeau et al., 1996), and intact *S. suis* organisms have been observed, by transmission electron microscopy (Chapter 5), within mononuclear and epithelial cells from the crypt epithelium of the palatine tonsils of the animals used in this study.

The number of CD8 positive cells was also increased in all infected animals. CD8-positive cells represent cytotoxic T lymphocytes, which classically are part of cellular immunity, and kill infected cells or tumour cells. They can also participate in the regulation of CD4 cells through the production of cytokines (Vukmanovic et al., 2000). In pigs, the CD8 molecule can be expressed alone, as in the cytotoxic T lymphocytes, or in association with CD4, as in CD4/CD8 double positive (DP) cells, which are believed to represent memory T lymphocytes (Zuckermann, 1999). The CD8 molecule can also be expressed in γδ TCR and NK cells (Zuckermann et al., 1998). Since the antibody used in this study does not differentiate between CD8 single and DP cells, we can only speculate on the role CD8-positive cells in the defence against *S. suis*. These cells could have participated in a cytotoxic response, or in the modulation of CD4 cells and macrophages, or even as memory cells, as the possibility of these animals having been previously infected with other bacteria with epitopes shared by *S. suis* serotype 2 cannot be excluded.

In this study the number of CD3-positive cells in the crypt epithelium of infected animals was higher than control animals only at 48h of infection and later, but no significant difference was detected, despite the fact the CD4 and CD8 T lymphocytes subsets increased at an earlier stage. Several factors could explain these apparently conflicting results. Although all sections of palatine tonsil were cut serially, since sections stained for *S. suis* and control Abs were intercalated throughout, the fields analysed for each lymphocyte marker were therefore not from immediately adjacent matching sections. Also, the sum of all T cell subsets, i.e., CD4, CD8 and γδ T lymphocytes, does not absolutely correspond to the number of CD3 positive cells, since any DP cell will have been counted twice, and γδ T lymphocytes and natural killer cells may also express the CD8 molecule.

The increase in the area positive for myeloid marker in infected crypts was not surprising since neutrophils and macrophages are the first cells recruited in response to injury and bacterial infection. The intense positive staining of the crypt epithelial cells, however, was intriguing, since reaction of the porcine myeloid marker with epithelial cells has not been described previously. MAC-387, a myeloid cell marker, stains the epithelium of several human mucosae, such as from the oropharynx, tonsils, oesophagus, vagina and cervix (Brandtzaeg et al., 1987; Brandtzaeg et al., 1988; Eversole et al., 1992). In the oral epithelium, MAC-387 is up-regulated in response to pathogen invasion and immunopathological processes (Eversole et al., 1993). Calprotectin, the protein of mucosal epithelial cells that is recognized by MAC-387, has anti-microbial properties and is suggested to participate in the innate defence (Steinbakk et al., 1990; Brandtzaeg et al., 1995; Clohessy and Golden, 1995). This protein is also associated with the cytoskeleton (Clark et al., 1990). In these porcine tonsils myeloid-positive epithelial staining occurred mainly within reticulated areas of the epithelium, suggesting that the protein recognized by the porcine myeloid marker might be associated with the cytoskeleton. Reticulated areas of the stratified tonsillar crypt epithelium of pigs have altered CK expression, comparable to that of basal cells (Middleton, unpublished data), as demonstrated also in human tonsil (Sato et al., 1990). Further investigations are needed to determine whether calprotectin is present in the porcine epithelium of the mucosal surfaces and, if present, to identify its role in defence and in the cell cytoskeleton. Alternatively, leakage of the specific protein from leukocytes to epithelial cells could give a false positive reaction (Brandtzaeg et al., 1992).

Although the number of MHC-II positive cells in the crypt epithelium of *S. suis* infected pigs was higher than controls at 18h and 72h post-infection, no significant difference was observed. The varying morphology of the cells positive for MHC-II marker in the tonsillar crypt epithelium was compatible with macrophages, lymphocytes and DCs. MHC-II molecule in pigs is expressed by B lymphocytes, macrophages, DCs, a fraction of T lymphocytes, and endothelial cells (Lunney and Pescovitz, 1987; Wilson et al., 1996; Makala et al., 1998). Expression of the MHC-II marker by a few epithelial cells cannot be ruled out, since the epithelial cells in reticulated areas can assume bizarre

shapes, becoming difficult to distinguish from non-epithelial cells. Dual staining using MHC-II along with a CK marker would be necessary to confirm this. MHC-II positive epithelial cells were not detected in studies of pig intestine (Vega-Lopez et al., 1993; Wilson et al., 1996), in contrast to that of humans and rodents (Brandeis et al., 1994).

Although the number of animals in this study was small, important observations were made in relation to the *S. suis* infection at the level of the crypt epithelium of the palatine tonsil. Cells of the innate immune system, i.e. neutrophils, macrophages, and likely epithelial cells, participate in the initial phase of *S. suis* infection. Within the first 24h post-infection, there is an increase in the number of B, CD4 and CD8 T lymphocytes, suggesting potential for the initiation of humoral and cellular responses in the crypt epithelium. γδ T lymphocytes are not increased, suggesting that they do not participate in the early response against *S. suis* between 18h and 72h; however, it cannot be ruled out that they did participate in the early detection of *S. suis,* by secretion of cytokines that recruit other cells. Also, in this epithelium, γδ T lymphocytes are not the dominant T cell subset, unlike in the peripheral blood of 4 week-old pigs. These observations will be useful for future investigations on cytokine expression and signalling in the crypt epithelium in response to *S. suis* infection, as a measure of the type of immune responses taking place; and they will aid in the understanding of the initial phases of the pathogenesis of this disease, and in the development of efficient methods of mucosal vaccination.

5 – An Ultrastructural Study of the Early Interaction of *Streptococcus suis* Serotype 2 and the Crypt Lymphoepithelium of the Palatine Tonsils of Pigs

5.1- Introduction

Streptococcus suis serotype 2, a widely distributed pathogen of pigs, is a common cause of meningitis, arthritis, and septicaemia, and is a major cause of economic losses in the swine industry (Staats et al., 1997). Pigs can carry pathogenic and non-pathogenic strains of *S. suis* in the palatine tonsils, but, for reasons not yet understood, only some animals will succumb to infection. Other animals become healthy carriers and are a potential source of infection in a herd (Clifton-Hadley et al., 1984). Numerous virulence factors are described for *S. suis*; however, their roles in inducing infection and disease have not been well elucidated. Information about the pathogenesis of this disease is limited, and focused mainly on the development of meningitis (Williams and Blakemore, 1990, Gottschalk and Segura, 2000). From the upper respiratory tract, *S. suis* gains access to other organs likely through the palatine tonsils, as demonstrated by the isolation of *S. suis* from submandibular, retropharyngeal and parotid lymph nodes, and from the peripheral blood, following experimental application of this pathogen to the palatine tonsils (Williams et al., 1973). The means by which *S. suis* gain access to the peripheral blood from the palatine tonsils is not known, e.g., whether via macrophages or extracellularly. Additionally, it is unclear how *S. suis* traverse the upper respiratory mucosal barrier to cause disease (Gottschalk and Segura, 2000). Once in the blood, *S. suis* reaches the brain through the choroid plexus, likely carried by monocytes (Williams and Blakemore, 1990).

Two studies have tried to address these issues on how *S. suis* engineers its way across epithelial barriers: both were performed in epithelial cell lines and the results are conflicting (Norton et al., 1999, Lalonde et al., 2000). In the first study using a human

laryngeal epithelial cell line, both pathogenic and non-pathogenic *S. suis*, especially the former, were able to invade epithelial cells, but only pathogenic strains caused epithelial cell lysis. It was suggested therefore that epithelial cell invasion and lysis are the mechanisms by which pathogenic *S. suis* breach the mucosal barrier (Norton et al., 1999). In the other study, human lung and cervical carcinoma cell lines, and also canine and porcine kidney cell lines, were used to investigate the ability of *S. suis* to invade and damage epithelial cells (Lalonde et al., 2000). In contrast to the results described above, cell lysis occurred, but in the absence of cell invasion. The authors suggested that *S. suis* uses lysis as opposed to cell invasion to breach the epithelial barrier and spread to the bloodstream (Lalonde et al., 2000).

In vitro studies in cell line monolayers may not illustrate completely the interaction of *S. suis* with the stratified epithelium of the tonsillar crypts. Therefore, the purpose of this study was to investigate, by TEM, the interactions, *in vivo*, between *S. suis* and the palatine tonsillar crypt epithelium to elucidate the early steps of the pathogenesis of *S. suis* serotype 2 infection in pigs.

5.2 - Materials and Methods

5.2.1- *Streptococcus suis* strain

The strain SX426 of *S. suis* serotype 2 was used, as described in Chapter 4.

5.2.2 - Animals and samples

The animals used in this study were the same as for the immunohistochemical study of leukocyte subsets in the crypt epithelium of the palatine tonsils. Small blocks from the right palatine tonsil of these animals obtained at necropsy were fixed in 5% glutaraldehyde and post-fixed in 2% aqueous osmium tetroxide. The tissues were dehydrated in alcohol and embedded in epon resin.

5.2.3 - Immunohistochemistry on Epon Sections

An immunoperoxidase technique was performed on epon sections to screen for blocks of tissue containing *S. suis* serotype 2, as immunohistochemical observations

indicated that the distribution of *S. suis* throughout the tonsil was irregular and unpredictable, rendering difficult location of the organisms by electron microscopy alone. One-micron sections of the epon blocks were cut, transferred to slides, and the resin removed by a saturated solution of sodium hydroxide in ethanol (Polak and Van Noorden, 1997). The slides were rehydrated and stained with a polyclonal rabbit anti-*S. suis* serotype 2 antibody (donated by Dr M. Gottschalk, Univ. of Montreal, St. Hyacinthe, Can) at 1/8000 dilution in a solution of 5% goat serum, followed by a biotinylated goat anti-rabbit secondary antibody at 1/400 (Vector Laboratories) and the ABC technique as previously described in Chapter 3, except that the 3,3 diaminobenzidine tetra-hydrochloride (Sigma) incubation period varied from 3 to 10 minutes, until the section turned slightly brown. Counterstaining with hematoxylin varied from 30 s to 1 min, until the section turned slightly blue. Rabbit anti-parainfluenza 3 virus (donated by Dr D. Haines, University of Saskatchewan, Can.) at 1/8000 dilution was used as a negative substitution control antibody.

5.2.4 - Transmission Electron Microscopy and Immunogold Techniques

Thin sections (0.1μm) from *S. suis*-positive blocks and blocks from control animals were contrasted with uranyl acetate and lead citrate and examined with a Philips EM 410 transmission electron microscope (TEM). A post-embedding immuno-electron microscopy technique (modified from Polak and Van Noorden, 1997) was applied to tissues from one of the infected animals (pig 23) to confirm that the bacteria seen on TEM were indeed *S. suis* serotype 2. Nickel grids with the thin sections were floated for 30 minutes on a solution of 5% goat serum and 0.1% Tween in PBS (goat blocker solution), drained and incubated overnight at 4°C in rabbit anti-*S. suis* polyclonal antibody at dilutions of 1/1000, 1/4000 and 1/8000 in goat blocker solution. The following day, the grids were rinsed in five changes of PBS, then in three changes of PBS with 0.1% ovalbumin (PBSO), and subsequently incubated for one hour in a 1/50 solution of goat anti-rabbit IgG conjugated with 10 nm gold particles (British BioCell) diluted in PBSO. The grids were then rinsed three times in PBSO, five times in PBS and seven times in distilled water. All washings were for 5 minutes, under agitation on a magnetic stirrer. Grids were then dried and contrasted with uranyl acetate and lead

citrate. A pure culture of *S. suis* serotype 2 (strain SX426) was embedded in agar, processed and stained for TEM as described above, and used as a positive control. Normal rabbit serum was used as a negative substitution control.

5.3 - Results

5.3.1- Immunohistochemistry on Epon Sections:

A weak and dubious reaction with *S. suis* serotype 2 antibody, possibly representing cross-reactivity, was observed in the crypt epithelium and lumen of 3 control and 3 infected animals. A strong positive reaction was observed in 5 of 8 infected pigs (Fig. 5.1), seen both as individual cocci and as large foci. Frequently, this reaction seemed to involve the whole cytoplasm of cells in the crypt epithelium, but it was not possible to determine the identity of these cells. The positive reaction for *S. suis* was mostly confined to the crypt epithelium and lumen, with scant positive reactivity in the subepithelial lymphoid tissue.

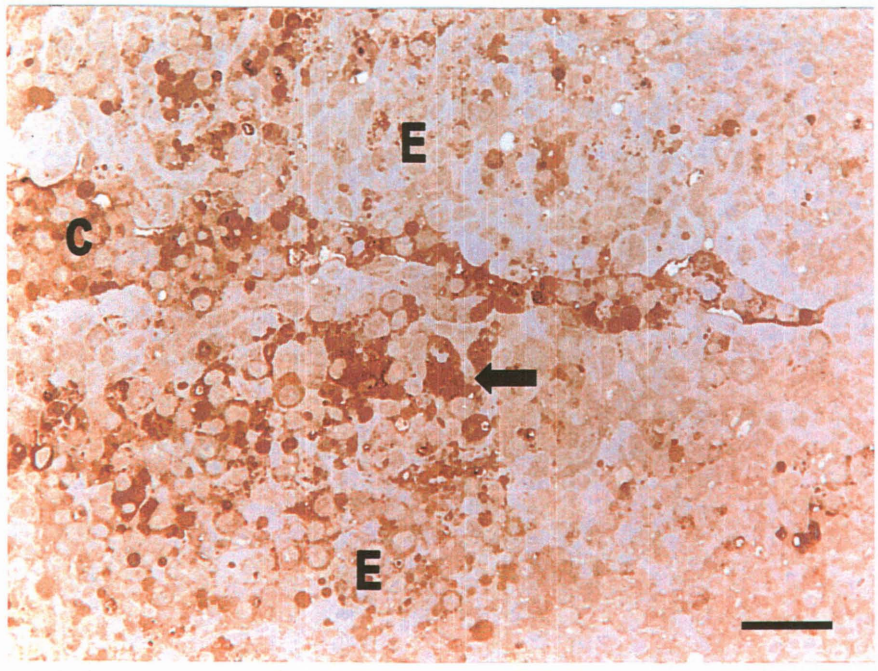


Fig. 5.1 – Strong positive reaction with *S. suis* antibody is observed in the crypt lumen and epithelium. Notice the large clumps of positive reaction (arrow). C – lumen, E – crypt epithelium. Pig 23, infected with *S. suis*, 72h post-infection, ABC technique on epon section. Bar = 30 μm.

5.3.2 – Ultrastructural Findings

Bacteria with morphology consistent with *S. suis* were observed in the crypt lumen and epithelium of most of the infected animals (Fig. 5.2 & 5.3).

Intracellular bacteria, usually within vacuoles, were found mostly in macrophages, but also in neutrophils and, less frequently, in epithelial cells. Bacteria were usually intact in neutrophils (Fig. 5.4), as well as in epithelial cells (Figs. 5.5 & 5.6). Epithelial cells containing bacteria were found both in the superficial and deeper layers of the epithelium. The cytoplasm of these epithelial cells was variably electron-dense or electron-lucent (Figs. 5.5, 5.6 & 5.7).

Bacteria present within macrophages were often disintegrating (Fig. 5.8a); however intact bacteria were also seen in these cells (Fig. 5.8b). As early as 18h after infection, macrophages containing bacteria were noticed straddling or subjacent to the basal lamina (Fig. 5.9).

In one infected animal, a small area of necrosis characterized by swollen organelles, mitochondria with electron-dense deposits, and faded or fragmented nuclei, was observed in the superficial crypt epithelium. Coccal organisms consistent with *S. suis* were intimately associated with this lesion (Fig. 5.10).

In the tonsil of pig 23, on which the immunogold technique was performed, many uniform electron-dense particles, corresponding to the gold particles used, were adherent to the capsule and microfilaments of the cocci, confirming that the bacteria observed were *S. suis* serotype 2 (Fig. 5.11).

Very few cocci were observed in the crypt epithelium of control animals. The morphology of those seen was different from the cocci seen in infected animals: while in the infected animals the capsule usually possessed numerous microfilaments (Fig. 5.3), in control animals filaments were absent or were very scant (Fig. 5.12).

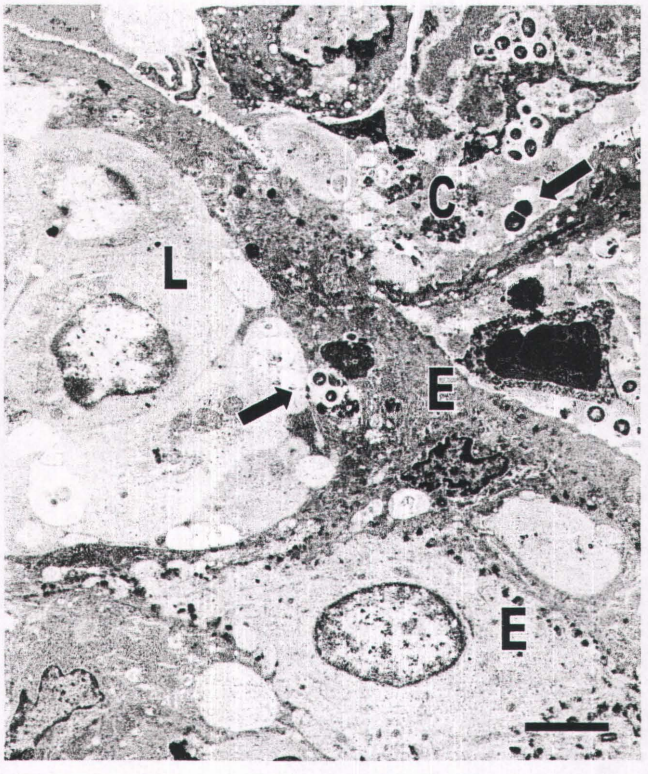


Fig. 5.2 – *Streptococcus suis* in the palatine tonsil. *S. suis* are observed in the crypt lumen and epithelium (arrows). C- crypt lumen, E – epithelial cell, L –lymphocyte. Pig 22, infected with *S. suis*, 72 h post-infection. Bar = 3 μm.

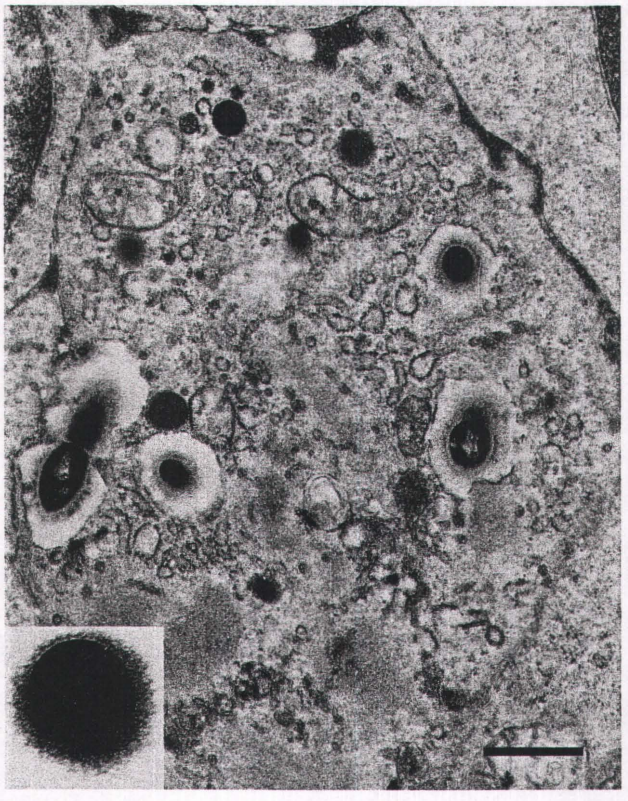


Fig. 5.3 - Cocci are round to oval, single or double, strongly electron-dense, with a moderately electron-lucent core and capsule, and numerous electron-dense capsular microfilaments. Pig 22, infected with *S. suis*, 72 h post-infection. Bar = 1 μm.
Insert - pure culture of *S. suis* serotype 2 (SX 426) in agar.

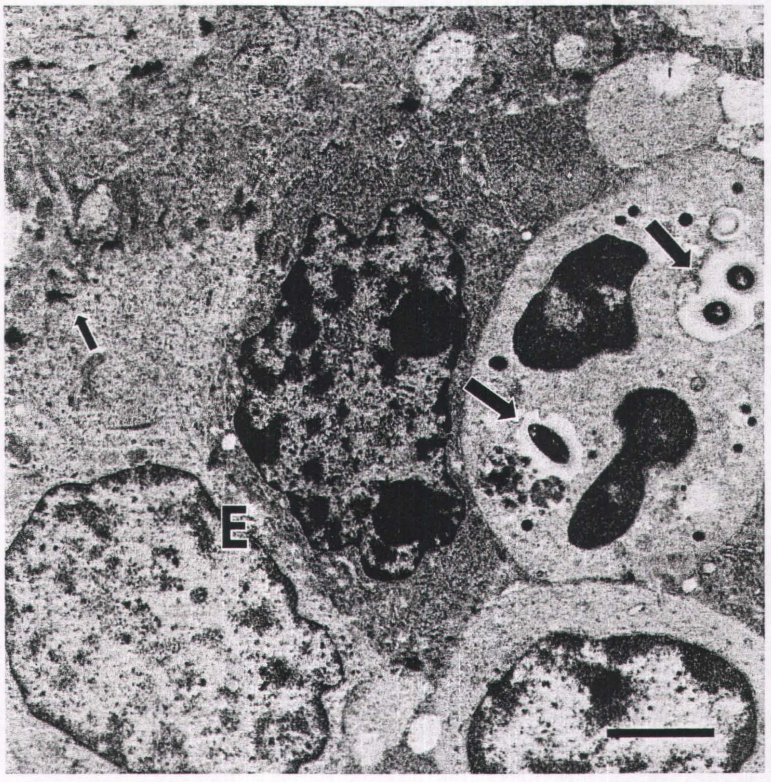


Fig. 5.4 – Intact bacteria (large arrows) are seen within a phagosome of a neutrophil within the crypt epithelium. Note the desmosomes (small arrow) of an epithelial cell (E). Pig 22, infected with *S. suis*, 72 h post-infection. Bar = 2 μm.

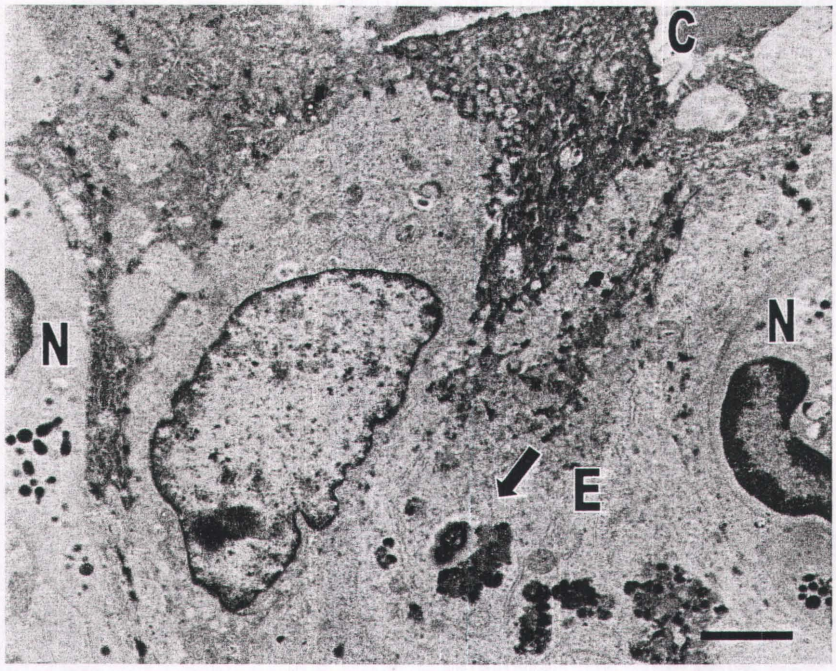


Fig. 5.5 – Diplococcal bacteria (arrow) within a vacuole containing electron-dense debris are present in an epithelial cell (E) subjacent to the luminal surface. This cell has an electron lucent cytoplasm and moderate amount of RER. N – neutrophil. C – crypt lumen. Pig 5, infected with *S. suis*, 18 h post-infection. Bar = 2 μm.

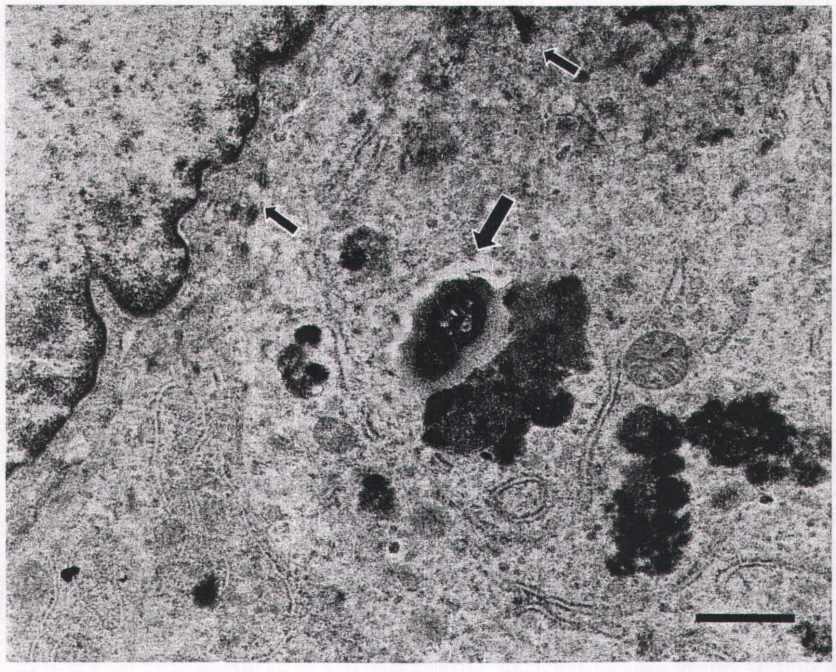


Fig. 5.6 - Higher magnification of Fig. 5.5. The membrane of the intra-epithelial vacuole containing the diplococci is discontinuous and possibly ruptured (large arrow). The presence of desmosomes (small arrows) confirms the epithelial nature of this cell. Pig 5, infected with *S. suis*, 18 h post-infection. Bar = 1 μm.

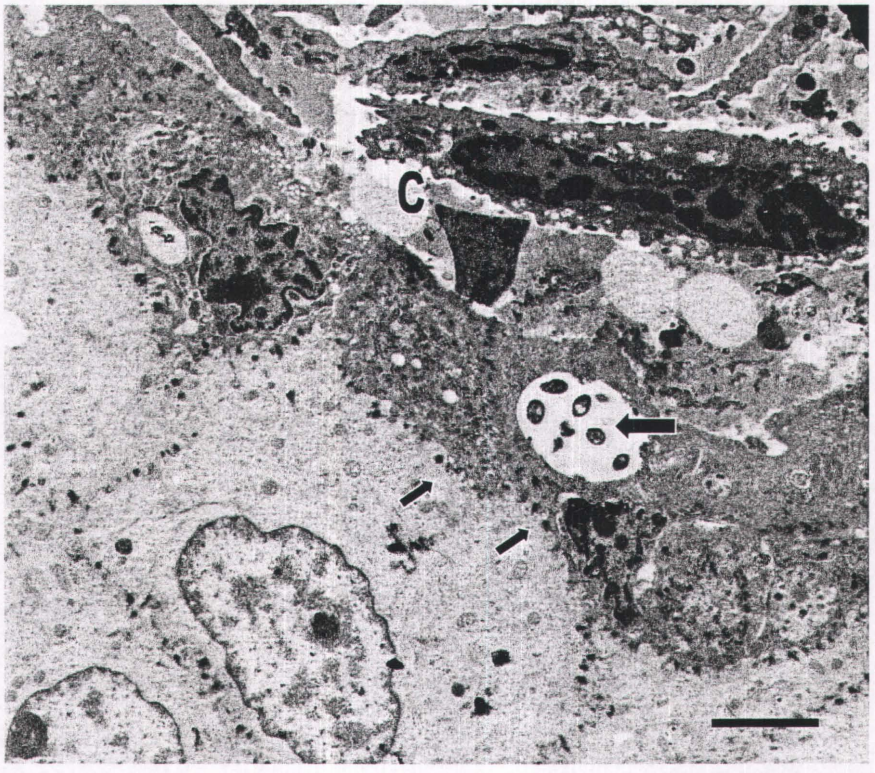
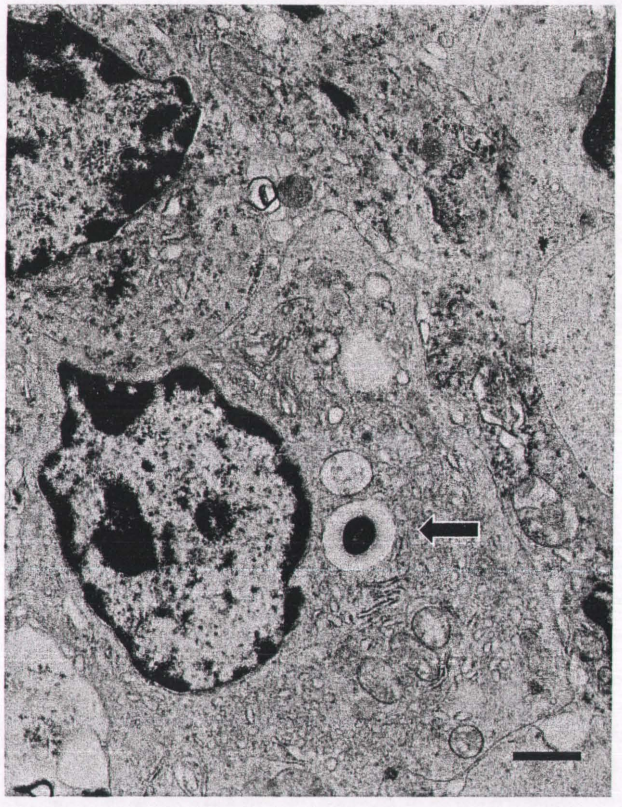


Fig. 5.7 – Many bacteria are observed in a vacuole of a superficial electron-dense epithelial cell (large arrow). Desmosomes (small arrows). C – crypt lumen. Pig 22, infected with *S. suis*, 72 h post-infection. Bar = 3 μm.

a



b

Fig. 5.8 – Streptococci (arrow) are present within phagosomes of macrophages. These bacteria are either disintegrating (**a**) or intact (**b**). **a -** Pig 22, infected with *S. suis*, 72 h post-infection . **b -** Pig 23, infected with *S. suis*, 72 h post-infection. Bars = 1 μm.

Fig. 5.9 - A mononuclear cell containing a coccal organism (small dark arrow) is straddling the basal lamina. A portion of the cytoplasm of this cell is in the crypt epithelium (large dark arrow) and the remaining is in the subepithelial lymphoid tissue. White arrows – basal lamina, LT – lymphoid tissue, E – epithelial cell - Pig 22, infected with *S. suis*, 72 h post-infection. Bar = 2 μm.

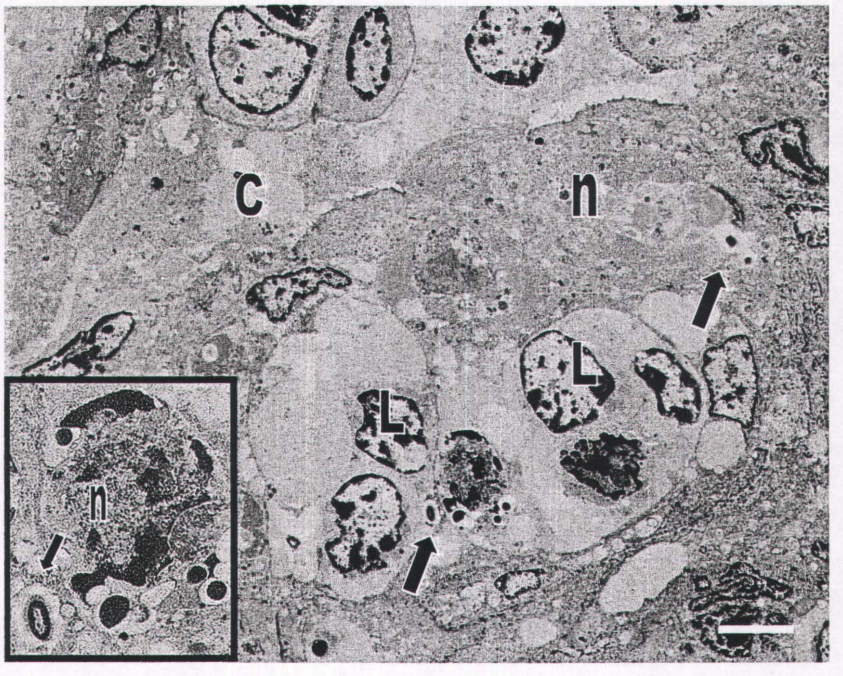


Fig. 5.10 - An area interpreted as necrotic (n), characterized by swollen organelles, mitochondria with electron-dense deposits, and faded or fragmented nuclei, is seen in the crypt epithelium in association with *Streptococcus suis* (arrows). C – crypt lumen, L – lymphocytes. Pig 23, infected with *S. suis*, 72 h post-infection. Bar = 5 μm.
Insert – *S. suis* (small arrow) in close association with a necrotic cell (n).

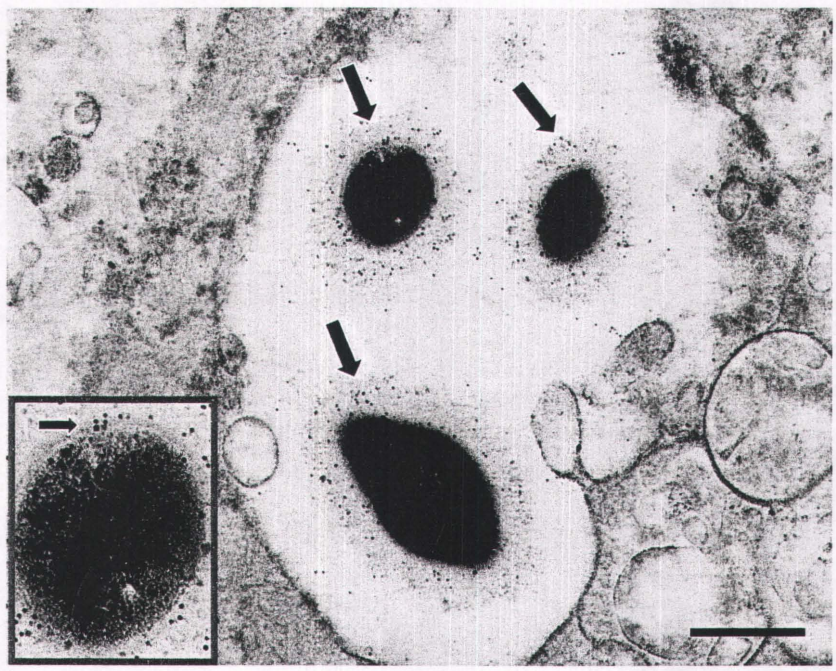


Fig. 5.11 – Intracellular *Streptococcus suis* serotype 2 are identified specifically by the deposition of homogeneously spherical electron-dense gold particles (arrows) in the capsule. Pig 23, infected with *S. suis*, 72 h post-infection. Bar = 0.5 μm. Insert – higher magnification. Post-embedding electron microscopy immunogold technique.

Fig. 5.12 - Cocci with very scant capsular microfilaments (arrows) are seen within a superficial epithelial cell of a control pig. Their morphology differs from the cocci seen in infected animals, which were rich in capsular microfilaments, as seen in Fig. 5.3. C- crypt lumen, E- epithelial cell. Pig 1, control. Bar = 1 μm.

5.4- Discussion

The use of IHC to screen for tissue blocks containing *S. suis* was a major aid to locating these bacteria by TEM. A good correlation between IHC of 1 μm sections and detection of *S. suis* by TEM was observed when the positive reaction for *S. suis* was strong. The weak IHC reaction in 3 control animals likely represents cross-reactivity with other streptococcal species from the commensal flora. Weak cross-reactivity of the *S. suis* polyclonal antibody with other species of streptococci has been observed (Middleton, Gottschalk and Willson, unpublished results).

The strain SX426 used in this study is virulent, since the infected animals developed clinical signs as described in Chapter 4. The thick capsule and numerous short filaments (fimbriae) of the intracellular bacteria observed in infected pigs is consistent with the morphology described for *S. suis* (Jacques et al., 1990), and with that of the positive control of a pure culture of *S. suis* serotype 2 in agar (Fig. 5.3).

Encapsulated *S. suis* are able to survive in neutrophils and macrophages *in vitro*, whereas non-encapsulated *S. suis* are killed (Wibawan and Lammler, 1994, Brazeau et al., 1996). In the present study it was shown that this phenomenon occurs also *in vivo*, as intact bacteria were observed inside neutrophils and macrophages. Alternatively, this could be interpreted as merely the uptake phase of phagocytosis; however, since at all time points post-infection the majority of intracellular bacteria seen in neutrophils appeared intact, this was likely due to resistance to the killing phase of phagocytosis. Although the bacteria were frequently disintegrating in macrophages (Fig. 5.8a), a few of these cells within the crypt epithelium (Fig. 5.8b), and also apparently crossing the basal lamina (Fig. 5.9), contained intact bacteria, suggesting some degree of bacterial resistance to the killing phase of phagocytosis, and the potential for spread by this means. This finding supports the hypothesis that macrophages transport *S. suis* to other tissues; however, it is possible that these intracellular bacteria may be destroyed later in the subepithelial lymphoid tissue and tonsillar draining lymph nodes.

S. suis organisms were also observed in vacuoles in epithelial cells throughout the full depth of the stratified crypt epithelium (Figs. 5.5 & 5.7). Few studies have investigated the interactions of *S. suis* and epithelial cells, and the results are contradictory (Norton et al., 1999, Lalonde et al., 2000). In this study, *S. suis* was demonstrated within crypt tonsillar epithelial cells using an *in vivo* model of infection, supporting the finding of epithelial cell invasion observed in a study using a laryngeal epithelial cell line (Norton et al., 1999).

There was no evidence of *S. suis* killing by epithelial cells, since only intact bacteria were seen in these cells. Two possible outcomes following uptake of *S. suis* by epithelial cells are: delivery to other cells, such as neutrophils and macrophages; or *S. suis*-mediated killing of the host epithelial cells with subsequent engulfment of the bacteria by phagocytes. The presence of *S. suis* in an apparently partially ruptured vacuole in an epithelial cell (Fig. 5.6), and of a necrotic focus in close association with these bacteria (Fig. 5.10), suggests that *S. suis* may induce necrosis and thus engineer escape from the cell. Suilysin, one of the virulence factors of *S. suis*, is a haemolysin that interacts with membrane cholesterol to cause cell lysis (Jacobs et al., 1994). As the

S. suis serotype 2 strain used in this experiment was suilysin positive, this toxin could have played a role in the focal necrosis and invasion of *S. suis* across the epithelium. Suilysin was involved in the lysis of different epithelial cell lines infected with *S. suis* serotype 2 and was suggested as a virulence factor that is involved in the process of tissue invasion by this organism (Norton et al., 1999, Lalonde et al., 2000).

This study suggested that *S. suis* invades epithelial cells of the tonsillar crypt epithelium *in vivo*, and probably induces focal necrosis of these cells, suggesting that this is the initial step in the pathogenesis of this infection. Also, this study supports the belief that *S. suis* disseminates to other tissues intracellularly via macrophages, as intact bacteria were seen within macrophages that apparently were crossing the basal lamina into the subepithelial tonsillar lymphoid tissue.

6- Morphological and Functional Study of the Crypt Epithelium of the Palatine Tonsils of Pigs

6.1 – Introduction

The palatine tonsils are part of MALT, a specialized compartment of the immune system located at the mucosal surfaces. They are in a strategic position, close to the entrance of both the respiratory and alimentary tracts, where most of the pathogens gain access to the body. Numerous pathogens utilize the palatine tonsils as a portal of entry, and as a site of persistence and multiplication (Al Sultan and Aitken, 1985; Stoddart et al., 1988; Shoo et al., 1990; Stenfors and Raisanen, 1991; Kelly, 1993; Frankel et al., 1997; Bruschke et al., 1998; Chiers et al., 1999; Oirschot, 1999). The tonsils have an important role in the defence, as illustrated by the presence of antibody-producing cells against several pathogens, such as *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus*, against diphtheria toxoid, and the lipopolysaccharide of *Escherichia coli* in the tonsils of human patients (Bernstein et al., 1999). Additionally, evidence indicates that they play an important role in the defence against Epstein-Barr virus and poliovirus infections in people (Ogra, 1971; Hirao et al., 1996).

The crypt epithelium of the palatine tonsil forms the first barrier against the external environment, and it is in close contact with the subjacent lymphoid tissue. This epithelium is highly infiltrated by lymphoid cells, creating areas of reticulation, and its role in transporting particulate material and pathogens from the crypt lumen to the underlying lymphoid tissue have been demonstrated (Williams and Rowland, 1972; Williams et al., 1973; Gebert, 1995; Perry and Whyte, 1998). This function is comparable to the role of the specialized M cells of MALT, such as in the PP of the intestine. A number of authors have suggested that M cells exist in the crypt epithelium of the palatine tonsil, e.g. in pigs (Belz and Heath, 1996), rabbits (Olah and Everett,

1975; Gebert, 1995), dogs (Belz and Heath, 1995), and humans (Howie, 1980). Only one of these studies, which was performed in rabbits, associated morphology and function to investigate the presence of M cells in the palatine tonsils (Gebert, 1995). The others were based solely on morphological findings, such as the presence of microvilli or microfolds on the apical cytoplasm, numerous cytoplasmic vesicles, and indentation of the plasmalemma by lymphocytes (Olah and Everett, 1975; Howie, 1980; Belz and Heath, 1995; Belz and Heath, 1996). There is however still controversy as to whether M cells are present in the palatine tonsils of people (Gebert and Pabst, 1999). These cells have been described in a few studies on the human palatine tonsils (Howie, 1980; Finzi et al., 1993); however other investigations have not confirmed their presence (Perry et al., 1988; Perry, 1994; Claeys et al., 1996). It has been suggested that M cells are present exclusively in MALT with simple epithelium, such as GALT or BALT, while in tissues with stratified squamous epithelium, such as the palatine tonsils, other cells, e.g. DCs, may perform the role of antigen uptake and transport to the underlying lymphoid tissue (Neutra et al., 1996). Considering this controversy, there is a clear need for further studies on the crypt epithelium of the palatine tonsils to investigate whether a specialized cell for antigen transport, comparable to the M cell, is indeed present in this tissue.

In Chapter 5, the early interaction of *S. suis* with the tonsillar crypt epithelium was investigated by TEM. In addition to neutrophils and macrophages, *S. suis* was present within epithelial cells. Although earlier studies described the uptake of *S. suis* (Williams et al., 1973) and particulate material (Williams and Rowland, 1972) by the crypt epithelial cells, no correlations with the ultrastructure were performed, and these studies predated the first description of M cells (Owen and Jones, 1974). In the present chapter, structure and function of the crypt epithelium are correlated in an ultrastructural study of this epithelium. The structure of epithelial cells in the crypts of the palatine tonsils of pigs is described and compared with that described in the literature for M cells. Function is evaluated by analysing the ultrastructure of the epithelial cells containing *S. suis* following experimental pharyngeal infection with these bacteria, and by examining these cells for the presence of features that characterize M cells.

6.2- Material and Methods

6.2.1 - *Streptococcus suis* strain

The strain SX 426 of *S. suis* serotype 2 was used, as described in Chapter 4.

6.2.2 - Animals and samples

The animals of this investigation were the same used for the immunohistochemical study of leukocyte subsets in the crypt epithelium of the palatine tonsils in Chapter 4, i.e., piglets experimentally infected with *S. suis* and non- infected piglets. The tissues used were the same as in Chapter 5, and the method of fixation and of processing the tissues for TEM are as described in that chapter.

6.3- Results

6.3.1 – Morphology of the crypt epithelium

The crypt epithelium of the palatine tonsils was stratified, squamous non-keratinized with approximately five to six layers, and was variably infiltrated by non-epithelial cells, creating areas of reticulation (Fig. 6.1).

The crypt epithelium could be divided into three layers: the basal layer, the intermediate layer, and the superficial layer, each of which is described below.

6.3.1.1- Basal Layer

The basal epithelial cells were connected to the basal lamina by hemidesmosomes and to adjacent epithelial cells by desmosomes. These cells and their nuclei were usually cylindrical, less frequently cuboidal, with an electron-lucent or electron-dense cytosol and nucleus. Nuclear chromatin was dispersed and the nucleolus could be readily observed. The cytoplasm was rich in mitochondria and free ribosomes, with a small amount of RER, and bundles of filaments arranged in the direction of the long axis of the cell (Fig. 6.2). Basal cells deformed by lymphoid cells often assumed bizarre shapes (Fig. 6.3).

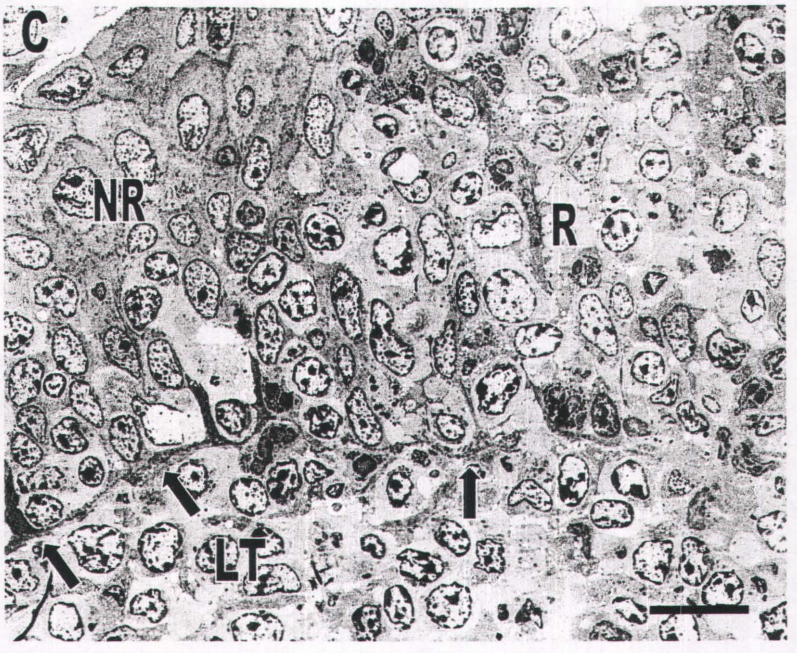


Fig. 6.1 – Full depth section of the tonsillar crypt epithelium. Note the marked infiltration of leukocytes in the epithelium, or reticulation, distorting the morphology of epithelial cells. The boundary between epithelium and lymphoid tissue (arrows) is sometimes effaced by the high infiltration of leukocytes. NR - non-reticulated area, R - reticulated area, C- crypt lumen, LT - lymphoid tissue. Bar = 15 μm.

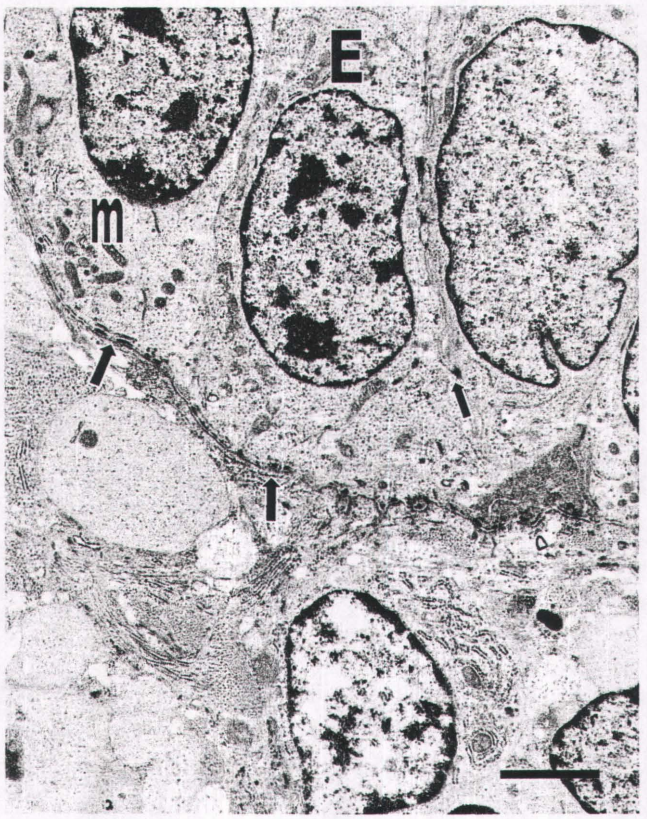


Fig. 6.2 – Basal layer of the crypt epithelium and subjacent lymphoid tissue. The basal epithelial cells are connected to the basal lamina by hemidesmosomes (arrows) and to each other by desmosomes (small arrow). The cytoplasm of these cells is rich in mitochondria (m) and the nucleus has dispersed chromatin.
E – epithelial cell. Bar = 3 μm.

6.3.1.2- Intermediate Layer

In the intermediate layer, the cells were polyhedral or cylindrical, and were frequently elongated, star-shaped or irregularly-shaped when distorted by lymphoid cells. The nuclei were round or oval, the nuclear chromatin dispersed, and one or two nucleoli were present. These epithelial cells were connected to each other by desmosomes; sometimes the cytoplasmic membrane of adjacent epithelial cells interdigitated. The cytosol and nucleus of the epithelial cells of the intermediate layer were either electron-lucent or electron-dense. The cytoplasm was rich in free ribosomes, had a variable number of mitochondria, with bundles of filaments dispersed in the cytosol, and generally there was minimal amount of RER (Fig. 6.4). Many other epithelial cells, on

the other hand, were rich in RER and vesicles, most of which were electron-dense. Their overall shape, as well as the nuclear outline, was frequently distorted by lymphoid cells. The nuclear membrane was frequently ruffled, and one or two nucleoli could be seen (Fig. 6.5).

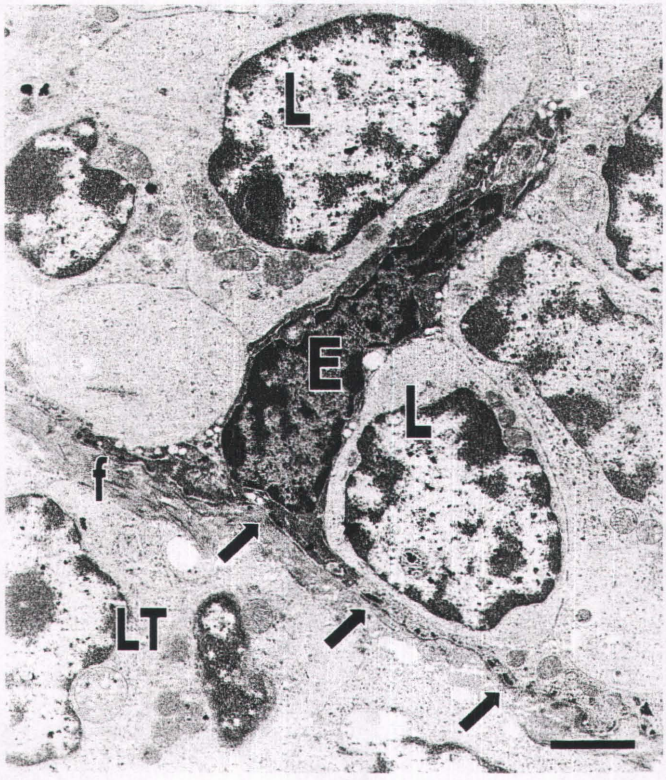


Fig. 6.3 – Reticulated area in the basal layer of the crypt epithelium. This electron-dense crypt epithelial basal cell (E) is deformed by the infiltration of lymphoid cells (L). Note the close contact of the epithelial cell with the lymphoid tissue below, from which it is separated only by the basal lamina and a thin layer of collagen fibres (f). Arrows – hemidesmosomes. LT – lymphoid tissue. Bar = 2 μm.

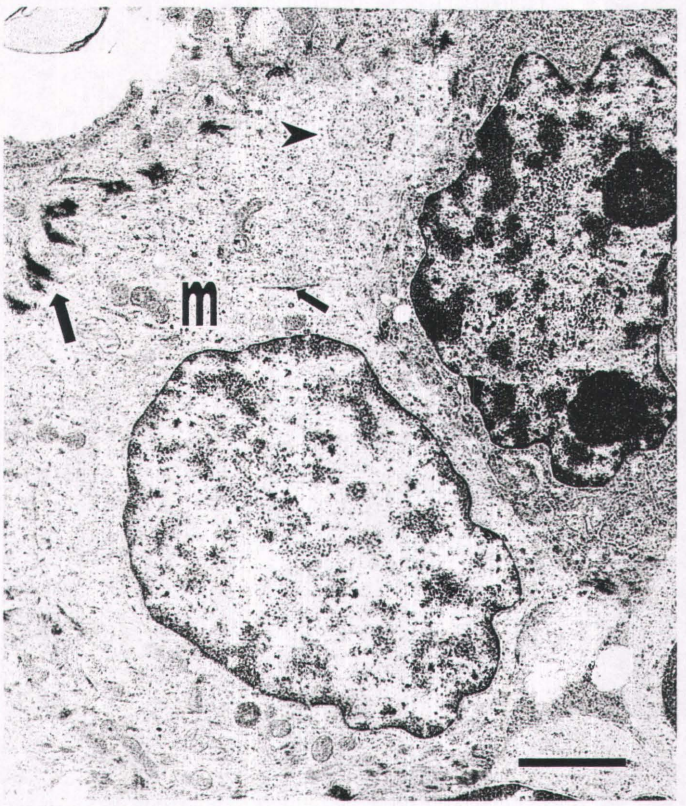


Fig. 6.4– Epithelial cell of the intermediate layer of the crypt epithelium. Note the desmosomes (large arrow) confirming the epithelial nature of this cell. The cytoplasm has sparse RER (arrow head), scattered mitochondria (m) and a few filament bundles (small arrow). The nuclear chromatin is dispersed. Bar = 2 μm.

6.3.1.3- Superficial Layer

In this layer the cells varied from cuboidal, bulging into the crypt lumen (Fig. 6.6a), to more typical flat squamous cells (Fig. 6.7). The cuboidal cells had finger-like specializations (microvilli) of the apical cytoplasmic membrane. Microvilli varied from numerous to sparse, and were homogeneous or heterogeneous in size (Fig. 6.6a). The majority of the cells had microvilli that were 0.3 to 0.6 μm in length and 0.1 to 0.2 μm in thickness, while others had more blunted microvilli with a length of 0.2 μm and thickness of 0.1 μm. The microvilli were covered with small, fine, hair-like structures, which formed the glycocalyx (Fig. 6.6b). Less frequently, instead of microvilli, apical irregular projections or folds were seen (Fig. 6.8). The cuboidal superficial cells were rich in organelles and frequently had numerous vesicles which were located mainly in

the apical portion of the cytoplasm. These cells had variable amount of RER, and a moderate number of mitochondria (Fig. 6.6a). In some, a prominent Golgi apparatus (Fig. 6.6a) and secondary lysosomes were seen. The vesicles in these superficial cells occasionally presented an electron-dense core (Fig. 6.8). The filaments were scattered throughout the cytoplasm or, less often, arranged as bundles. The nucleus of the superficial cells was usually indented, the chromatin dispersed, and with or without a nucleolus (Fig. 6.6a).

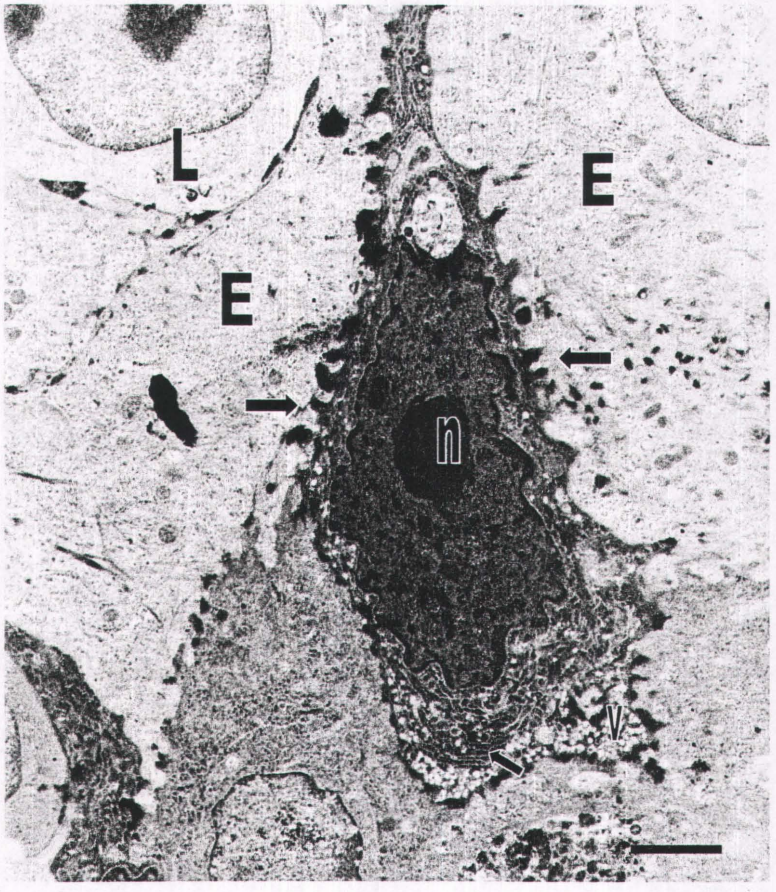


Fig. 6.5 – Electron-dense epithelial cell in the intermediate layer of the crypt epithelium. This cell has a cytoplasm rich in RER (small arrow) and vesicles (v), and the nucleus has a big nucleolus (n). Note the desmosomes (arrow) confirming the epithelial nature of this cell. E - epithelial cell, L - lymphocyte. Bar = 2 μm.

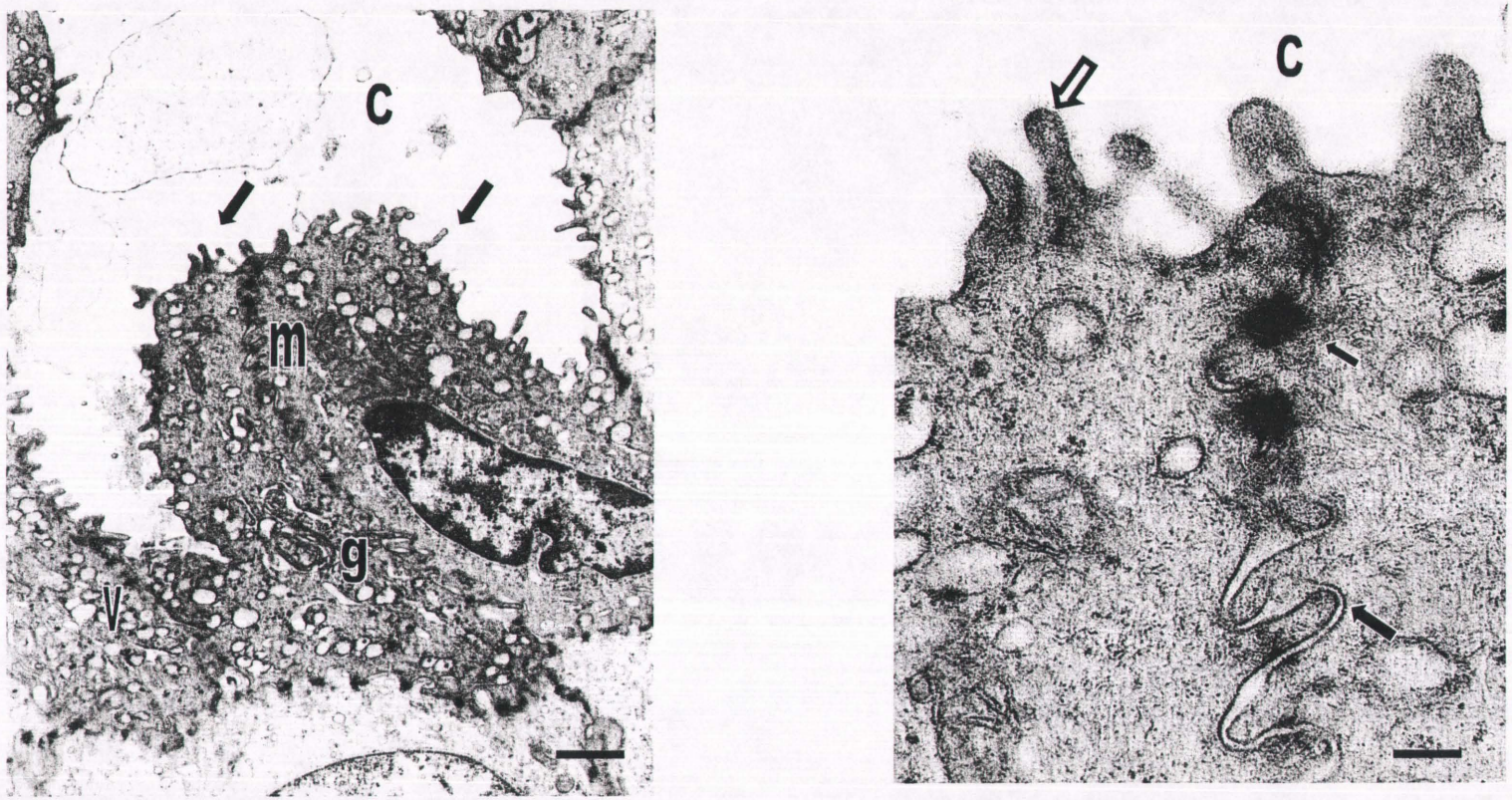
In contrast to the cuboidal superficial cells, the cytoplasm of the squamous cells was packed with filaments oriented parallel to the surface, and was poor in organelles (Fig. 6.7b). Microvilli were lacking, or if present, were blunted and sparse.

Similar to the epithelial cells of the other layers, superficial epithelial cells varied from electron-dense to electron-lucent. The electron-dense cells were either cuboidal, and rich in vesicles and RER (Fig. 6.9), or squamous, poor in organelles, and shedding into the crypt lumen (Fig. 6.7). Superficial cells were linked to each other and to the epithelial cells of the intermediate layer by desmosomes and interdigitations of the cytoplasmic membrane (Fig. 6.6b).

Several types of non-epithelial cells, lymphocytes, plasma cells, macrophages and neutrophils were present in the crypt epithelium. Lymphocytes were the predominant infiltrating cell and they occurred in small or larger groups of cells embraced by epithelial cells (Fig. 6.10), sometimes also in close association with macrophages. Less often, solitary lymphocytes were observed.

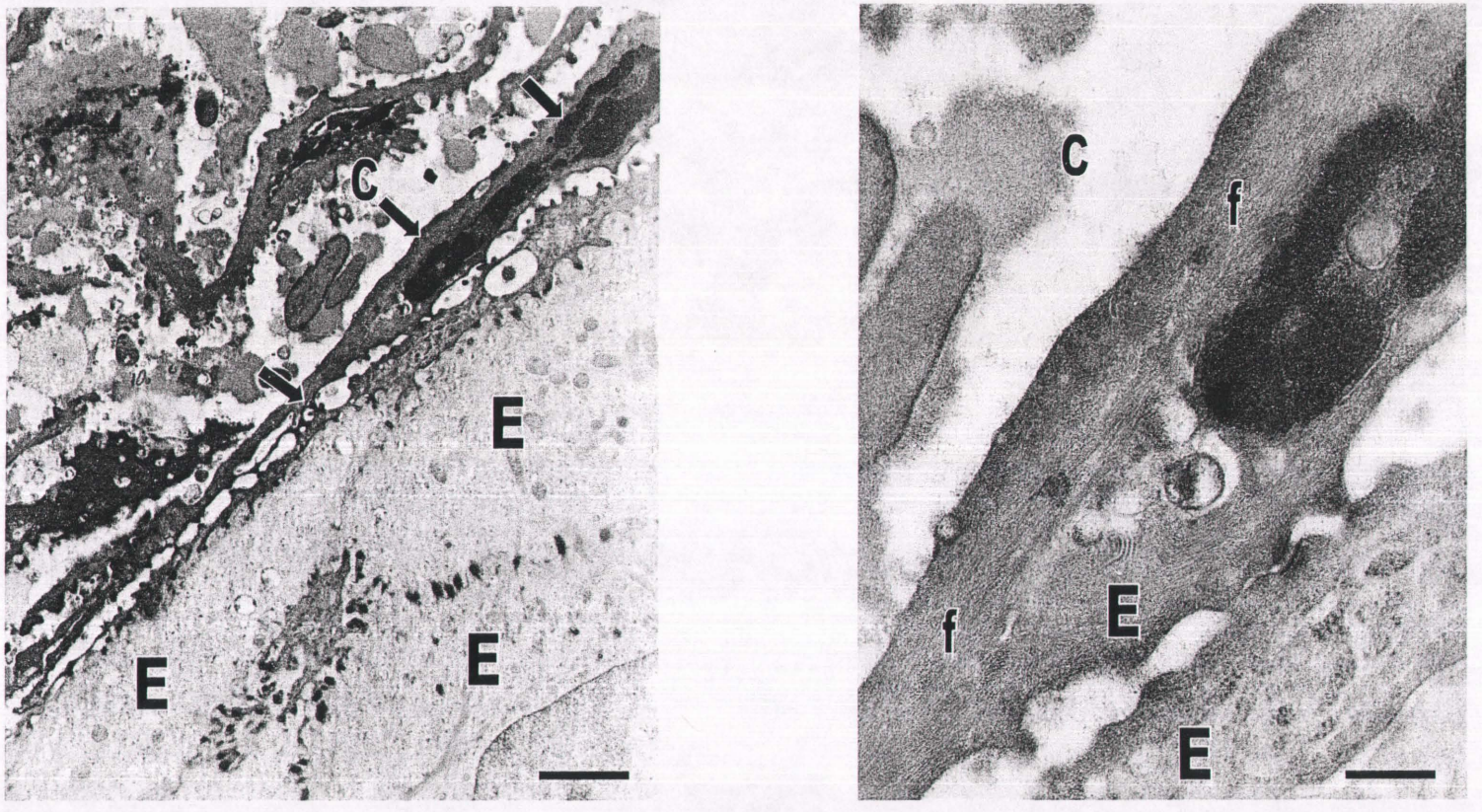
6.3.2 – Epithelial cells containing *S. suis*

As described in Chapter 5, bacteria with morphology consistent with *S. suis* were observed in a few epithelial cells, in both the superficial and deeper layers of the crypt epithelium. The two superficial cells containing bacteria lacked microvilli (Figs. 5.2 & 5.7), and one of them (Figs. 5.7 & 6.11) had a discrete fold on the apical surface. These superficial cells were both electron-dense, had well-developed RER and vesicles, one of them was embracing lymphocytes (Fig. 5.2), but the other was not (5.7 & 6.11). Two other epithelial cells containing *S. suis* were in the intermediate layer, immediately subjacent to superficial cells. One was electron-lucent, with a well-developed RER, had no vesicles, and apparently was not associated with lymphocytes (Figs. 5.5, 5.6, 6.12). The other was instead electron-dense, also had a well-developed RER, few vesicles, and was embracing two lymphocytes.



a b

Fig. 6.6 - Superficial epithelial cells of the crypt epithelium. **a -** Cuboidal cells are bulging into the lumen. Numerous microvilli of different sizes are present apically (arrows). The cytoplasm is rich in vesicles (v), has a moderate amount of mitochondria (m), and the nucleus is indented. The Golgi apparatus (g) is prominent in one of these cells. Bar = 1 μm. **b** - Interdigitations (arrow) and desmosomes (small arrow) connect neighbouring cells. Small hair-like structures, the glycocalyx (open arrow), can be seen covering the microvilli. Bar = 0.2 μm. C – crypt lumen.



a **b**

Fig. 6.7 – A squamous superficial epithelial cell in the crypt epithelium (arrows). **a** – This cell has no microvilli, has a pyknotic nucleus and it is detaching into the lumen. Bar = 2 μm. **b** – Higher magnigication of **a**. The cytoplasm of this superficial cell is packed with filaments (f) oriented parallel to the plasmalemma, and has very few organelles. Bar = 0.4 μm. C – crypt lumen, E – epithelial cell.

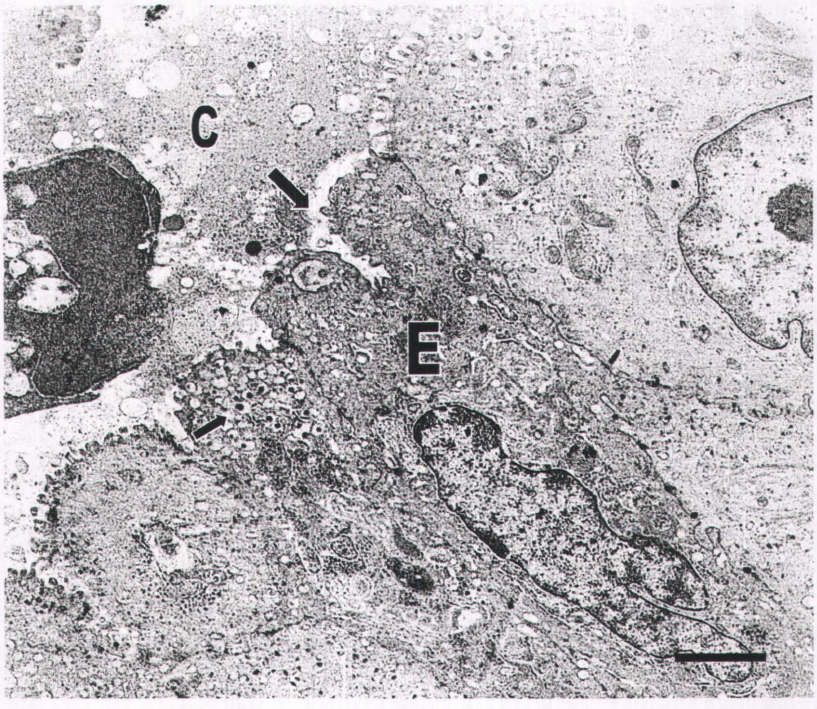


Fig. 6.8 – A superficial epithelial cell (E) unlike the adjacent cells with microvilli, has a fold (arrow) of the apical cytoplasm. Vesicles in an adjacent epithelial cell (small arrow) have an electron-dense core. C – crypt lumen. Bar = 2 μm.

Fig. 6.9 – A portion of an electron-dense epithelial cell is seen between electron-lucent superficial cells. The cytoplasm of this cell is very rich in vesicles (v) and RER (arrow). C – crypt lumen. Bar = 2 μm.

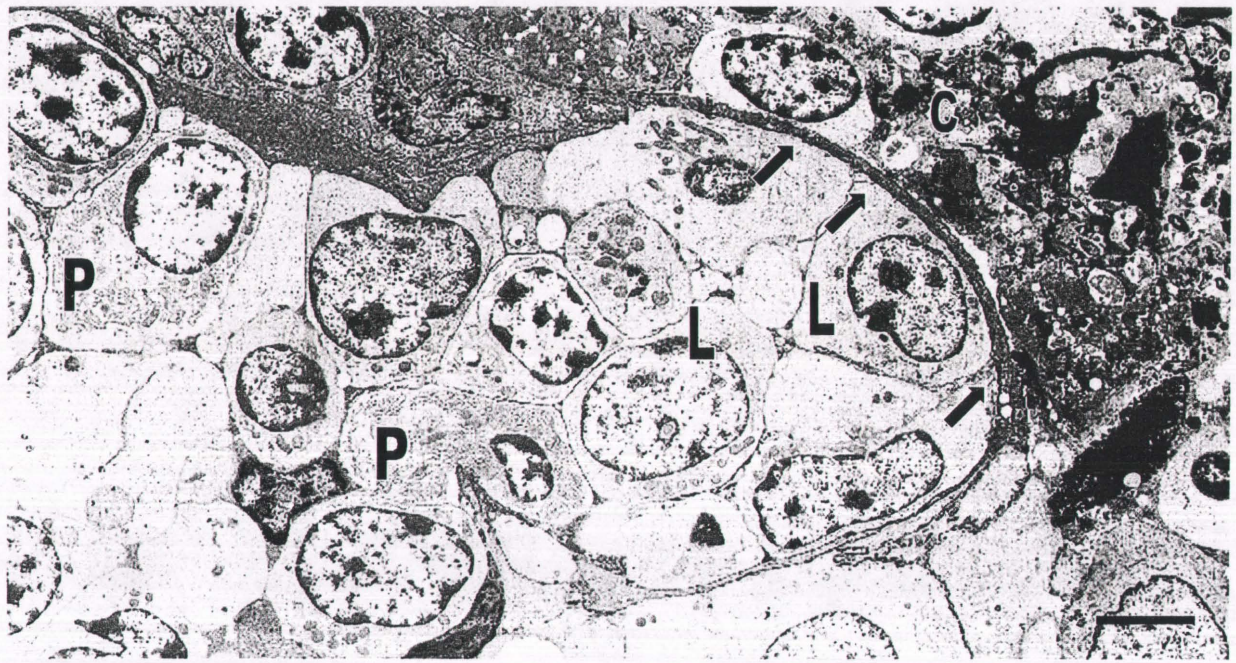


Fig. 6.10 – A large group of lymphocytes infiltrating the crypt epithelium. Note the very thin cytoplasm of the superficial epithelial cell (arrows) that embraces these lymphocytes, allowing them to be closely opposed to the lumen (C). L – Lymphocyte, P -plasma cell. Bar = 5 μm.

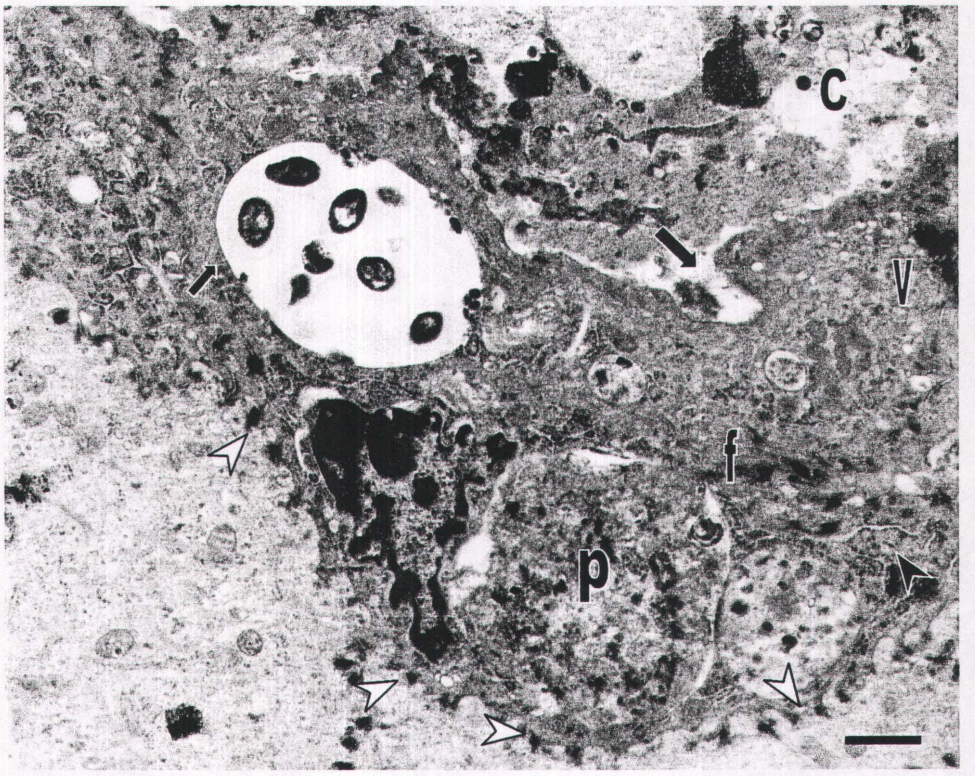


Fig. 6.11 – Higher magnification of Fig. 5.7 from Chapter 5. This superficial epithelial cell containing *S. suis* (small arrow) is electron-dense, rich in RER (arrow head), vesicles (v), phagolysosomes (p), and bundles of filaments (f). The apical membrane of this epithelial cell forms a discrete fold (large arrow). Desmosomes –white arrow heads, C – crypt lumen. Bar = 1μm.

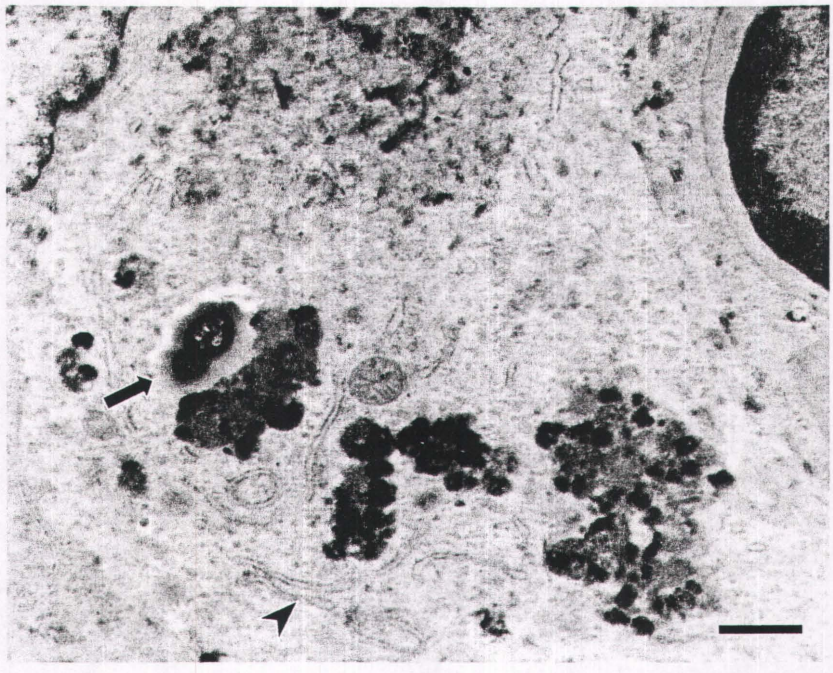


Fig. 6.12 – Higher magnification of Fig. 5.5 from Chapter 5. Epithelial cell from the intermediate layer containing *S. suis* (arrow). This cell is electron lucent, has moderate amount of RER (arrow head), and lacks vesicles. Bar = 1 μm.

6.4 - Discussion

This study agrees with previous descriptions of the overall structure of the tonsillar crypt epithelium of pigs (Belz and Heath, 1996), dogs (Belz and Heath, 1995), rabbits (Olah and Everett, 1975), and humans (Perry et al., 1988; Perry, 1994). The organization of this epithelium is unusual and differs from other stratified, squamous non-keratinized epithelia such as the oral, esophageal, and vaginal mucosae (Ham and Cormarck, 1979). A unique feature of the tonsillar crypt epithelium is the morphology of the superficial cells. In contrast to the cells of the classical stratified squamous non-keratinized epithelia that are flat, poor in organelles, and have nuclei frequently pyknotic (Farbman, 1988), most superficial cells of the crypt epithelium are cuboidal, possess microvilli, are rich in organelles, and have an intact nucleus. Microvilli are present in small number on most of the superficial epithelial cells of the body, but on cells with intense absorptive capacity, like enterocytes and proximal tubular cells of the kidney, they are markedly developed, as well as on cells involved in transepithelial transport (transcytosis) of large molecules (Griepp and Robbins, 1988). The presence of well-developed microvilli on the superficial cells of the crypt epithelium, in association with numerous cytoplasmic vesicles, suggests a high capacity for uptake of material from the lumen.

In this study, however, both the superficial and intermediate layers demonstrated heterogeneity of the cell morphology. Cells rich in RER, indicative of protein synthesis for export, and rich in vesicles, suggestive of either uptake or secretion of material, were seen amidst cells rich in free ribosomes, poor in RER, and lacking vesicles, features implying protein synthesis for intracellular consumption. These differences in cell morphology have not been described previously in the porcine species. The observations here suggest that for the tonsillar crypt epithelium additional roles may exist beyond the simple protective function of other stratified, squamous non-keratinized epithelia. These other roles do not appear to be restricted to only a few superficial cells such as the putative M cells, but pertain also to cells of the other layers of the crypt epithelium.

Uptake and transport of material from the crypt lumen to the underlying lymphoid tissue have been suggested previously as functions for the putative M cells in

the palatine tonsils of pigs (Belz and Heath, 1996). Another possible role for the crypt epithelial cells which are rich in RER and vesicles and that are not restricted only to the superficial layer, is the secretion of cytokines in response to pathogens or infiltrating lymphoid cells. Epithelial cells can secrete a variety of cytokines, such as IL-1, IL-6, TGF-α and -β, in response to other locally produced cytokines, lymphocyte infiltration, injury or infection. Epithelial cells also may secrete cytokines constitutively. These molecules are believed to have regulatory roles in epithelial proliferation, in the control of bacterial flora, and in the growth and differentiation of non-epithelial cells, especially lymphocytes that infiltrate the epithelium. Intestinal epithelial cells of rats and humans secrete IL-6; keratinocytes constitutively secrete several colony-stimulating factors (CSF); and human bronchial epithelial cells and rat type II alveolar epithelial cells constitutively secrete granulocyte CSF and granulocyte-macrophage CSF (Stadnyk, 1997). Finally, epithelial cells positive for IL-1 β have been observed in the crypt epithelium of the palatine tonsils of humans (Von Gaudecker et al., 1996).

In the present study function was associated with morphology by analysing the features of epithelial cells that contained bacteria with morphology consistent with *S. suis* (refer to Chapter 5) after pharyngeal experimental infection with this pathogen. The sole common feature of the few epithelial cells containing *S. suis* was a well-developed RER. These cells were located throughout all layers of the epithelium, either had vesicles or not, and were variably in close association with lymphocytes. No microvilli were observed in the superficial cells containing bacteria, and one of them (Fig. 6.11) had discrete folds on the apical surface instead.

Morphological features described for M cells such as well-developed microvilli, presence of folds in the apical membrane, numerous vesicles, prominent Golgi apparatus and RER were observed in this study in many cells of the crypt epithelium, and interestingly were not limited to cells in close contact with lymphocytes. To date no consistent morphological criteria for the identification of M cells have been developed. They are numerous only in the rabbit (Olah and Everett, 1975), otherwise they are difficult to find (Howie, 1980; Belz and Heath, 1996). They are in intimate contact with lymphoid cells (Olah and Everett, 1975; Howie, 1980; Belz and Heath, 1996). The

apical cytoplasm is rich in vesicles, and RER and Golgi apparatus are well-developed (Olah and Everett, 1975; Howie, 1980; Belz and Heath, 1996). Sometimes the apical membrane is folded (Belz and Heath, 1996). The microvilli may be regularly or irregularly distributed (Howie, 1980; Belz and Heath, 1996), and short, long, or of variable length (Olah and Everett, 1975; Belz and Heath, 1995; Belz and Heath, 1996).

In pigs, the putative M cells have been described as being few. They have a variable morphology; some have long irregular microvilli 3-9 μm long while others are covered by uniform closely packed microvilli 0.3-1 μm long and 0.1μm wide. Superficial epithelial cells, not considered M cells, are described to have short microvilli up to 0.25 μm long and 0.06 μm wide (Belz and Heath, 1996). Long microvilli were not seen in the present study. On the majority of superficial cells, the microvilli were 0.3-0.6 μm long and 0.1-0.2 μm wide, which is within the range described for the M cells of pigs (Belz and Heath, 1996), and only a few cells had blunted microvilli 0.2 μm long and 0.1 μm thick. Taken together, if the morphological concepts described in the literature for the identification of M cells are applied in this study, the palatine tonsils of pigs could be interpreted as having numerous M cells in the crypt epithelium, contradicting what has been described for this species (Belz and Heath, 1996).

Although the number of epithelial cells containing *S. suis* was too low for drawing definite conclusions, they did not all possess the morphological criteria described for M cells. However, this bacterium could have mediated its own uptake to enter into the epithelial cells, as occurs with invasive bacteria such as *Yersinia* spp. which has a membrane protein called invasin that mediates attachment and entry into nonphagocytic cells (Finlay and Falkow, 1997), or *Neisseria gonorrhoea* that induces focal polymerization of the actin of the host cell, inducing its own engulfment (Nassif et al., 1999). Hence, further studies using inert substances such as liposomes or latex microspheres are necessary to evaluate if the cellular uptake is performed exclusively by one type of cell that could be classified as M cell.

Considering the findings of the present study and comparing with those described in pigs (Belz and Heath, 1996), two opposing speculations may be made: pigs either have numerous M cells in the crypt epithelium, contrary to findings of Belz & Heath

(1996), or M cells are not present in this epithelium. The criteria for the identification of M cells in tissues with stratified epithelium are still uncertain, and until definitive criteria are developed, the best way to identify these cells is functionally, i.e. by their ability to take up particulate material from the crypt lumen and to transport it to neighbouring cells. Hence, the existence of M cells in the palatine tonsils of pigs remains putative.

7 – General Discussion

This study was performed to investigate the crypt lymphoepithelium of the palatine tonsils, which is the barrier between the external environment and the underlying lymphoid tissue, and consequently is the first line of defence of this organ and a site of potential interaction of microbes and antigens with the immune system. In this research, the crypt epithelium of the palatine tonsils of pigs was investigated in healthy market-weight pigs and in piglets experimentally infected with *S. suis* serotype 2. This pathogen is well recognized to utilize the palatine tonsils as a portal of entry and also as a site of persistence in carrier animals. In fact, the strategic position of the palatine tonsils, close to the entrance of upper respiratory and alimentary tracts, where most pathogens gain access to the body, makes these lymphoid organs a frequent portal of entry and site of multiplication and persistence of numerous pathogens in domestic animals and humans. The importance of these organs is unquestionable in the initial steps of the pathogenesis of many infectious diseases. Consequently, they are also potentially important organs in the development of effective mucosal vaccines, which are now being widely investigated for protection against infectious diseases of domestic animals and humans. Investigation of the palatine tonsils of pigs, in addition to contributing to the understanding of porcine infectious diseases and mucosal vaccination in this species, provides information on comparative pathology. Investigations of the palatine tonsils of humans have been restricted to tissues from patients suffering from chronic tonsillitis; therefore, they do not represent a normal physiological state. Pigs are generally considered a very suitable species to use as a model in human medical science (Swindle et al., 1992). The similarities in physiology and anatomy, and low maintenance costs, make the pig a species of choice in human medical research.

This research was divided into four parts: 1) characterization of the lymphocyte subpopulations in the crypt epithelium of the palatine tonsil of 6-month-old market pigs; 2) analysis of the early changes that occur in leukocyte subsets of the crypt epithelium in response to *S. suis* experimental infection; 3) investigation of the transport of *S. suis* across the crypt epithelium in the early stages of this infection; and 4) examination of the crypt epithelium for the presence of M cells.

For the investigation of the lymphocyte subsets in the crypt epithelium of the palatine tonsil of pigs an immunohistochemical technique using monoclonal antibodies against lymphocyte markers CD3, CD4, CD8α, CD8β, λ-Ig-Lc and γδ TCR was used, and qualitative and quantitative studies were performed by image analysis of the slides stained for these markers. Other studies described in the literature are restricted to either qualitative, i.e. descriptive immunohistological studies on the distribution of leukocytes in the palatine tonsils (Jonjic et al., 1987; Ramos et al., 1992), or solely quantitative as in investigations using FACS technology (Yang and Parkhouse, 1996; Zuckermann and Gaskins, 1996). Although these approaches are extremely useful, either one used alone does not provide complete information on the tissue being studied. In qualitative studies, variations that occur among animals are not evaluated, whereas in merely quantitative studies using FACS technology, although precise information is obtained on the proportions of the different lymphocyte subsets and in the variations among animals, distribution in the various compartments of the lymphoid tissue is not addressed. Although the isolation of specific compartments of a lymphoid tissue (such as performed in the intestine) can be carried out prior to FACS analysis (Solano-Aguilar et al., 2000), contamination among the different compartments is very common, thus compromising the analysis.

In Chapter 3, it was demonstrated that the crypt epithelium is infiltrated by a variety of lymphocyte subsets and has the potential to participate in both humoral and cellular immune responses. Also there is variation in the number in each leukocyte subset among different animals and among different crypts within the same animal. The variations among animals were unexpected since the animals used were apparently healthy, with the same age and came from the same source. Further studies are indicated

using more animals and/or tissue samples to evaluate these variations more fully. Lymphocyte subsets in the crypt epithelium vary among different age groups, as can be concluded from the results of Chapters 3 and 4, which were performed in 6-month-old and 3-week-old pigs, respectively. It would be interesting to investigate whether different rearing conditions, such as high health status herds and conventional herds, influence the composition of lymphocyte subsets in the crypt epithelium.

It was notable that the population of DP cells, believed to represent memory cells in pigs, was low (less than 17%) in the crypt epithelium, in contrast with results of FACS studies on the whole tonsils of pigs of the same age (Zuckermann and Gaskins, 1996), and with the fact that memory cells are expected to dominate in epithelial surfaces (Mackay, 1993), including the crypt epithelium of the palatine tonsils of humans (Ruco et al., 1995). Studies using antibodies against activation markers, which are now available for pigs, are necessary to confirm whether the majority of CD4 cells in the crypt epithelium of the palatine tonsils of pigs are indeed naïve cells. This is an interesting question since the palatine tonsils are closely related to the thymus ontogenetically and may have epithelial corpuscles comparable to the thymic Hassall's corpuscles (Slipka, 1988; Perry and Slipka, 1993).

In Chapter 3, a high number of γδ T lymphocytes (approximately half of the T lymphocyte population) was observed in the crypt epithelium. Although these cells are known to represent a high proportion of lymphocytes in the peripheral blood of pigs, they were described to be only 2.5% of the tonsillar lymphocytes of 4-week-old pigs (Yang and Parkhouse, 1996). Hence, it seems that in the palatine tonsils these cells are concentrated mainly in the crypt epithelium, and this is indeed apparent in the histological sections stained for γδ TCR. γδ T lymphocytes are found in higher proportions in the epithelial surfaces and as yet their functions and what they recognize are not clear (Boismenu and Havran, 1997; Born et al., 1999). More studies are needed to understand the biology of γδ T lymphocytes, and since they are numerous in pigs, these animals represent a good model for this study.

In Chapter 4, again using an immunohistochemical technique and monoclonal antibodies against a variety of the leukocyte markers, it was demonstrated that the

populations of leukocytes in the crypt epithelium do respond to bacterial infection. Additionally, the substantially lower number of lymphocyte subsets in 3-week-old compared to 6-month-old pigs may be associated with lower resistance to *S. suis* infection at younger age. The increased positivity for the myeloid marker at 24h after oral infection with *S. suis* was not unexpected since neutrophils and macrophages contribute to the innate immune system and are generally involved in the early response to pathogens. As described in Chapter 5, *S. suis* was observed by TEM within neutrophils and macrophages of the crypt epithelium 18h post-infection. The rapid response is believed to be due to the presence of receptors on these cells that recognize a few highly conserved structures on the surface of pathogenic microbes. These structures are called pathogen-associated molecular patterns (PAMP), and include teichoic acid from Gram-positive bacteria, LPS on the surface of Gram-negative bacteria, yeast wall mannans, and double-stranded RNA from virus (Medzhitov and Janeway Jr., 2000). Teichoic acid is present in the cell wall of *S. suis* (Elliott et al., 1977).

The positive reaction with the porcine myeloid marker in epithelial cells in reticulated areas of the crypt epithelium was intriguing. It is possible that this reaction reflects a peculiarity of the crypt lymphoepithelium. Vimentin, a cytoskeletal protein normally confined to mesenchymal cells, including macrophages, is also present in M cells in the palatine tonsils of rabbits (Gebert et al., 1995). In humans, a variety of epithelial cells, e.g. from the oropharynx, tonsils, oesophagus, vagina and cervix, have shown a positive reaction for the myeloid marker MAC-387 (Brandtzaeg et al., 1987) and it has been suggested to be associated with innate defence against pathogens (Brandtzaeg et al., 1995). The significance of this myeloid positive reaction in the crypt epithelium of the palatine tonsils of pigs should be investigated further. It may simply be a false positive reaction due to leakage of the specific protein from leukocytes to epithelial cells, or an epithelial protein in common with a myeloid cell protein. If the latter is true, there may be a role for this specific protein in the intra-epithelial defence against pathogens, given the fact that this staining was increased in the crypt epithelium of the animals infected with *S. suis*.

Lymphocytes were also seen to increase in number in the crypt epithelium in response to *S. suis*. The B cell subset represented by λ-Ig-Lc positive cells was increased at 18h following oral infection with *S. suis*, and at 24h an increase in CD4 and CD8 subsets was noticed. This response was very rapid considering that cells of the acquired immune system take 3 to 5 days to proliferate and differentiate in response to an infection (Medzhitov and Janeway Jr., 2000). One possibility is that they infiltrated from the subjacent lymphoid tissue under the influence of cytokines or chemokines. The increase in the B cell subset may indicate a non-specific polyclonal response to virulence factors secreted by *S. suis*. Suilysin, a virulence factor present in the *S. suis* strain used, can trigger the production of IL-6 by porcine monocytes and alveolar macrophages (Lun et al., 2001). Therefore, IL-6 production may explain the early increase in B lymphocytes. IL-6 is a growth factor for differentiated B lymphocytes (Abbas et al., 2000b). Several speculations can be made on the early increase of T subsets. It could have been triggered by superantigens, which are microbial proteins that are potent T cell mitogens. Superantigens are present in group A streptococci (Norrby-Teglund et al., 1994); however it is unknown if they are present in *S. suis*. The increase in CD4 and CD8 subsets could represent a memory type of response, if these animals had been previously infected with other bacteria with epitopes shared by *S. suis* serotype 2. The CD8 response alone may represent an innate response of NK cells, which can express CD8 molecule (Zuckermann et al., 1998). Experiments evaluating longer periods post-infection would be necessary to analyse whether the T cell response observed was specific to *S. suis* and also to determine the direction of this response, i.e. whether a Th1 or Th2 type of response. Taken together, these findings suggest that there is a potential to initiate humoral and cellular immune responses in the crypt epithelium of the palatine tonsils. Considering that *S. suis* was observed inside neutrophils, macrophages and epithelial cells (Chapter 5), suggesting resistance to intracellular killing, a cellular immune response would appear necessary for effective clearance of this bacterial pathogen.

In Chapter 5 the early stage of *S. suis* infection was investigated in the crypt epithelium following experimental pharyngeal infection with this pathogen. This study

supports the *in vitro* evidence that *S. suis* resists the killing phase of phagocytosis, since intact bacteria were observed within neutrophils and macrophages (Wibawan and Lammler, 1994; Brazeau et al., 1996); it also substantiates the hypothesis that *S. suis* gains access to other tissues inside macrophages (Williams and Blakemore, 1990). The observation of *S. suis* within epithelial cells of the crypt epithelium demonstrated that *in vivo* this pathogen utilizes epithelial cell invasion in the early stage of the infection. Hence, effective methods of preventing *S. suis* infection could involve the development of mucosal vaccines that induce IgA production in the upper respiratory and digestive tracts in a way to hamper the epithelial invasion. Alternatively, the blocking of potential receptors for *S. suis* in the crypt epithelium, such as the sugar Galα1-4Gal present in the porcine pharyngeal epithelium which is recognized by the 18 kDa adhesin of *S. suis* (Haataja et al., 1993), could be developed as a method to prevent *S. suis* infection.

The hypothesis that M cells are present in the crypt epithelium of the palatine tonsils of pigs could not be confirmed in the study associating morphology and function (Chapter 6). The few epithelial cells containing *S. suis* (the criterion used to define function), did not present a common morphological feature that allowed characterization as M cells. Since the possibility that this pathogen mediated its own uptake cannot be ruled out, further studies are needed using particulate material to investigate whether M cells are present in this epithelium. However, the ultrastructural features of several epithelial cells from all different layers of the crypt suggest that this epithelium is highly active, and likely has a role not only in uptake of antigens but also in the secretion of cytokines to maintain this unique microenvironment of intimate contact between lymphocytes and epithelial cells. A logical next step in investigating this epithelium is to examine the expression of cytokines in healthy and experimentally infected pigs.

This research has demonstrated that the crypt epithelium of the palatine tonsils and the intra-epithelial leukocyte populations are highly dynamic and respond quickly to exposure to *S. suis* and perhaps to other pathogens. As the immune events in this first barrier zone may influence the outcome of infection, further studies on the palatine tonsils may provide invaluable information on the pathogenesis of infectious diseases. Because of the strategic position of the palatine tonsils in the upper aero-digestive tract

where most of the pathogens gain access, and because the tonsils can seed distant tissues with primed lymphocytes, such information will be extremely useful for the development of mucosal vaccines for both animals and humans.

8 - References

1. Abbas, A K, Lichtman, A H, and Pober, J S. 2000a. Antigen receptors and accessory molecules of T lymphocytes. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 102-121.

2. Abbas, A K, Lichtman, A H, and Pober, J S. 2000b. B cell activation and antibody production. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 182-207.

3. Abbas, A K, Lichtman, A H, and Pober, J S. 2000c. Cytokines. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 235-269.

4. Abbas, A K, Lichtman, A H, and Pober, J S. 2000d. Effector mechanisms of cell-mediated immunity. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 291-308.

5. Abbas, A K, Lichtman, A H, and Pober, J S. 2000e. Effector mechanisms of humoral immunity. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 309-334.

6. Abbas, A K, Lichtman, A H, and Pober, J S. 2000f. General properties of immune response. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 3-16.

7. Abbas, A K, Lichtman, A H, and Pober, J S. 2000g. Immunity to microbes. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 343-362.

8. Abbas, A K, Lichtman, A H, and Pober, J S. 2000h. Innate immunity. *In*: Abbas, A K, Lichtman, A H, and Pober, J S (eds.). Cellular and molecular immunology, 4th ed. W.B. Saunders Company: Philadelphia, pp. 270-290.

9. Abitorabi, M A, Mackay, C R, Jerome, E H, Osorio, O, Butcher, E C, and Erle, D J. 1996. Differential expression of homing molecules on recirculating lymphocytes from sheep gut, peripheral, and lung lymph. J Immunol 156: 3111-3117.

10. Ackerman, M R, Cheville, N F, and Gallagher, J E. 1991. Colonization of the pharyngeal tonsil and respiratory tract of the gnotobiotic pig by a toxigenic strain of *Pasteurella multocida* type D. Vet Pathol 28: 267-274.

11. Al Sultan, I I and Aitken, I D. 1985. The tonsillar carriage of *Pasteurella haemolytica* in lambs. J Comp Pathol 95: 193-201.

12. Allan, C H, Mendrick, D L, and Trier, J S. 1993. Rat intestinal M cells contain acidic endosomal-lysosomal compartments and express class II major histocompatibility complex determinants. Gastroenterology 104: 698-708.

13. Amass, S F. 1997. Demonstration of vertical transmission of *Streptococcus suis* in swine by genomic fingerprinting. J Clin Microbiol 35: 1595-1596.

14. Amass, S F, Clark, L K, and Wu, C C. 1995. Source and timing of *Streptococcus suis* infection in neonatal pigs: implications for early weaning procedures. Swine Health and Production 3: 189-193.

15. Amass, S F, Wu, C C, and Clark, L K. 1996. Evaluation of antibiotics for the elimination of tonsilar carrier state of *Streptococcus suis* in pigs. J Vet Diagn Invest 8: 64-67.

16. Amerongen, H M, Weltzin, R, Farnet, C M, Michetti, P, Haseltine, W A, and Neutra, M R. 1991. Transepithelial transport of HIV-1 by intestinal M cells: a mechanism for transmission of AIDS. J Acquir Immune Defic Syndr 4: 760-765.

17. Amerongen, H M, Weltzin, R, Mack, J A, Winner, L S 3, Michetti, P, Apter, F M, Kraehenbuhl, J P, and Neutra, M R. 1992. M cell-mediated antigen transport and monoclonal IgA antibodies for mucosal immune protection. Ann N Y Acad Sci 664: 18-26.

18. Appel, M J G. 1969. Pathogenesis of canine distemper. Am J Vet Res 30: 1167-1182.

19. Arends, J P, Hartwig, N, Rudolphy, M, and Zanen, H C. 1997. Carrier rate of *Streptococcus suis* capsular type 2 in palatine tonsils of slaughtered pigs. J Clin Microbiol 20: 945-947.

20. Beard, P M, Rhind, S M, Sinclair, M C, Wildblood, L A, Stevenson, K, McKendrick, I J, Sharp, J M, and Jones, D G. 2000. Modulation of γδ T cells and CD1 in *Mycobacterium avium* subsp. *paratuberculosis* infection. Vet Immunol Immunopathol 77: 311-319.

21. Belz, G T. 1998a. An unusual structure of venules in tonsils of the soft palate of young pigs. J Anat 192: 131-135.

22. Belz, G T. 1998b. Intercellular and lymphatic pathways associated with tonsils of the soft palate in young pigs. Anat Embriol 197: 331-340.

23. Belz, G T and Heath, T J. 1995. The epithelium of canine palatine tonsils. Anat Embryol 192: 189-194.

24. Belz, G T and Heath, T J. 1996. Tonsils of the soft palate of young pigs: crypt structure and lymphoepithelium. Anat Rec 245: 102-113.

25. Benkirane, R, Gottschalk, M G, Jacques, M, and Dubreuil, J D. 1998. Immunochemical characterization of an IgG-binding protein of *Streptococcus suis*. FEMS Immunol Med Microbiol 20: 121-127.

26. Bentley, R W, Leigh, J A, and Collins, M D. 1991. Intrageneric structure of *Streptococcus* based on comparative analysis of small-subunit rRNA sequences. Int J Syst Bacteriol 41: 487-494.

27. Bernstein, J M, Gorfien, J, and Brandtzaeg, P. 1999. The immunobiology of the tonsils and adenoids. *In*: Ogra, P L, Mestecky, J, Lamm, M E, Strober, W, Bienenstock, J, and McGhee, J R (eds.). Mucosal immunology, 2nd ed. Academic Press: San Diego, pp. 1339-1362.

28. Berthelot-Hérault, F, Morvan, H, Kéribin, A-M, Gottschalk, M, and Kobisch, M. 2000. Production of muramidase-released protein (MRP), extracellular factor (EF) and suilysin by field isolates of *Streptococcus suis* capsular types 2, 1/2, 9, 7 and 3 isolated from swine in France. Vet Res 31: 473-479.

29. Bevanger, L, Bergh, K, Caugant, D A, and Froholm, L O. 1998. Identification of nasopharyngeal carriage of an outbreak strain of *Neisseria meningitidis* by pulsed-field gel electrophoresis versus phenotypic methods. J Med Microbiol 47: 993-998.

30. Bianchi, A T, Zwart, R J, Jeurissen, S H, and Moonen Leusen, H W. 1992. Development of the B- and T-cell compartments in porcine lymphoid organs from birth to adult life: an immunohistological approach. Vet Immunol Immunopathol 33: 201-221.

31. Bienenstock, J, McDermott, M R, and Clancy, R L. 1999. Respiratory tract defenses: role of mucosal lymphoid tissues. *In*: Ogra, P L, Mestecky, J, Lamm, M E, Strober, W, Bienenstock, J, and McGhee, J R (eds.). Mucosal immunology, 2nd ed. Academic Press: San Diego, pp. 283-292.

32. Binns, R M and Licence, S T. 1985. Patterns of migration of labelled blood lymphocyte subpopulations: evidence for two types of Peyer's patches in the young pig. *In*: Klaus, G G B (ed.). Microenvironments in the lymphoid system. Plenum Press: New York, pp. 661-667.

33. Blouin, C, Higgins, R, Gottschalk, M, and Simard, J. 1994. Evaluation of the antibody response in pigs vaccinated against *Streptococcus suis* type 2 using a double-antibody sandwich enzyme-linked immunosorbent assay. Can J Vet Res 58: 49-54.

34. Blue, M L, Daley, J F, Levine, H, and Schlossman, S F. 1985. Coexpression of T4 and T8 on peripheral blood T cells demonstrated by two-color fluorescence flow cytometry. J Immunol 134: 2281-2286.

35. Boismenu, R and Havran, W L. 1997. An innate view of gamma-delta T cells. Curr Opin Immunol 9: 57-63.

36. Borghesi, C, Regoli, M, Bertelli, E, and Nicoletti, C. 1996. Modifications of the follicle-associated epithelium by short-term exposure to a non-intestinal bacterium. J Pathol 180: 326-332.

37. Born, W, Cady, C, Jones-Carson, J, Mukasa, A, Lahn, M, and O'Brien, R. 1999. Immunoregulatory functions of γδ T cells. Adv Immunol 71: 77-144.

38. Boulnois, G J, Paton, J C, Mitchel, T J, and Andrew, P W. 1991. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. Mol Microbiol 5: 2611-2616.

39. Bouma, A, Zwart, R J, De Bruin, M G M, De Jong, M C M, Kimman, T G, and Bianchi, A T. 1997. Immunohistological characterization of the local cellular response directed against pseudorabies virus in pigs. Vet Microbiol 58: 145-154.

40. Brandeis, J M, Sayegh, M H, Gallon, L, Blumberg, R S, and Carpenter, C B. 1994. Rat intestinal epithelial cells present major histocompatibility complex allopeptides to primed T cells. Gastroenterology 107: 1537-1542.

41. Brandtzaeg, P. 1984. Immune functions of human nasal mucosa and tonsils in health and disease. *In*: Bienenstock, J (ed.). Immunology of the lung and upper respiratory tract. McGraw-Hill: New York, pp. 28-95.

42. Brandtzaeg, P. 1997. Mucosal immunity in the female genital tract. J Reprod Immunol 36: 23-50.

43. Brandtzaeg, P, Baekkevold, E S, Farstad, I N, Jahnsen, F L, Johansen, F, Nilsen, E M, and Yamanaka, T. 1999a. Regional specialization in the mucosal immune system: what happens in the microcompartments? Immunol Today 20: 141-151.

44. Brandtzaeg, P, Dale, I, and Fagerhol, M K. 1987. Distribution of a formalin-resistant myelomonocytic antigen (L1) in human tissues. II. Normal and aberrant occurrence in various epithelia. Am J Clin Pathol 87: 700-707.

45. Brandtzaeg, P, Dale, I, and Gabrielsen, T O. 1992. The leucocyte protein L1 (calprotectin): usefulness as an immunohistochemical marker antigen and putative biological function. Histopathology 21: 191-196.

46. Brandtzaeg, P, Baekkevold, E S, Farstad, I N, Jahnsen, F L, Johansen, F-E, Nilsen, E M, Yamanaka, T. 1999a. Regional specialization in the mucosal immune system: what happens in the microcompartments? Immunol Today 20: 141-151.

47. Brandtzaeg, P, Farstad, I N, and Haraldsen, G. 1999b. Regional specialization in the mucosal immune system: primed cells do not always home along the same tract. Immunol Today 20: 267-277.

48. Brandtzaeg, P, Gabrielsen, T, Dale, I, Muller, F, Steinbakk, M, and Fagerhol, M K. 1995. The leucocyte protein L1 (calprotectin): a putative nonspecific defence factor at epithelial surfaces. Adv Exp Med Biol 371A: 201-206.

49. Brandtzaeg, P and Halstensen, T S. 1992. Immunology and immunopathology of tonsils. Adv Otorhinolaryngol 47: 64-75.

50. Brandtzaeg, P, Jones, D B, Flavell, D J, and Fagerhol, M K. 1988. Mac 387 antibody and detection of formalin resistant myelomonocytic L1 antigen. J Clin Pathol 41: 963-970.

51. Brandtzaeg, P, Surjan, L, Jr., and Berdal, P. 1978. Immunoglobulin systems of human tonsils I. control subjects of various ages: quantification of Ig-producing cells, tonsillar morphometry and serum Ig concentrations. Clin Exp Immunol 31: 367-381.

52. Brassard, J, Gottschalk, M, and Quessy, S. 2001. Decrease of the adhesion of *Streptococcus suis* serotype 2 mutants to embryonic bovine tracheal cells and porcine tracheal rings. Can J Vet Res 65: 156-160.

53. Brazeau, C, Gottschalk, M, Vincelette, S, and Martineau-Doize, B. 1996. *In vitro* phagocytosis and survival of *Streptococcus suis* capsular type 2 inside murine macrophages. Microbiology 142: 1231-1237.

54. Briskin, M, Winsor-Hines, D, Shyjan, A, Cochran, N B S W J, McEvoy, L M, Butcher, E C, Kassam, N, Mackay, C R, Newman, W, and Ringler, D J. 1997. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. Am J Pathol 151: 97-110.

55. Brown, H R, Goller, N L, Rudelli, R D, Dymecki, J, and Wisniewski, H M. 1989. Postmortem detection of measles virus in non-neural tissues in subacute sclerosing panencephalitis. Ann Neurol 26: 263-268.

56. Bruschke, C J M, Weerdmeester, K, Van Oirschot, J T, and Van Rijn, P A. 1998. Distribution of bovine virus diarrhoea virus in tissues and white blood cells of cattle during acute infection. Vet Microbiol 64: 23-32.

57. Bucy, R P, Chen, C H, Cihak, J, Lösch, U, and Cooper, M D. 1988. Avian T cells expressing γδ receptors localize in the splenic sinusoids and the intestinal epithelium. J Immunol 141: 2200-2205.

58. Bukowski, J F, Morita, C T, and Brenner, M B. 1999. Human gamma-delta T lymphocytes recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. Immunity 11: 57-65.

59. Charland, N, Harel, J, Kobisch, M, Lacasse, S, and Gottschalk, M. 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. Microbiology 144 Part 2: 325-332.

60. Charland, N, Kobisch, M, Martineau-Doize, B, Jacques, M, and Gottschalk, M. 1996. Role of capsular sialic acid in virulence and resistance to phagocytosis of *Streptococcus suis* capsular type 2. FEMS Immunol Med Microbiol 14: 195-203.

61. Charland, N, Nizet, V, Rubens, C E, Kim, K S, Lacouture, S, and Gottschalk, M. 2000. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect Immun 68: 637-643.

62. Chen, W, Alley, M R, Manktelow, B W, Hopcroft, D, and Bennett, R. 1991. The potential role of the ovine pharyngeal tonsil in the respiratory tract immunity: a scanning and transmission electron microscopy study of its epithelium. J Comp Pathol 104: 47-56.

63. Cheng, A F, Khin-Thi-Oo, Li, E K, and French, G L. 1987. Septic arthritis caused by *Streptococcus suis* serotype 2. J Infect 14: 237-241.

64. Cheung, A K. 1995. Investigation of pseudorabies virus DNA and RNA in trigeminal ganglia and tonsil tissues of latently infected swine. Am J Vet Res 56: 45-50.

65. Chiers, K, Haesebrouck, F, van Overbeke, I, Charlier, G, and Ducatelle, R. 1999. Early *in vivo* interactions of *Actinobacillus pleuropneumoniae* with tonsils of pigs. Vet Microbiol 68: 301-306.

66. Childers, N K, Denys, F R, McGee, N F, and Michalek, S M. 1990. Ultrastructural study of liposome uptake by M cells of rat Peyer's patch: an oral vaccine system for delivered of purified antigen. Reg Immunol 3: 8-16.

67. Chu, R M, Glock, R D, and Ross, R F. 1979. Gut-associated lymphoid tissues of young swine with emphasis on dome epithelium of aggregated lymph nodules (Peyer's patches) of the small intestine. Am J Vet Res 40: 1720-1728.

68. Claeys, S, Cuvelier, C, Quatacker, J, and Van Cauwenberge, P. 1996. Ultrastructural investigation of M-cells and lymphoepithelial contacts in naso-pharyngeal associated lymphoid tissue (NALT). Acta Otolaryngol Suppl (Stockh) 523: 40-42.

69. Clark, B R, Kelly, S E, and Fleming, S. 1990. Calgranulin expression and association with keratinocyte cytoskeleton. J Pathol 160: 25-30.

70. Clark, M A, Jepson, M A, Simmons, N L, and Hirst, B H. 1994. Differential surface characteristics of M cells from mouse intestinal Peyer's and caecal patches. Histochem J 26: 271-280.

71. Clarke, D, Almeyda, J, Ramsay, I, and Drabu, Y J. 1991. Primary prevention of *Streptococcus suis* meningitis. Lancet 338: 1147-1148.

72. Clifton-Hadley, F A. 1984. Studies of *Streptococcus suis* type 2 infection in pigs. Vet Res Comm 8: 217-227.

73. Clifton-Hadley, F A and Alexander, T J L. 1980. The carrier site and carrier rate of *Streptococcus suis* type II in pigs. Vet Rec 107: 40-41.

74. Clifton-Hadley, F A, Alexander, T J L, Enright, M R, and Guise, J. 1984. Monitoring herds for *Streptococcus suis* type 2 by sampling tonsils of slaughter pigs. Vet Rec 115: 562-564.

75. Clohessy, P A and Golden, B E. 1995. Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence. Scand J Immunol 42: 551-556.

76. Croitoru, K and Bienenstock, J. 1994. Characteristics and functions of mucosa-associated lymphoid tissue. *In*: Ogra, P L, Lamm, M E, McGhee, J R, Mestecky, J, Strober, W, and Bienenstock, J (eds.). Handbook of mucosal immunology. Academic Press, Inc.: San Diego, pp. 141-149.

77. Davies, P R and Ossowicz, C J. 1991. Evaluation of methods used for detecting *Streptococcus suis* type 2 in tonsils, and investigation of the carrier state in pigs. Res Vet Sci 50: 190-194.

78. Davis, W C, Marusic, S, Lewin, H A, Splitter, G A, Perryman, L E, McGuire, T C, and Gorham, J R. 1987. The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiaton antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. Vet Immunol Immunopathol 15: 337-376.

79. Davis, W C, Zuckermann, F A, Hamilton, M J, Barbosa, J I R, Saalmuller, A, Binns, R M, and Licence, S T. 1998. Analysis of monoclonal antibodies that recognize γδ T / null cells. Vet Immunol Immunopathol 60: 305-316.

80. de Jong, M F. 1999. Progressive and nonprogressive atrophic rhinitis. *In*: Straw, B E, D'Allaire, S, Mengeling, W L, and Taylor, D J (eds.). Diseases of swine, 8th ed. Iowa State University Press: Ames, pp. 355-384.

81. del Campo Sepúlveda, E M, Altman, E, Kobisch, M, D'Allaire, S, and Gottschalk, M. 1996. Detection of antibodies against *Streptococcus suis* capsular type 2 using a purified capsular polysaccharide antigen-based indirect ELISA. Vet Microbiol 52: 113-125.

82. Delves, P J and Roitt, I M. 2000a. The immune system - first of two parts. N Engl J Med 343: 37-49.

83. Delves, P J and Roitt, I M. 2000b. The immune system - second of two parts. N Engl J Med 343: 108-117.

84. Denham, S, Zwart, R J, Whittall, J T D, Pampusch, M, Corteyn, A H, Bianchi, A T, Murtaugh, M P, Parkhouse, M E, Tlaskalova, H, Sinkora, J, Sinkora, M, and Rehakova, Z. 1998. Monoclonal antibodies putatively identifying porcine B cells. Vet Immunol Immunopathol 60: 317-328.

85. Deusch, K, Lüling, F, Reich, K, Classen, M, Wagner, H, and Pfeffer, K. 1991. A major fraction of human intraepithelial lymphocytes simultaneously expresses the γδ T cell receptor, the CD8 acessory molecule and preferentially uses the $V_{\delta}1$ gene segment. Eur J Immunol 21: 1053-1059.

86. Devriese, L A, Hommez, J, Pot, B, and Haesebrouck, F. 1994. Identification and composition of the streptococcal and enterococcal flora of tonsils, intestines and faeces of pigs. J Appl Bacteriol 77: 31-36.

87. Dick, C P, Johnson, R P, and Yamashiro, S. 1989. Sites of persistence of feline calicivirus. Res Vet Sci 47: 367-373.

88. Dillender, M J and Lunney, J K. 1993. Characteristics of T lymphocyte cell lines established from NIH minipigs challenge inoculated with *Trichinella spiralis*. Vet Immunol Immunopathol 35: 301-319.

89. Dungworth, D L. 1993. The respiratory system. *In*: Jubb, K V F, Kennedy, P C, and Palmer, N (eds.). Pathology of domestic animals, 4th ed. Academic Press: San Diego, pp. 539-699.

90. Elliott, S D. 1966. Streptococcal infection in young pigs. I- An immunochemical study of the causative agent (PM streptococcus). J Hyg Camb 64: 205-212.

91. Elliott, S D, McCarty, M, and Lancefield, R C. 1977. Teichoic acids from group D streptococci with special reference to strains from pig meningitis (*Streptococcus suis*). J Exp Med 145: 490-499.

92. Ermak, T H, Dougherty, E P, Bhagat, H R, Kabok, Z, and Pappo, J. 1995. Uptake and transport of copolymer biodegradable microspheres by rabbit Peyer's patch M cells. Cell Tissue Res 279: 433-436.

93. Eversole, L R, Miyasaki, K T, and Christensen, R E. 1992. The distribution of normal antimicrobial protein, calprotectin, in normal oral keratinocytes. Archs Oral Biol 37: 963-968.

94. Eversole, L R, Miyasaki, K T, and Christensen, R E. 1993. Keratinocyte expression of calprotectin in oral inflammatory mucosal diseases. J Oral Pathol Med 22: 303-307.

95. Farbman, A I. 1988. The oral cavity. *In*: Weiss, L (ed.). Cell and tissue biology, 6th ed. Urban & Schwarzenberg: Baltimore, pp. 573-593.

96. Fedorka-Cray, P J, Kelley, L C, Stabel, T J, Gray, J T, and Laufer, J A. 1995. Alternate routes of invasion may affect pathogenesis of *Salmonella typhimurium* in swine. Infect Immun 63: 2658-2664.

97. Fekadu, M, Shaddock, J H, Chandler, F W, and Baer, G M. 1983. Rabies virus in the tonsils of a carrier dog. Arch Virol 78: 37-47.

98. Ferrante, A, Rowan-Kelly, B, and Paton, J C. 1984. Inhibition of *in vitro* human lymphocyte response by the pneumococcal toxin pneumolysin. Infec Immun 46: 585-589.

99. Field, H I, Buntain, D, and Done, J T. 1954. Studies on piglet mortality. 1. Streptococcal meningitis and artritis. Vet Rec 66: 453-455.

100. Finlay, B B and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev 61: 136-169.

101. Finzi, G, Cornaggia, M, Capella, C, Fiocca, R, Bosi, F, Solcia, E, and Samloff, L M. 1993. Cathepsin E in follicle associated epithelium of intestine and tonsils: localization to M cells and possible role in antigen processing. Histochemistry 99: 201-211.

102. Francois, B, Gissot, V, Ploy, M C, and Vignon, P. 1998. Recurrent septic shock due to *Streptococcus suis*. J Clin Microbiol 36: 2395.

103. Frankel, S S, TennerRacz, K, Racz, P, Wenig, B M, Hansen, C H, Heffner, D, Nelson, A M, Pope, M, and Steinman, R M. 1997. Active replication of HIV-1 at the lymphoepithelial surface of the tonsil. Am J Pathol 151: 89-96.

104. Galina, L, Pijoan, C, Sitjar, M, Christianson, W T, Rossow, K, and Collins, J E. 1994. Interaction between *Streptococcus suis* serotype 2 and porcine reproductive

and respiratory syndrome virus in specific pathogen-free piglets. Vet Rec 134: 60-64.

105. Gaskell, R M and Povey, R C. 1979. Feline viral rhinotracheitis: sites of virus replication and persistence in acutely and persistently infected cats. Res Vet Sci 27: 167-174.

106. Gebert, A. 1995. Identification of M-cells in the rabbit tonsil by vimentin immunohistochemistry and in vivo protein transport. Histochem Cell Biol 104: 211-220.

107. Gebert, A. 1996. M-cells in the rabbit tonsil exhibit distinctive glycoconjugates in their apical membranes. J Histochem Cytochem 44: 1033-1042.

108. Gebert, A. 1997a. M cells in the rabbit palatine tonsil: the distribution, spatial arrangement and membrane subdomains as defined by confocal lectin histochemistry. Anat Embryol 195: 353-358.

109. Gebert, A. 1997b. The role of M cells in the protection of mucosal membranes. Histochem Cell Biol 108: 455-470.

110. Gebert, A and Hach, G. 1993. Differential binding of lectins to M cells and enterocytes in the rabbit cecum. Gastroenterology 105: 1350-1361.

111. Gebert, A and Pabst, R. 1999. M cells at locations outside the gut. Semin Immunol 11: 165-170.

112. Gebert, A and Posselt, W. 1997. Glycoconjugate expression defines the origin and differentiation pathway of intestinal M cells. J Histochem Cytochem 45: 1341-1350.

113. Gebert, A, Rothkotter, H J, and Pabst, R. 1994. Cytokeratin 18 is an M-cell marker in porcine Peyer's patches. Cell Tissue Res 276: 213-221.

114. Gebert, A, Rothkotter, H J, and Pabst, R. 1996. M cells in Peyer's patches of the intestine. Int Rev Cytol 167: 91-159.

115. Gebert, A, Willfuhr, B, and Pabst, R. 1995. The rabbit M-cell marker vimentin is present in epithelial cells of the tonsil crypts. Acta Otolaryngol Stockh 115: 697-700.

116. Giannasca, P J, Giannasca, K T, Falk, P, Gordon, J I, and Neutra, M R. 1994. Regional differences in glycoconjugates of intestinal M cells in mice: potential targets for mucosal vaccines. Am J Physiol 267: G1108-G1121.

117. Gottschalk, M, Higgins, R, and Quessy, S. 1999a. Dilemma of the virulence of *Streptococcus suis* strains. J Clin Microbiol 37: 4202-4203.

118. Gottschalk, M, Lacouture, S, and Odierno, L. 1999b. Immunomagnetic isolation of *Streptococcus suis* serotypes 2 and 1/2 from swine tonsils. J Clin Microbiol 37: 2877-2881.

119. Gottschalk, M, Lebrun, A, Wisselink, H, Dubreuil, J D, Smith, H, and Vecht, U. 1998. Production of virulence-related proteins by Canadian strains of *Streptococcus suis* capsular type 2. Can J Vet Res 62: 75-79.

120. Gottschalk, M, Petitbois, S, Higgins, R, and Jacques, M. 1991. Adherence of *Streptococcus suis* capsular type 2 to porcine lung sections. Can J Vet Res 55: 302-304.

121. Gottschalk, M and Segura, M. 2000. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet Microbiol 76: 259-272.

122. Graeme-Cook, F, Bhan, A K, and Harris, N L. 1993. Immunohistochemical characterization of intraepithelial and subepithelial mononuclear cells of the upper airways. Am J Pathol 143: 1416-1422.

123. Griebel, P J, Kennedy, L, Graham, T, Davis, W C, and Reynolds, J D. 1992. Characterization of B-cell phenotypic changes during ileal and jejunal Peyer's patch development in sheep. Immunology 77: 564-570.

124. Griepp, E B and Robbins, E S. 1988. Epithelium. *In*: Weiss, L (ed.). Cell and tissue biology, 6th ed. Urban & Schwarzenberg: Baltimore, pp. 113-153.

125. Grutzkau, A, Hanski, C, Hahn, H, and Riecken, E O. 1990. Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterocolitica* and other enteroinvasive bacteria. Gut 31: 1011-1015.

126. Haataja, S, Tikkanen, K, Liukkonen, J, Francois-Gerard, C, and Finne, J. 1993. Characterization of a novel bacterial adhesion specificity of *Streptococcus suis* recognizing blood group P receptor oligosaccharides. J Biol Chem 268: 4311-4317.

127. Haataja, S, Tikkanen, K, Nilsson, U, Magnusson, G, Karlsson, K A, and Finne, J. 1994. Oligosaccharide-receptor interaction of the Galα1-4Gal binding adhesins of *Streptococcus suis*. J Biol Chem 269: 27466-27472.

128. Hagedorn-Olsen, T, Nielsen, N C, Friis, N F, and Nielsen, J. 1999. Progression of *Mycoplasma hyosynoviae* infection in three pig herds. Development of tonsillar carrier state, arthritis and antibodies in serum and synovial fluid in pigs from birth to slaughter. J Vet Med A 46: 555-564.

129. Ham, A W and Cormarck, D H. 1979a. Epithelial tissue. *In*: Histology, 8th ed. J. B. Lippincott Company: Philadelphia, pp. 187-209.

130. Ham, A W and Cormarck, D H. 1979b. The digestive system. *In*: Ham, A W and Cormarck, D H (eds.). Histology, 8th ed. J. B. Lippincott Company: Philadelphia, pp. 645-693.

131. Hathaway, L J and Kraehenbuhl, J P. 2000. The role of M cells in mucosal immunology. Cell Mol Life Sci 57: 323-332.

132. Haverson, K, Saalmuller, A, Alvarez, B, Alonso, F, Bailey, M, Bianchi, A T J, Boersma, W J A, Chen, Z, Davis, W C, Dominguez, J, Engelhardt, H, Ezquerra, A, Grosmaire, L S, Hamilton, M J, Hollemweguer, E, Huang, C A, Khanna, K V, Kuebart, G, Lackovic, G, Ledbetter, J A, Lee, R, Llanes, D, Lunney, J K, McCullough, K C, Molitor, T, Nielsen, J, Niewold, T A, Pescovitz, M D, Perez de la Lastra, J, Rehakova, Z, Salmon, H, Schnitzlein, W M, Seebach, J, Simon, A, Sinkora, J, Sinkora, M, Stokes, C R, Summerfield, A, Sver, L, Thacker, E, Valpotic, I, Yang, H, Zuckermann, F A, and Zwart, R. 2001. Overview of the third international workshop on swine leukocyte differentiation antigens. Vet Immunol Immunopathol 80: 5-23.

133. Hein, W R and Mackay, C R. 1991. Prominence of γδ T cells in the ruminant immune system. Immunol Today 12: 30-34.

134. Higgins, R and Gottschalk, M. 1999a. Distribution of *Streptococcus suis* capsular types in 1998. Can Vet J 40: 277.

135. Higgins, R and Gottschalk, M. 1999b. Streptococcal diseases. *In*: Straw, B E, D'Allaire, S, Mengeling, W L, and Taylor, D J (eds.). Diseases of swine, 8th ed. Iowa State University Press: Ames, pp. 563-578.

136. Hill, A F, Zeidler, M, Ironside, J, and Collinge, J. 1997. Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. Lancet 349: 99-100.

137. Hirao, M, Harabuchi, Y, Kataura, A, and Imai, S. 1996. Immunological role of the human palatine tonsil in Epstein-barr virus persistence. Acta Otolaryngol (Stockh) 523: 158-160.

138. HogenEsch, H and Felsburg, P J. 1990. Ultrastructure and alkaline phosphatase activity of the dome epithelium of canine Peyer's patches. Vet Immunol Immunopathol 24: 177-186.

139. Holt, M E, Enright, M R, and Alexander, T J L. 1988. Immunisation of pigs with live cultures of *Streptococcus suis* type 2. Res Vet Sci 45: 349-352.

140. Howie, A J. 1980. Scanning and transmission electron microscopy on epithelium of human palatine tonsils. J Pathol 130: 91-98.

141. Iglesias, J G, Trujano, M, and Xu, J. 1992. Inoculation of *Streptococcus suis* type 2 alone or in combination with pseudorabies virus. Am J Vet Res 53: 364-367.

142. Inman, L R and Cantey, J R. 1983. Specific adherence of *Escherichia coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patch in *Escherichia coli* diarrhea in the rabbit. J Clin Invest 71: 1-8.

143. Jacobs, A A C, Loeffen, P L W, van den Berg, A J G, and Storm, P K. 1994. Identification, purification, and caracterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect Immun 62: 1742-1748.

144. Jacobs, A A C, van den Berg, A J G, and Loeffen, P L W. 1996. Protection of experimentally infected pigs by suilysin, the thiol-activated haemolysin of *Streptococcus suis*. Vet Rec 139: 225-228.

145. Jacques, M, Gottschalk, M, Foiry, B, and Higgins, R. 1990. Ultrastructural study of surface components of *Streptococcus suis*. J Bacteriol 172: 2833-2838.

146. Jepson, M A, Mason, C M, Bennett, M K, Simmons, N L, and Hirst, B H. 1992. Co-expression of vimentin and cytokeratins in M cells of rabbit intestinal lymphoid follicle-associated epithelium. Histochem J 24: 33-39.

147. Jepson, M A, Simmons, N L, Hirst, G L, and Hirst, B H. 1993a. Identification of M cells and their distribution in rabbit intestinal Peyer's patches and appendix. Cell Tissue Res 273: 127-136.

148. Jepson, M A, Simmons, N L, O'Hagan, D T, and Hirst, B H. 1993b. Comparison of poly (DL-lactide-co-glycolide) and polystyrene microsphere targeting to intestinal M cells. J Drug Target 1: 245-249.

149. Jepson, M A, Simmons, N L, Savidge, T C, James, P S, and Hirst, B H. 1993c. Selective binding and transcytosis of latex microspheres by rabbit intestinal M cells. Cell Tissue Res 271: 399-405.

150. Jonjic, N, Jonjic, S, Rukavina, D, and Koszinowski, U H. 1987. Distribution of T-lymphocyte subsets in porcine lymphoid tissues. Immunology 60: 395-401.

151. Josefsen, T D and Landsverk, T. 1997. Increase in gamma-delta cells in the ruminal mucosa of reindeer calves (*Rangifer tarantus tarantus* l.) induced by baled grass silage. Vet Immunol Immunopathol 60: 197-202.

152. Kaiser, J. 2002. Xenotransplantation. Cloned pigs may help overcome rejection. Science 295: 27-28.

153. Kato, T and Owen, R L. 1999. Structure and function of intestinal mucosal epithelium. *In*: Ogra, P L, Mestecky, J, Lamm, M E, Strober, W, Bienenstock, J, and McGhee, J R (eds.). Mucosal immunology, 2nd ed. Academic Press: San Diego, pp. 115-132.

154. Kaufmann, S H E. 1990. Heat shock proteins and the immune response. Immunol Today 11: 129-135.

155. Kawashima, K, Narita, M, and Yamada, S. 1999. Changes in macrophage and lymphocyte subpopulations of lymphoid tissues from pigs infected with the porcine reproductive and respiratory syndrome virus (PRRSV). Vet Immunol Immunopathol 71: 257-262.

156. Kelly, W R. 1993. The liver and biliary system. *In*: Jubb, K V F, Kennedy, P C, and Palmer, N (eds.). Pathology of domestic animals, 4th ed. Academic Press: San Diego, pp. 319-406.

157. Kelsall, B and Strober, W. 1999. Gut-associated lymphoid tissue - antigen handling and T-lymphocyte responses. *In*: Ogra, P L, Mestecky, J, Lamm, M E, Strober, W, Bienenstock, J, and McGhee, J R (eds.). Mucosal immunology, 2nd ed. Academic Press: San Diego, pp. 293-317.

158. Kerneis, S, Bogdanova, A, Kraehenbuhl, J P, and Pringault, E. 1997. Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. Science 277: 949-952.

159. Kilpper-Balz, R and Schleifer, K H. 1987. *Streptococcus suis* sp. nov., nom. rev. Int J Syst Bacteriol 37: 160-162.

160. King, D P, Hyde, D M, Jackson, K A, Novosad, D M, Ellis, T N, Putney, L, Stovall, M Y, Van Winkle, L S, Beaman, B L, and Ferrick, D A. 1999. Cutting edge: protective response to pulmonary injury requires γδ T lymphocytes. J Immunol 162: 5033-5036.

161. Kohbata, S, Yokoyama, H, and Yabuuchi, E. 1986. Cytopathogenic effect of *Salmonella typhi* GIFU 10007 on M cells of murine ileal Peyer's patches in ligated ileal loops: an ultrastructural study. Microbiol Immunol 30: 1225-1237.

162. Kraehenbuhl, J P and Neutra, M R. 1992. Molecular and cellular basis of immune protection of mucosal surfaces. Physiol Rev 72: 853-879.

163. Kumar, P, Timoney, J F, and Sheoran, A S. 2001. M cells and associated lymphoid tissue of the equine nasopharyngeal tonsil. Equine Vet J 33: 224-230.

164. Kuper, C F, Koornstra, P J, Hameleers, D M H, Biewenga, J, Spit, B J, Duijvestijn, A M, van Breda Vriesman, P J C, and Sminia, T. 1992. The role of nasopharyngeal lymphoid tissue. Immunol Today 13: 219-224.

165. Lackovic, G, Tomaskovic, M, Njari, B, Krsnik, B, Rode, B, and Valpotic, I. 1999. Distribution of immune cells expressing CD3a, CD21 and S-100 protein markers in the porcine gut-associated lymphoid tissues. Eur J Histochem 43: 39-46.

166. Lalonde, M S M, Lacouture, S, and Gottschalk, M. 2000. Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. Microbiology 146: 1913-1921.

167. Landsverk, T. 1988. Phagocytosis and transcytosis by follicle-associated epithelium of the ileal Peyer's patch in calves. Imunol Cell Biol 66: 261-268.

168. Langford, P, Williams, A E, Kroll, J S. 1991. Superoxide dismutases of pathogenic and non-pathogenic *Streptococcus suis* type 2 isolates. FEMS Microbiol Lett 77: 347-350.

169. Liebler, E M, Paar, M, and Pohlenz, J F. 1991. M cells in the rectum of calves. Res Vet Sci 51: 107-114.

170. Liebler, E M, Pohlenz, J F, and Cheville, N F. 1988. Gut-associated lymphoid tissue in the large intestine of calves. II. electron microscopy. Vet Pathol 25: 509-515.

171. Lun, S C, Perez-Casal, J, and Willson, P. 2001. Suilysin: a potential virulence factor of type II *Streptococcus suis*? Can Soc Microbiol (CSM proceedings) 90.

172. Lunney, J K and Butler, J E 1998. Immunogenetics. *In*: Rothschild, M F and Ruvinsky, A (eds). The genetics of the pig. CAB International: Oxon, pp. 163-197.

173. Lunney, J K and Pescovitz, M D. 1987. Phenotypic and functional characterization of pig lymphocyte populations. Vet Immunol Immunopathol 17: 135-144.

174. Lunney, J K, Walker, K, Goldman, T, Aasted, B, Bianchi, A, Binns, R, Licence, S, Bischof, R, Brandon, M, Blecha, F, Kielian, T L, McVey, D S, Chu, R M, Carr, M, Howard, C, Sopp, P, Davis, W, Dvorak, P, Dominguez, J, Canals, A, Sanchez Vizcaino, J M, Kim, Y B, Laude, H, Mackay, C R, Magnusson, U, McCullough, K, Misfeldt, M, Murtaugh, M, Molitor, T, Choi, C, Pabst, R, Parkhouse, R M, Denham, S, Yang, H, Pescovitz, M, Pospisil, R, Tlaskalova, H, Saalmuller, A, Weiland, E, Salmon, H, Sachs, D, Arn, S, Shimizu, M, Stokes, C, Stevens, K, Valpotic, I, Zuckermann, F, and Husman, R. 1994. Overview of the first international workshop to define swine leukocyte cluster of differentiation (CD) antigens. Vet Immunol Immunopathol 43: 193-206.

175. MacInnes, J I and Desrosiers, R. 1999. Agents of the "suis-ide diseases" of swine: *Actinobacillus suis*, *Haemophilus parasuis* and *Streptococcus suis*. Can J Vet Res 63: 83-89.

176. Mackay, C R. 1993. Immunological memory. Adv Immunol 53: 217-265.

177. Maher, D. 1991. *Streptococcus suis* septicaemia presenting as severe acute gastro-enteritis. J Infec 22: 303-304.

178. Makala, L H C, Haverson, K, Stokes, C R, Bailey, M, and Bland, P W. 1998. Isolation and characterization of pig Peyer's patch dendritic cells. Vet Immunol Immunopathol 61: 67-81.

179. Marcial, M A and Madara, J L. 1986. *Cryptosporidium*: cellular localization, structural analysis of absorptive cell-parasite membrane-membrane interactions in guinea pigs, and suggestion of protozoan transport by M cells. Gastroenterology 90: 583-594.

180. Marshall, D J, Moxley, R A, and Kelling, C L. 1996. Distribution of virus and viral antigen in specific pathogen-free calves following inoculation with noncytopathic bovine viral diarrhea virus. Vet Pathol 33: 311-318.

181. McGhee, J R, Lamm, M E, and Strober, W. 1999. Mucosal immune responses - an overview. *In*: Ogra, P L, Mestecky, J, Lamm, M E, Strober, W, Bienenstock, J, and McGhee, J R (eds.). Mucosal immunology, 2nd ed. Academic Press: San Diego, pp. 485-506.

182. McGhee, J R, Mestecky, J, Dertzbaugh, M T, Eldridge, J H, Hirasawa, M, and Kiyono, H. 1992. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 10: 75-88.

183. Medzhitov, R and Janeway Jr., C. 2000. Innate immunity. N Engl J Med 343: 338-344.

184. Mestecky, J. 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol 7: 265-276.

185. Mestecky, J, Moro, I, and Underdown, B J. 1999. Mucosal immunoglobulins. *In*: Ogra, P L, Mestecky, J, Lamm, M E, Strober, W, Bienenstock, J, and McGhee, J R (eds.). Mucosal immunology, 2nd ed. Academic Press: San Diego, pp. 133-152.

186. Meunier, P C, Cooper, B J, Appel, M J G, Lanieu, M E, and Slauson, D O. 1985. Pathogenesis of canine parvovirus enteritis: sequential virus distribution and passive immunization studies. Vet Pathol 22: 617-624.

187. Moll, R, Franke, W W, Schiller, D L, Geiger, B, and Krepler, R. 1982. The catalog of human cytokeratins: pattern of expression in normal epithelia, tumors and cultured cells. Cell 31: 11-24.

188. Morfitt, D C and Pohlenz, J F L. 1989. Porcine colonic lymphoglandular complex: distribution, structure, and epithelium. Am J Anat 184: 41-51.

189. Mosmann, T R and Sad, S. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 17: 138-146.

190. Nadal, D, Albini, B, Chen, C, Schläpfer, E, Bernstein, J M, and Ogra, P L. 1991. Distribution and engraftment patterns of human tonsillar mononuclear cells and immunoglobulin-secreting cells in mice with severe combined immunodeficiency: role of the epstein-barr virus. Int Arch Allergy Appl Immunol 95: 341-351.

191. Nandoskar, A, Ferrante, A, Bates, E J, Hurst, N, and Paton, J C. 1986. Inhibition of human monocyte respiratory burst, degranulation, phospholipid methylation and bactericidal activity by pneumolysin. Immunology 59: 515-520.

192. Narita, M, Inui, S, and Shimizu, Y. 1984. Tonsillar changes in pigs given psudorabies (Aujeszky's disease) virus. Am J Vet Res 45: 247-251.

193. Nassif, X, Pujol, C, Morand, P, and Eugene, E. 1999. Interactions of pathogenic *Neisseria* with host cells. Is it possible to assemble the puzzle? Mol Microbiol 32: 1124-1132.

194. Neutra, M R. 1998. Current concepts in mucosal immunity V. Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. Gastrointest Liver Physiol 37: G785-G791.

195. Neutra, M R, Frey, A, and Kraehenbuhl, J P. 1996a. Epithelial M cells: gateways for mucosal infection and immunization. Cell 86: 345-348.

196. Neutra, M R, Pringault, E, and Kraehenbuhl, J P. 1996b. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 14: 275-300.

197. Nickel, R, Schummer, A, and Seiferle, E. 1979. Digestive system. *In*: The viscera of domestic mammals, 2nd ed. Verlag Paul Parey: Berlin, pp. 21-203.

198. Norrby-Teglund, A, Newton, D, Kotb, M, Holm, S E, and Norgren, M. 1994. Superantigenic properties of group a streptococcal exotoxin SpeF (MF). Infect Immun 62: 5227-5233.

199. Norton, P M and Leigh, J A. 1997. Virulence mechanisms of *Streptococcus suis*. Adv Exp Med Biol 418: 823-825.

200. Norton, P M, Rolph, C, Ward, P N, Bentley, R W, and Leigh, J A. 1999. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunol Med Microbiol 26: 25-35.

201. Ogra, P L. 1971. Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to poliovirus. New Engl J Med 284: 59-64.

202. Ohmann, H B. 1983. Pathogenesis of bovine viral diarrhoea-mucosal disease: distribution and significance of BVDV antigen in diseased calves. Res Vet Sci 34: 5-10.

203. Oirschot, J T. 1999. Classical swine fever (hog cholera). *In*: Straw, B E, D'Allaire, S, Mengeling, W L, and Taylor, D J (eds.). Diseases of swine, 8th ed. Iowa State University Press: Ames, pp. 159-172.

204. Okwumabua, O, Staats, J, and Chengappa, M M. 1995. Detection of genomic heterogeneity in *Streptococcus suis* isolates by DNA restriction fragment lengh polymorphisms of rRNA genes (ribotyping). J Clin Microbiol 33: 968-972.

205. Olah, I and Everett, N B. 1975. Surface epithelium of the rabbit palatine tonsil: scanning and transmission electron microscopic study. J Reticuloendothelial Soc 18: 53-62.

206. Olah, I and Glick, B. 1992. Follicle-associated epithelium and medullary epithelial tissue of the bursa of Fabricius are two different compartments. Anat Rec 233: 577-587.

207. Olah, I, Surjan, L, Jr., and Toro, I. 1972. Electronmicroscopic observations on the antigen reception in the tonsillar tissue. Acta Biol Acad Scientiarum Hungaricae 23: 61-73.

208. Olofsson, K, Hellstrom, S, and Hammarstrom, M L. 1998. The surface epithelium of recurrent infected palatine tonsils is rich in γδ T cels. Clin Exp Immunol 111: 36-47.

209. Ortolani, C, Forti, E, Radin, E, Cibin, R, and Cossarizza, A. 1993. Cytofluorimetric identification of two populations of double positive (CD4+,CD8+) T lymphocytes in human peripheral blood. Biochem Biophys Res Commun 191: 601-609.

210. Owen, R L. 1977. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of the Peyer's patches in the normal unobstructed mouse intestine: an ultastructural study. Gastroenterology 72: 440-451.

211. Owen, R L, Apple, R T, and Bhalla, D K. 1986a. Morphometric and cytochemical analysis of lysosomes in rat Peyer's patch follicle epithelium: their reduction in volume fraction and acid phosphatase content in M cells compared to adjacent enterocytes. Anat Rec 216: 521-527.

212. Owen, R L and Jones, A L. 1974. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology 66: 189-203.

213. Owen, R L, Pierce, N F, Apple, R T, and Cray Jr, W C. 1986b. M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. J Infec Dis 153: 1108-1118.

214. Pabst, R. 1996. The respiratory immune system of pigs. Vet Immunol Immunopathol 54: 191-195.

215. Pappo, J. 1989. Generation and characterization of monoclonal antibodies recognizing follicle epithelial M cells in rabbit gut-associated lymphoid tissues. Cell Immunol 120: 31-41.

216. Pappo, J and Ermak, T H. 1989. Uptake and translocation of fluorescent latex particles by rabbit Peyer's patch follicle epithelium: a quantitative model for M cell uptake. Clin Exp Immunol 76: 144-148.

217. Pappo, J, Ermak, T H, and Steger, H J. 1991. Monoclonal antibody-directed targeting of fluorescent polystyrene microspheres to Peyer's patch M cells. Immunology 73: 277-280.

218. Pappo, J and Mahlman, R T. 1993. Follicle epithelial M cells are a source of interleukin-1 in Peyer's patches. Immunology 78: 505-507.

219. Paton, J C and Ferrante, A. 1983. Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. Infec Immun 41: 1212-1216.

220. Paton, J C, Rowan-Kelly, B, and Ferrante, A. 1984. Activation of human complement by the pneumococcal toxin pneumolysin. Infec Immun 43: 1085-1087.

221. Perez-Casal, J, Ayres-Price, J, Maguin, E, and Scott, J R. 1993. An M protein with a single C repeat prevents phagocytosis of *Streptococcus pyogenes*: use of temperature-sensitive shuttle vector to deliever homologus sequences to the chromosome of *S. pyogenes*. Mol Microbiol 8: 809-819.

222. Perry, L L, Feilzer, K, Portis, J L, and Caldwell, H D. 1998. Distinct homing pathways direct T lymphocytes to the genital and intestinal mucosae in *Chlamydia* -infected mice. J Immunol 160: 2905-2914.

223. Perry, M E. 1994. The specialised structure of crypt epithelium in the human palatine tonsil and its functional significance. J Anat 185: 111-127.

224. Perry, M E, Jones, M M, and Mustafa, Y. 1988. Structure of the crypt epithelium in human palatine tonsils. Acta Otolaryngol Stockh suppl. 454: 53-59.

225. Perry, M E and Slipka, J. 1993. Formation of the tonsillar corpuscle. Func Dev Morphol 3: 165-168.

226. Perry, M E and Whyte, A. 1998. Immunology of the tonsils. Immunol Today 19: 414-421.

227. Perseghin, P, Bezzi, G, Troupioti, P, and Gallina, M. 1995. *Streptococcus suis* meningitis in an italian blood donor. Lancet 346: 1305-1306.

228. Pescovitz, M D, Hsu, S M, Katz, S I, Lunney, J K, Shimada, S, and Sachs, D H. 1990. Characterization of porcine CD1-specific mab that distinguishes CD4/CD8 double-positive thymic from T lymphocytes. Tissue Antigens 35: 151-156.

229. Pescovitz, M D, Sakopoulos, A G, Gaddy, J A, Husmann, R J, and Zuckermann, F A. 1994. Porcine peripheral blood CD4+/ CD8+ dual expressing T-cells. Vet Immunol Immunopathol 43: 53-62.

230. Polak, J M and Van Noorden, S. 1997. Post-embedding immunocytochemistry for the transmission electron microscope. *In*: Polak, J M and Van Noorden, S (eds.). Introduction to immunocytochemistry, 2nd ed. New York, pp. 81-89.

231. Quessy, S, Busque, P, Higgins, R, Jacques, M, and Dubreuil, J D. 1997. Description of an albumin binding activity for *Streptococcus suis* serotype 2. FEMS Microbiol Lett 147: 245-250.

232. Quessy, S, Dubreuil, J D, Jacques, M, Malouin, F, and Higgins, R. 1994. Increase of capsular material thickness following *in vivo* growth of virulent *Streptococcus suis* serotype 2 strains. FEMS Microbiol Lett 115: 19-26.

233. Quiding-Jarbrink, M, Granstrom, G, Nordstrom, I, Holmgren, J, and Czerkinsky, C. 1995. Induction of compartmentalized B-cell responses in human tonsils. Infect Immun 63: 853-857.

234. Ramos, J A, Ramis, A J, Marco, A, Domingo, M, Rabanal, R, and Ferrer, L. 1992. Histochemical and immunohistochemical study of the mucosal lymphoid system in swine. Am J Vet Res 53: 1418-1426.

235. Rasmussen, S R, Aarestrup, F M, Jensen, N E, and Jorsal, S E. 1999. Associations of *Streptococcus suis* serotype 2 ribotype profiles with clinical disease and antimicrobial resistance. J Clin Microbiol 37: 404-408.

236. Raulet, D H. 1989. The structure, function, and molecular genetics of the γδ T cell receptor. Annu Rev Immunol 7: 175-207.

237. Reams, R Y, Glickman, L T, Harrington, D D, Bowersock, T L, and Thacker, H L. 1993. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part I. Epidemiologic factors and antibiotic susceptibility patterns. J Vet Diagn Invest 5: 363-367.

238. Reams, R Y, Glickman, L T, Harrington, D D, Thacker, H L, and Bowersock, T L. 1994. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part II: clinical signs, gross and microscopic lesions, and coexisting microorganisms. J Vet Diagn Invest 6: 326-334.

239. Richmond, I, Pritchard, G E, Ashcroft, T, Avery, A, Corris, P A, and Walters, E H. 1993. Bronchus associated lymphoid tissue (BALT) in human lung: its distribution in smokers and non-smokers. Thorax 48: 1130-1134.

240. Robertson, I D and Blackmore, D K. 1989. Prevalence of *Streptococcus suis* types 1 and 2 in domestic pigs in Australia and New Zealand. Vet Rec 124: 391-394.

241. Rojko, J L and Kociba, G J. 1991. Pathogenesis of infection by the feline leukemia virus. J Am Vet Med Assoc 199: 1305-1310.

242. Romagnani, S. 1997. The Th1/Th2 paradigm. Immunol Today 18: 263-266.

243. Rothkötter, H J, Möllhoff, S, and Pabst, R. 1999. The influence of age and breeding conditions on the number and proliferation of intraepithelial lymphocytes in pigs. Scand J Immunol 50: 31-38.

244. Ruco, L P, Uccini, S, Stoppacciaro, A, Pilozzi, E, Morrone, S, Gallo, A, De Vincentiis, M, Santoni, A, and Baroni, C D. 1995. The lymphoepithelial organization of the tonsil: an immunohistochemical study in chronic recurrent tonsillitis. J Pathol 176: 391-398.

245. Russel, M W, Moldoveanu, Z, White, P L, Sibert, G J, Mestecky, J, and Michalek, S M. 1996. Salivary, nasal, genital, and systemic antibody responses in monkeys immunized intranasally with bacterial protein antigen and the cholera toxin B subunit. Infect Immun 64: 1272-1283.

246. Saalmuller, A. 1998. Overview of the second international workshop to define swine cluster of differentiation (CD) antigens. Vet Immunol Immunopathol 60: 207-228.

247. Saalmuller, A, Hirt, W, and Reddehase, M J. 1989. Phenotypic discrimination between thymic and extrathymic CD4- CD8- and CD4+ CD8+ porcine T lymphocytes. Eur J Immunol 19: 2011-2016.

248. Saalmuller, A, Reddehase, M, Buhring, H J, Jonjic, S, and Koszinowski, U H. 1987. Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur J Immunol 17: 1297-1301.

249. Sanford, S E and Tilker, A M E. 1982. *Streptococcus suis* type II-associated diseases in swine: observations of a one-year study. J Am Vet Med Assoc 181: 673-676.

250. Sato, Y, Wake, K, and Watanabe, I. 1988. Changes in cells shapes and cytokeratins of epithelial cells during the infiltration of lymphocytes in the human palatine tonsils. Acta Otolaryngol Stockh suppl: 454: 48-52.

251. Sato, Y, Wake, K, and Watanabe, I. 1990. Differentiation of the crypt epithelium in human palatine tonsils: the microenvironment of the crypt epithelium as a lymphoepithelial organ. Arch Histol Cytol 53: 41-54.

252. Savidge, T C and Smith, M W. 1995. Evidence that membranous (M) cell genesis is immunoregulated. Adv Mucosal Immunol 239-241.

253. Schuh, J C L, Ohmann, H B, Babiuk, L A, and Doige, C E. 1992. Bovine herpesvirus-1- induced pharyngeal tonsil lesions in neonatal and weanling calves. J Comp Pathol 106: 243-253.

254. Schuh, J C L and Oliphant, L W. 1992. Development and immunophenotyping of the pharyngeal tonsil (adenoid) in cattle. J Comp Pathol 106: 229-241.

255. Schwartz, K J. 1999. Salmonellosis. *In*: Straw, B E, D'Allaire, S, Mengeling, W L, and Taylor, D J (eds.). Diseases of swine, 8th ed. Iowa State University Press: Ames, pp. 535-551.

256. Scicchitano, R, Stanisz, A, Ernst, P, and Bienenstock, J. 1988. A common mucosal immune system revisited. *In*: Husband, A J (ed.). Migration and homing of lymphoid cells. CRC Press: Boca Raton, pp. 1-34.

257. Segers, R P A M, Kenter, T, Haan, L A M, and Jacobs, A A C. 1998. Characterisation of the gene encoding suilysin from *Streptococcus suis* and expression in fields strains. FEMS Microbiol Lett 167: 255-261.

258. Segura, M, Stankova, J, and Gottschalk, M. 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumor necrosis factor alpha and interleukin-6 production by murine macrophages. Infect Immun 67: 4646-4654.

259. Serhir, B, Dubreuil, J D, Higgins, R, and Jacques, M. 1995. Purification and characterization of a 52-kilodalton immunoglobulin G-binding protein from *Streptococcus suis* capsular type 2. J Bacteriol 177: 3830-3836.

260. Serhir, B, Higgins, R, Foiry, B, and Jacques, M. 1993. Detection of immunoglobulin-G-binding proteins in *Streptococcus suis*. J Gen Microbiol 139: 2953-2958.

261. Sharon, N. 1996. Carbohydrate-lectin interactions in infectious disease. Adv Exp Med Biol 408: 1-7.

262. Shoo, M K, Wiseman, A, Allan, E M, Dalgleish, R G, Gibbs, H A, Al-Hendi, A B, and Selman, I E. 1990. Distribution of *Pasteurella haemolytica* in the respiratory tracts of carrier calves and those subsequently infected experimentally with *Dictyocaulus viviparus*. Res Vet Sci 48: 383-385.

263. Sicinski, P, Rowinski, J, Warchol, J B, Jarzabek, Z, Gut, W, Szczygiel, B, Bielecki, K, and Koch, G. 1990. Poliovirus type 1 enters the human host through intestinal M cells. Gastroenterology 98: 56-58.

264. Sinkora, J, Rehakova, Z, Samankova, L, Haverson, K, Butler, J E, Zwart, R, and Boersma, W. 2001. Characterization of monoclonal antibodies recognizing immunoglobulin κ and λ chains in pigs by flow cytometry. Vet Immunol Immunopathol 80: 79-91.

265. Slipka, J. 1988. Palatine tonsils - their evolution and ontogeny. Acta Otolaryngol Stockh suppl:454: 18-22.

266. Smith, H E, Rijnsburger, M, Stockhofe-Zurwieden, N, Wisselink, H J, Vecht, U, and Smits, M A. 1997. Virulent strains of *Streptococcus suis* serotype 2 and highly virulent strains of *Streptococcus suis* serotype 1 can be recognized by a unique ribotype profile. J Clin Microbiol 35: 1049-11053.

267. Smith, H E, Vecht, U, Gielkens, A L J, and Smits, M A. 1992. Cloning and nucleotide sequence of the gene encoding the 136-kilodalton surface protein

(muramidase-released protein) of *Streptococcus suis* type 2. Infect Immun 60: 2361-2367.

268. Smith, H E, Vecht, U, Wisselink, H J, Stockhofe-Zurwieden, N, Biermann, Y, and Smits, M A. 1996. Mutants of *Streptococcus suis* types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs. Infect Immun 64: 4409-4412.

269. Solano-Aguilar, G I, Vengroski, K G, Beshah, E, Douglass, L W, and Lunney, J K. 2001. Characterization of lymphocyte subsets from mucosal tissues in neonatal swine. Dev Comp Immunol 25: 245-263.

270. Solano-Aguilar, G I, Vengroski, K G, Beshah, E, and Lunney, J K. 2000. Isolation and purification of lymphocyte subsets from gut-associated lymphoid tissue in neonatal swine. J Immunol Methods 241: 185-199.

271. Staats, J J, Feder, I, Okwumabua, O, and Chengappa, M M R. 1997. *Streptococcus suis*: past and present. Vet Res Commun 21: 381-407.

272. Staats, J J, Plattner, B L, Nietfeld, J, Dritz, S, and Chengappa, M M. 1998. Use of ribotyping and hemolysin activity to identify highly virulent *Streptococcus suis* type 2 isolates. J Clin Microbiol 36: 15-19.

273. Stabel, J R. 2000. Transitions in immune responses to *Mycobacterium paratuberculosis*. Vet Microbiol 77: 465-473.

274. Stadnyk, A W. 1997. Cytokine production by epithelial cells. FASEB 8: 1041-1047.

275. Stanley, A C, Huntley, J F, Jeffrey, M, and Buxton, D. 2001. Characterization of ovine nasal-associated lymphoid tissue and identification of M cells in the overlying follicle-associated epithelium. J Comp Pathol 125: 262-270.

276. Steinbakk, M, Naess-Andresen, C-F, Lingaas, E, Dale, I, Brandtzaeg, P, and Fagerhol, M K. 1990. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. Lancet 336: 763-765.

277. Stenfors, L E and Raisanen, S. 1991. *In vivo* attachment of beta-haemolytic streptococci to tonsillar epithelial cells in health and disease. Acta Otolaryngol Stockh 111: 562-568.

278. Stevens, D L, Tanner, M H, Winship, J, Swarts, R, Ries, K M, Schlievert, P M, and Kaplan, E. 1989. Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. N Engl J Med 321: 1-7.

279. Stoddart, M E, Gaskell, R M, Harbour, D A, and Pearson, G R. 1988. The sites of early viral replication in feline infectious peritonitis. Vet Microbiol 18: 259-271.

280. Suarez, A, Guttler, A, Stratz, M, Staendner, L H, Timmis, K N, and Guzman, C A. 1997. Green fluorescent protein-based reporter systems for genetic analysis of bacteria including monocopy applications. Gene 196: 69-74.

281. Summerfield, A, McNeilly, F, Walker, I, Allan, G, Knoetig, S M, and McCullough, K C. 2001. Depletion of CD4+ and CD8 high+ T-cells before the onset of viremia during classical swine fever. Vet Immunol Immunopathol 78: 3-19.

282. Summerfield, A, Rziha, H J, and Saalmuller, A. 1996. Functional characterization of porcine CD4+CD8+ extrathymic T lymphocytes. Cell Immunol 168: 291-296.

283. Swindle, M M, Moody, D C, and Phillips, L D. 1992. Swine as a model in biomedical research, 1st ed. Iowa State University Press: Ames.

284. Tang, X, Hori, S, Osamura, R Y, and Tsutsumi, Y. 1995. Reticular crypt epithelium and intra-epithelial lymphoid cells in the hyperplastic human palatine tonsils: an immunohistochemical analysis. Pathol Int 45: 34-44.

285. Tanimoto, T and Ohtsuki, Y. 1996. Evaluation of antibodies reactive with porcine lymphocytes and lymphoma cells in formalin-fixed, paraffin-embedded, antigen-retrived tissue sections. Am J Vet Res 57: 853-859.

286. Taylor, D J. 1999a. *Actinobacillus pleuropneumoniae*. *In*: Straw, B E, D'Allaire, S, Mengeling, W L, and Taylor, D J (eds.). Diseases of swine, 8th ed. Iowa State University Press: Ames, pp. 343-354.

287. Taylor, D J. 1999b. Miscellaneous bacterial infections. *In*: Straw, B E, D'Allaire, S, Mengeling, W L, and Taylor, D J (eds.). Diseases of swine, 8th ed. Iowa State University Press: Ames, pp. 613-642.

288. Thanawongnuwech, R, Halbur, P G, and Thacker, E L. 2000. The role of pulmonary macrophages in porcine reproductive and respiratory syndrome virus infection. Anim Health Res Rev 1: 95-102.

289. Thibodeau, V, Frost, E H, Chenier, S, and Quessy, S. 1999. Presence of *Yersinia enterocolitica* in tissues of orally-inoculated pigs and the tonsils and feces of pigs at slaughter. Can J Vet Res 63: 96-100.

290. Torremorell, M, Calsamiglia, M, and Pijoan, C. 1998. Colonization of suckling pigs by *Streptococcus suis* with particular reference to pathogenic serotype 2 strains. Can J Vet Res 62: 21-26.

291. Torres-Medina, A. 1981. Morphologic characteristics of the epithelial surface of aggregated lymphoid follicles (Peyer's patches) in the small intestine of newborn gnotobiotic calves and pigs. Am J Vet Res 42: 232-236.

292. van Keulen, L J, Schreuder, B E, Meloen, R H, Mooij Harkes, G, Vromans, M E, and Langeveld, J P. 1996. Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. J Clin Microbiol 34: 1228-1231.

293. Vecht, U, Wisselink, H J, Dijk, J E v, and Smith, H E. 1992. Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. Infect Immun 60: 550-556.

294. Vecht, U, Wisselink, H J, Jellema, M L, and Smith, H E. 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect Immun 59: 3156-3162.

295. Vega-Lopez, M A, Telemo, E, Bailey, M, Stevens, K, and Stokes, C R. 1993. Immune cell distribution in the small intestine of the pig: immunohistological evidence for an organized compartmentalization in the lamina propria. Vet Immunol Immunopathol 37: 49-60.

296. Von Gaudecker, B, Sticherling, M, and Sterry, W. 1996. Immunohistochemical localization of cytokines and receptor-associated molecules in human tonsils and skin. Acta Otolaryngol Stockh suppl 523: 71-74.

297. Vukmanovic, M, Vyas, B, Gorak, P, Noble, A, and Kemeny, D M. 2000. Human Tc1 and Tc2/Tc0 CD8 T-cell clones display distinct cell surface and functional phenotypes. Blood 95: 231-240.

298. Wagner, N, Löhler, J, Kunkel, E J, Ley, K, Leung, E, Krissansen, G, Rajewsky, K, and Müller, W. 1996. Critical role of β7 integrins in formation of gut-associated lymphoid tissue. Nature 382: 366-370.

299. Walker, R I, Schmauder-Chock, E A, and Parker, J L. 1988. Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches. Can J Microbiol, 34: 1142-1147.

300. Wardley, R C and Povey, R C. 1977. The pathology and sites of persistence associated with three different strains of feline calicivirus. Res Vet Sci 23: 15-19.

301. Wassef, J S, Keren, D F, and Mailloux, J L. 1989. Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of shigellosis. Infect Immun 57: 858-863.

302. Waters, W R, Palmer, M V, Pesch, B A, Oslen, S C, Wannemuehler, M J, and Whipple, D L. 2000. Lymphocyte subset proliferative responses of *Mycobacterium bovis*-infected cattle to purifed protein derivative. Vet Immunol Immunopathol 77: 257-273.

303. Weltzin, R, Lucia-Jandris, P, Michetti, P, Fields, B N, Kraehenbuhl, J P, and Neutra, M R. 1989. Binding and transepithelial transport of immunoglobulins by intestinal M cells: demonstration using monoclonal IgA antibodies against enteric viral proteins. J Cell Biol 108: 1673-1685.

304. Wibawan, I W T and Lammler, C. 1994. Relation between encapsulation and various properties of *Streptococcus suis*. J Vet Med B 41: 453-459.

305. Williams, A E. 1990. Relationship between intracelullar survival in macrophages and pathogenicity of *Streptococcus suis* type 2 isolates. Microb Pathog 8: 189-196.

306. Williams, A E and Blakemore, W F. 1990. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. J Infect Dis 162: 474-481.

307. Williams, D M, Lawson, G H K, and Rowland, A C. 1973. Streptoccoccal infection in piglets: the palatine tonsil as portals of entry for *Streptococcus suis*. Res Vet Sci 15: 352-362.

308. Williams, D M and Rowland, A C. 1972. The palatine tonsils of the pig - an afferent route to the lymphoid tissue. J Anat 113: 131-137.

309. Wilson, A D, Haverson, K, Southgate, K, Bland, P W, Stokes, C R, and Bailey, M. 1996. Expression of major histocompatibility complex class II antigens on normal porcine intestinal endothelium. Immunology 88: 98-103.

310. Wilson, C, Sama, A, Clark, M A, Hirst, B H, and Wilson, J A. 1998. Identification of cell subpopulations in human tonsillar epithelium. J Anat 192: 465-466.

311. Windsor, R S and Elliott, S D. 1975. Streptococcal infection in young pigs. IV- an outbreak of strptococcal meningitis in weaned pigs. J Hyg Camb 75: 69-78.

312. Wisselink, H J, Smith, H E, Stockhofe-Zurwieden, N, Peperkamp, K, and Vecht, U. 2000. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in European countries. Vet Microbiol 74: 237-248.

313. Wisselink, H J, Vecht, U, Stockhofe-Zurwieden, N, and Smith, H E. 2001. Protection of pigs against challenge with virulent *Streptococcus suis* serotype 2 strains by a muramidase-released protein and extracellualr factor vaccine. Vet Rec 148: 473-477.

314. Wolf, J L, Kauffmann, R S, Finberg, R, Dambrauskas, R, Fields, B N, and Trier, J S. 1983. Determinants of reovirus interactions with the intestinal M cells and absorptive cells of murine intestine. Gastroenterology 85: 291-300.

315. Wood, R L, Pospischil, A, and Rose, R. 1989. Distribution of persistent *Salmonella typhimurium* infection in internal organs of swine. Am J Vet Res 50: 1015-1020.

316. Wunschmann, A, Kremmer, E, and Baumgartner, W. 2000. Phenotypical characterization of T and B cell areas in lymphoid tissues of dogs with spontaneous distemper. Vet Immunol Immunopathol 73: 83-98.

317. Wünschmann, A, Alldinger, S, Kremmer, E, and Baumgärtner, W. 1999. Identification of CD4+ and CD8+ T cell subsets and B cells in the brain of dogs with spontaneous acute, subacute-, and chronic-demyelinating distemper encephalitis. Vet Immunol Immunopathol 67: 116.

318. Yamanaka, N, Sambe, S, Harabuchi, Y, and Kataura, A. 1983. Immunological study of tonsil. Distribution of T cell subsets. Acta Otolaryngol Stockh 96: 509-516.

319. Yamanaka, N, Yokoyama, M, Kawaguchi, T, Tamaki, K, and Ishii, H. 1996. Role of γδ T cells in the palatine tonsil. Acta Otolaryngol Stockh suppl 523: 90-93.

320. Yang, H and Parkhouse, M E. 1996. Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues. Immunology 89: 76-83.

321. Zhou, F, Kraehenbuhl, J P, and Neutra, M R. 1995. Mucosal IgA response to rectally administered antigen formulated in IgA-coated liposomes. Vaccine 13: 637-644.

322. Zuckermann, F. 1999. Extrathymic CD4/CD8 double positive T cells. Vet Immunol Immunopathol 72: 55-66.

323. Zuckermann, F A. 2000. Aujeszky's disease virus: opportunities and challenges. Vet Res 31: 121-131.

324. Zuckermann, F A and Gaskins, H R. 1996. Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associated lymphoid tissue. Immunology 87: 493-499.

325. Zuckermann, F A and Husmann, R J. 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87: 500-512.

326. Zuckermann, F A, Pescovitz, M D, Aasted, B, Dominguez, J, Trebichavsky, I, Novikov, B, Valpotic, I, Nielsen, J, Arn, S, Sachs, D H, Lunney, J K, Boyd, P, Walker, J, Lee, R, Davis, W C, Barbosa, I R, and Saalmuller, A. 1998. Report on the analyses of mab reactive with porcine CD8 for the second international swine CD workshop. Vet Immunol Immunopathol 60: 291-303.

327. Zuckermann, F A, Schnitzlein, W M, Thacker, E, Sinkora, J, and Haverson, K. 2001. Characterization of monoclonal antibodies assigned to the CD45 subgroup of the third international swine CD workshop. Vet Immunol Immunopathol 80: 165-174.