

STUDIES OF THE PALATINE TONSILS IN THE PIG AND OF THEIR  
ROLE IN THE PATHOGENESIS OF PIGLET STREPTOCOCCAL INFECTION

1 INTRODUCTION

Anatomy and Histology of the Palatine Tonsils

by

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Pathology of the Palatine Tonsils

The Palatine Tonsils and Disease Pathogenesis

Streptococci in Pigs

II PLAN OF WORK AND ORIGINAL MATERIALS AND METHODS

Plan of Work

Source and Management of Pigs

Pathogenesis of Pigs

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*Dorothy M. Williams*

*I declare that this thesis was composed by me, that it is a record of original work carried out by me, and that it has not been submitted in any form to any other university.*

*Dorothy M. Williams*

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take origin. Examination by SUMMARY microscopy revealed that the tonsillar crypt epithelial cells participate in phagocytosis.

A histological study was made on tonsils from porcine fetuses in late gestation up to aged adults to provide a concept of normality as a background to later experimental studies. In late gestation, lymphocytic follicles were frequently observed in the tonsils. Germinal centres were noted by four weeks of age, thereafter increasing markedly in number, size and activity. The interfollicular lymphoid tissue developed in cellularity, presenting lymphocytes, macrophages, plasma cells and large pyroninophilic cells by one month of age. Germ-free and dibirotic animals of this age showed an essentially similar tonsillar structure. In pigs up to one year of age there was little variation in this pattern but from then on germinal centre activity decreased markedly. Senile changes included atrophy of the branching tonsillar crypt system and an increase in the connective tissue stroma, replacing much of the lymphoid parenchyma.

To define the afferent and efferent pathways to the palatine tonsils, non-infective studies employing vital dyes were carried out. Intra-vital dye injections failed to illustrate any afferent route to the tonsils from adjacent areas, while confirming the tonsillar efferent lymphatic drainage to the submandibular lymph nodes. Topical application of India ink to the oral surface of the tonsils defined an afferent route through the tonsillar crypt epithelium to the lymphoid parenchyma. Ink was distributed throughout the interfollicular tissue, occasionally within germinal centres, and later in trabeculae and the capsule from which the efferent lymphatics

take origin. Examination by electron-microscopy revealed that the tonsillar crypt epithelial cells participate in phagocytosis.

Infective studies with Streptococcus suis were carried out to investigate the role of the palatine tonsils in the pathogenesis of piglet streptococcal meningitis and arthritis caused by this organism. Application of Strep. suis to the oral surface of the tonsils produced clinical disease in one of four piglets infected at ten days of age; inapparent infection occurred in the other three in which the organism was confined to the tonsils and their drainage lymph nodes. Strep. suis or its antigenic breakdown products, specifically identified by immunofluorescent microscopy, were located in the tonsillar crypt lumina, epithelium, interfollicular tissue and, rarely, in germinal centres, of all four infected piglets. Histological examination failed to reveal any abnormalities of the tonsils. In a second experiment, phagocytosis of bacteria by epithelial cells of the tonsillar crypts was demonstrated by electron-microscopy.

Inapparent infection was established in three piglets aged six weeks. The distribution of Strep. suis was essentially similar to that described above except for an exaggerated presence of Strep. suis antigen in the majority of the tonsillar germinal centres.

Attempts to identify specific humoral antibody to Strep. suis in four older pigs infected systemically with the organism proved unsuccessful. Similarly immunofluorescent methods failed to demonstrate specific antibody-containing cells in the tissues of these four pigs and in the spleens of mice given Strep. suis intra-

peritoneally.

## CHAPTER I

### INTRODUCTION

The term "lympho-epithelial tissue" denotes lymphoid aggregations with an intimate anatomical epithelial relationship: much of this tissue is included within mucous membranes, particularly of the alimentary tract, and the thymus also has a prominent compartment of epithelial cells. The palatine tonsils, discrete organs forming a major part of the oro-pharyngeal lymphoid tissue, belong to this category. The function of lympho-epithelial tissues has been widely studied in recent years within the broad field of immunobiology but in mammalia it has yet to be explicitly defined. In this thesis, therefore, aspects of development and function of the porcine palatine tonsils are considered, and their role in the pathogenesis of piglet streptococcal infection.

### Anatomy and Histology of the Palatine Tonsils

The palatine tonsils of the pig are paired oval lymphoid organs situated on either side of the median furrow of the soft palate where they form bilateral raised areas. On the surface of these are apparent the numerous orifices of the tonsillar crypts created by invaginations of the oral mucous membrane (Sisson, 1953). They are part of the ring of lymphatic tissue around the oro- and naso-pharynx (Trautmann & Fiebiger, 1952).

Levin (1930) briefly described the histological structure of the palatine tonsils of the adult pig. The oral surface is clothed by stratified squamous epithelium which invaginates to form deep

branching crypts. Arranged about the crypts are numerous germinal centres containing large pale cells with irregular nuclei. Mitotic figures are fairly frequently observed in the centres but rarely in the dense lymphocytic aggregations compactly filling the reticular tissue (interfollicular lymphoid areas) through which many fibrous trabeculae run. The crypt epithelium may be infiltrated by lymphocytes.

Heavy infiltration of lymphocytes may often be seen into the loosely arranged (reticulated) areas of epithelium which occur within the crypt system (Trautmann & Fiebiger, 1952). Afferent lymphatics are absent from the tonsils, nor are there lymphatic sinuses such as are recognised in lymph nodes. Small lymphatics originate in the tonsils to anastomose with well-developed vessels in the trabeculae and the aboral capsule which divides the tonsils from the tissues of the soft palate. Macrophages and lymphocytes are contained in the interfollicular areas and plasma cells are present in very variable numbers. These are frequently aggregated in the subepithelial zones, and may penetrate the epithelium itself (personal observations - Chapter III).

The lympho-epithelial tissue of the pharyngeal ring has been studied in the various domestic animals with particular reference to histological structure (Chukalovjkaya, 1956) and classified according to crypt form, presence or absence of a capsule creating either limited or diffuse lymphoid tissue, and of trabeculae which produce lobulation. The palatine tonsils of the pig may therefore be described as limited, lobulated, polycryptic organs. Microscopically, however, they present essentially similar features to those described for the palatine (faucial) tonsils of man (Parkinson, 1951; Bloom &

Fawcett, 1968) and other animals (Trautmann & Fiebiger, 1952). The major point of species difference is that in the pig the anatomical location of the palatine tonsils in the soft palate compared with the lateral walls of the isthmus faucium, often within tonsillar fossae, in all other domestic species (Trautmann & Fiebiger, 1952; Sisson, 1953) and in man (Parkinson, 1951; Bloom & Fawcett, 1968).

Although the actual density of lymphocytes in the nodules

### Ontogeny of the Palatine Tonsils

#### Foetal:

The development of the palatine tonsils in the foetal pig was described by Levin (1930): the tonsillar area in the soft palate becomes defined just prior to mid-gestation at 45 - 50 days when there is diffuse proliferation of the basal epithelium on either side of the mid-line concurrent with a subepithelial condensation of undifferentiated mesenchymal tissue. By 55 days, clumps of basal epithelium can be observed budding into the subepithelial tissue and becoming, from 70 days onwards, hollow epithelium-lined crypts. Nodules, lymphoid in character, first develop at this stage, enclosing the tips of the early crypt forms which later begin to branch. Although he noted perivascular aggregations of lymphocytes, Levin dismissed the possibility that these were of haematogenous origin. The mature foetus at 114 days possesses tonsillar lymphocytic follicles and without germinal centres.

Patten (1933) was apparently unaware that the anatomical location of the palatine tonsils of the pig is within the soft palate and that, unlike other species, tonsillar fossae are not a feature of the pig. No mention was made of the development of the epithelial crypt system nor of the lymphoid tissue.

In foetal pig palatine tonsils, no evidence of crypts or lymphoid cells was seen between 40 - 50 days of gestation (Binns, 1968) but around 60 days the first small lymphoid nodules were observed in association with uneven areas of the epithelium. Thereafter progressive development of the crypt system took place together with a pronounced increase in the amount of nodular lymphoid tissue up to full-term although the actual density of lymphocytes in the nodules did not change markedly from their first appearance.

Lymphoid follicles in the lingual tonsils of the foetal pig were observed on the 77th day of gestation but germinal centres and plasma cells were absent (Kruml, Kovaru, Ludvig & Trebichavsky, 1970). Kutilov (1956), however, did not observe follicles in neo-natal animals.

In the human foetus, the development of the palatine tonsils is summarised by Parkinson (1951). Lymphocytes are first noted at 4 months and are considered to arise locally from the subepithelial primitive mesenchymal cells. Neither germinal centres nor plasma cells are present in foetal life but lymphocytic infiltration of the epithelium may be observed. A further account of tonsillar development in the human foetus (Arey, 1965) describes crypts developing progressively from the 3rd - 6th month as solid epithelial ingrowths which later branch and canalise. In the 3rd month, lymphocytes first appear with lymphoid nodules developing after 6 months. From the mesenchymal tissue immediately adjacent to the developing organ the aboral capsule and internal connective tissue trabeculae begin to form from the 5th - 6th month onwards.

Lymphocytopoeisis and the origin of the tonsillar lymphocytes are not detailed in the above accounts of tonsillar development in the

pig and in man. This is discussed in relation to the foetal rabbit tonsils studied by light and electron microscopy (Harrison & Patt, 1966; Harrison, Patt & Albright, 1970). Their observations led them to conclude that the tonsillar lymphocytes by a process of migration and differentiation originate from the basal cells of the epithelium: massive mitotic activity was noted in the basal epithelial layer on the 18th day of gestation and at 19 - 20 days lymphoid cells were recognised lying near the epithelium or at the tips of the budding epithelial crypts. A marked similarity in the various cytological features of the lymphocytes and epithelial cells was observed.

In a similar light microscopical study of the lympho-epithelial tissue of the iliac tonsil and appendix of the rabbit (Ackerman, 1966), no evidence was found to suggest epithelial involvement in lymphocytopoiesis but rather that the local underlying mesenchymal cells participated in the production of lymphocytes. This work agreed with earlier studies on bovine and human foetal palatine tonsils by James (1956) who observed mitosis in the local mesenchymal cells with the appearance of lymphocytes around the primordial crypts. James noted penetration of lymphocytes into the epithelium through openings in the reticulin fibres adjacent to the basement membrane. There was progressive formation of intercellular spaces within the epithelium prior to birth and in his studies no plasma cells or germinal centres were noted.

#### Post-natal:

The post-natal development of the porcine palatine tonsils is not well documented in the literature. Although Kutilov (1956) mentioned

that this deficiency necessitated his making a study of structure related to age preliminary to examining the pathology of the tonsils in the pig, he made only limited comment on his observations: no follicles were present at a few days of age but were noted towards the first month of life, at which time the crypt epithelium was still quite continuous.

Levin (1930) briefly described the macroscopic and microscopic appearance of adult palatine tonsils in the pig but made no reference to age changes in the structure or constituent components. Studying the development of the lympho-reticular system in the pig, Pestana, Hallenbeck and Shorter (1965), although no specific mention was made of the tonsils, found germinal centres to be abundant and well-defined at two days of age. Plasma cells were not identified in any of the lymphoid tissues examined.

More information is available relating to post-natal changes in the human palatine tonsils. Plasma cells may first be seen at around 3 weeks of age, germinal centres by 3 - 6 months; thereafter there is a marked increase in lymphocytes, plasma cells and lymphoid nodules which results in tonsillar enlargement; involution of the tonsillar tissue follows at 30 - 40 years of age; and only a semblance of the crypt structure may remain in old age (Parkinson, 1951). Marked proliferation of lymphoid tissue is observed in the first year of life and maximum size is attained in childhood (Minear, Arey & Milton, 1937). Atrophy commences at around 14 years of age, the lymphoid elements regressing as involution proceeds. Brown (1966) stated that maximum hypertrophy occurs at the age of 6. Slight involution at 20 has been noted (Tajima, 1967), becoming increasingly evident in later years. Tajima observed the greatest

number of follicles at 16 - 20 years but maximum activity, based on prevalence of mitotic figures and small lymphocytes, occurs between the ages of 6 - 10 years..

There is therefore general agreement on the occurrence of hypertrophy of the palatine tonsillar tissue in childhood, although the age of onset of involution or atrophy varies from author to author.

#### Function of the Palatine Tonsils

The palatine tonsils and other lympho-epithelial tissues form part of the larger lymphoid system which in mammals encompasses the thymus, spleen, lymph nodes and scattered lymphoid foci. The early work on the functions of this system rarely involved the palatine tonsils so that the hypotheses advanced regarding their specific role were based largely on comparisons with other lymphoid organs. In the palatine tonsils, morphological features common to lymph nodes have been described (Trautmann & Fiebiger, 1952; Bloom & Fawcett, 1968) but it has been pointed out that afferent lymphatic vessels are absent (Trautmann & Fiebiger, 1952; Koburg, 1967). It is perhaps invalid therefore to assume identical functions for the tonsils and lymph nodes.

Wright (1950) suggested that the importance of the tonsils and alimentary lympho-epithelial tissues, in lacking afferent vessels, must be related to surface exposure. He proposed that the tonsils are capable of participating in immune responses, basing this on the presence of germinal centres, plasma cells and lymphocytes, but he commented that tonsillectomy in the young does not have any apparent deleterious effect. He failed, however, to define specifically the role of the palatine tonsils.

A comprehensive review on the tonsils and associated structures (Parkinson, 1951) cited lymphocytopoiesis as the important tonsillar function. The possibility of an endocrine function was dismissed since tonsillectomy does not induce any recognised deficiency syndrome. Parkinson commented that the anatomical location and lympho-epithelial structure, both features facilitating contact with ingested antigens, produce in the tonsils an ideal situation for antibody production to be initiated, and stressed their morphological and cellular similarities to lymph nodes. No experimental evidence was however quoted to support the theory. A more recent re-postulate that the tonsils form an "antigen-priming mechanism" for the host (Kocsard-Varo, 1964) similarly lacked any presented experimental foundation.

Various hypotheses on tonsillar function were discussed by Eigler (1965) and Koburg (1966). The tonsils may simply be peripheral lymph nodes filtering and destroying material absorbed from the overlying mucous membrane; the tonsillar epithelium, together with the lymphoid parenchyma, may serve a defensive role as an excretory mechanism through epithelial desquamation and cellular migration into the crypts, causing destruction and elimination of potential pathogens; the tonsils may be involved in defence against haematogenous as well as ingested antigens; and lastly, perhaps they also initiate immune responses leading to production of antibody and specifically differentiated, "committed" lymphocytes to be disseminated throughout the body. This last theory receives their greatest support. They consider also that antigen penetration is favoured by the reticulated structure of areas of the crypt epithelium and enhanced by the vast surface epithelial area presented by the

extensive ramifying crypt system. The tonsils therefore "must be considered as being under continuous antigenic stimulation" (Koburg, 1967).

Without supportive experimental data, tonsillar function was summarised by Farocki (1967) as lymphocytopoiesis and antibody production, phagocytosis and destruction of bacteria, and induction of systemic immunity by localised infections. Antigenic release beyond the tonsils may stimulate a more generalised lymphoid tissue participation, perhaps only a minimal quantity of specific antibody being synthesised locally in the tonsils.

The first real contribution to tonsillar function came from autoradiographic studies on lymphocyte kinetics in the palatine tonsils of the rabbit (Koburg, 1967): this work confirmed local lymphocytopoiesis. Koburg (1967) suggested, based on experimental data, that antigenic stimuli initiate lymphocyte production from the tonsillar germinal centres, and that the lymphocytes migrate to the subepithelial zone and into the epithelium. On further antigen encounter in these sites, these cells return to the tonsillar lymphoid parenchyma to differentiate into plasma cells. Koburg (1970) demonstrated a secondary immune response in the popliteal lymph nodes of rabbits previously given surface tonsillar exposure to a specific antigen, thus lending support to the theory of "committed" lymphocyte production by the tonsils.

Studies on cultured human tonsillar lymphocytes have illustrated "blast-cell" (transitional cell) formation on the addition of specific antigens to which the donor host had been previously exposed (Oettgen, Silber, Meischer & Hirschhorn, 1966). Analysis of human tonsillar tissue cultures from patients between 7 months and 14 years of age

revealed 80% of the cell types to be small lymphocytes, 10% large lymphocytes, with 10% plasma cells and non-specified others (Goldfarb & Ullal, 1965).

In the pharyngeal tonsils (adenoids) of man, frequent IgG-containing plasma cells have been noted and a significant but smaller population of IgA cells (Crabbé & Heremans, 1967). IgM, IgG and IgA immunoglobulins have all been identified in germinal centres in human palatine tonsils (Burtin & Buffe, 1967). Specific antibody, indicated by the presence of haemolytic plaque-forming cells, has been noted in the tonsils as well as lymph nodes, after intravenous immunisation of rabbits with sheep red blood cells (Diamantstein & Hofferberth, 1969). Uptake of protein antigen into tonsillar macrophages was observed following intravenous administration (Sauer & Diamantstein, 1969).

The last decade has witnessed marked advances in the field of immunobiology and of particular interest here, in the ontogeny of immunological activity. The current concepts of the functions of the various lymphoid organs in the development of immune responses are well summarised by Good, Cooper, Peterson, Hoyer and Gabrielson (1967) and reviewed by Nossal and Ada (1971). Basically, there are two ontogenetically distinct lymphocyte populations: one, thymus-dependent, concerned with the expression of cell-mediated immunological activity; the other, dependent on the Bursa of Fabricius in birds but on tissues as yet undefined in the mammal, involved in antibody production and the development of germinal centres. Much attention has been focused on the lympho-epithelial tissues of the alimentary tract in seeking the mammalian equivalent of the Bursa (Perey, Frommel, Hong & Good, 1970; Silverstein & Prendergast, 1970; Weissman, 1970). Although it has been postulated that the palatine tonsils may exert a

bursal-type function (Peterson, Cooper & Good, 1965), no function other than lymphocytopoiesis can be ascribed with certainty to the tonsils (Fitchellius, 1969).

A radically different approach to lympho-epithelial tissues has been adopted by Harrison (1970a & b). Following implantation of foetal rabbit tonsils into neo-natally thymectomised mice, she described restoration of follicular organisation and repopulation of "thymus-dependent" areas of lymph nodes. A similar but lesser effect was obtained with implants contained within diffusion chambers. These experiments in her view favour a thymus-like function, supported by a humoral factor, for the tonsils but they have yet to be repeated by other workers.

It is therefore apparent that no one theory on the function of the palatine tonsils receives general support: although present evidence suggests an important immunological role, its exact nature awaits definition.

Fibrinous tonsillitis and haemorrhagic necrotic tonsillitis may be associated with purine pasteurellosis and anthrax respectively (Jubb & Kennedy, 1970). In hog cholera (swine fever) suppurative tonsillitis may occur, most likely the result of secondary bacterial invasion of necrotic lesions of viral origin (Dunn, 1970). The tonsillar epithelium participates in stomatitis-pharyngitis, usually characterised by a catarrhal inflammation which can lead to plugging of the tonsillar crypts with desquamated epithelial cells, leucocytes and bacteria, and in neurobacillosis of the oral cavity, coagulative necrotic tonsillitis is common (Jubb & Kennedy, 1970). While the tonsils and tonsillitis receive scarcely a mention in any veterinary pathology textbooks, Smith and Jones (1966) make a brief comment on

Pathology of the Palatine Tonsils

In the pig, tonsillitis is not a recognised specific disease entity as in man. Tonsillar lesions, however, have been frequently observed in association with systemic disease. In a survey by Kutilov (1956), the palatine tonsils were studied in over 300 pigs which had died or been slaughtered suffering from various systemic disease states, swine fever, influenza, pasteurellosis, salmonellosis and bronchopneumonia, and compared with a similar number from clinically healthy animals. No pathological lesions were noted in the material from this latter group, which showed tonsillar crypts often packed with lymphocytes and epithelial cell debris and an intact epithelium varyingly infiltrated with lymphocytes. Kutilov classified the different forms of tonsillitis, noted in just over half the diseased pigs, as serous, purulent, necrotic, ulcerative and chronic. No specific type was peculiar to any one systemic disease and in each case tonsillar lesions were considered to be secondary phenomena.

Fibrinous tonsillitis and haemorrhagic necrotic tonsillitis may be associated with porcine pasteurellosis and anthrax respectively (Jubb & Kennedy, 1970). In hog cholera (swine fever) suppurative tonsillitis may occur, most likely the result of secondary bacterial invasion of necrotic lesions of viral origin (Dunne, 1970). The tonsillar epithelium participates in stomatitis-pharyngitis, usually characterised by a catarrhal inflammation which can lead to plugging of the tonsillar crypts with desquamated epithelial cells, leucocytes and bacteria, and in necrobacillosis of the oral cavity, coagulative necrotic tonsillitis is common (Jubb & Kennedy, 1970). While the tonsils and tonsillitis receive scarcely a mention in many veterinary pathology textbooks, Smith and Jones (1966) make a brief comment on

the tonsils of domestic animals and state that "in the pig these are prone to become infected and to form chronic abscesses a few millimetres in diameter". Tonsillar abscesses have been noted following experimental reproduction of streptococcal cervical lymphadenitis in pigs (Boehm, 1969; Armstrong, Boehm & Ellis, 1970; Armstrong & Ellis, 1971) but no pathological investigations of the palatine tonsils in other streptococcal infections in the pig are recorded.

In man tonsillitis may be acute or chronic and is most frequently encountered in local tonsillar infections with beta-haemolytic streptococci of Lancefield's Group A. Acute tonsillitis may be catarrhal (Parkinson, 1951), follicular, or parenchymatous (Parkinson, 1951; Jackson & Jackson, 1959; Boyd, 1970); chronic tonsillitis is often but not necessarily a sequel to repeated acute episodes, and hyperplasia may or may not be evident (Schenck, 1950; Parkinson, 1951; Jackson & Jackson, 1959). Involvement of the palatine tonsils in man is not confined to streptococcal infections, occurring also in measles, scarlet fever, diphtheria, chickenpox, tuberculosis or tumours of the lymphoid system.

#### The Palatine Tonsils and Disease Pathogenesis

Studies on the pathogenesis of certain viral and bacterial diseases of the pig have brought to light early involvement of the palatine tonsils following oral infection. In the baby pig with Aujeszky's disease, caused by pseudo-rabies virus (PRV), the primary site of viral multiplication, within the first day post-infection, was found to be the palatine tonsils and the pharyngeal mucosa, with subsequent extension to the cervical chain of lymph nodes (Sabo, Rajcani & Blaskovic, 1968). Immunofluorescent techniques specific

for PRV revealed focal distribution of virus within the tonsillar crypt epithelium, some of which was undergoing necrosis. (They, 1965). suggested that perhaps this epithelial necrosis allowed spread of the virus particles. Following oral administration of streptococci of Cheville and Mengeling (1969), investigating the pathogenesis of chronic hog cholera (swine fever), observed by immunofluorescent microscopy limited focal involvement of the tonsillar crypt epithelium but generally none of the lymphoid parenchyma; otherwise viral antigen was rarely noted outwith the alimentary and renal tubular epithelium. In contrast virus was widespread in acute fatal cases, with strong specific fluorescence present in the tonsillar epithelium and in cells scattered throughout the lymphoid tissue. Electron-microscopy revealed in the cytoplasm of the cell types which fluoresced, including epithelial cells, small particles which were considered to be possibly hog cholera virus.

In a study similar to that on Aujeszky's disease but with African swine fever virus, entry and multiplication of the virus occurred primarily in the tonsils and submandibular lymph nodes, with subsequent viraemia producing widespread viral dissemination (Colgrove, Haelterman & Coggins, 1969). Immunofluorescent examination of the tonsils revealed viral antigen in macrophages and reticular cells of the interfollicular areas, very occasionally in the epithelium but never in the germinal centres.

The tonsils have been proposed as portals of entry for bacteria.

The association between certain streptococci and disease states of the pig has been recognized and these streptococci characterized. In a study of healthy piglets and calves in the first week of life numerous organisms were isolated from the tonsils and from other lymphoid tissue in the area (Payne & Derbyshire, 1963). In the pathogenesis of Salmonella cholerae suis it was postulated that

bacterial entry might be effected through the mucosa of the pharyngeal area, in addition to the lower small intestine and colon (Lawson, 1965). The organisms were frequently isolated from the tonsils from 48 hours post-infection. Following oral administration of streptococci of Lancefield's Groups D and E, these bacteria were almost invariably recovered from throat swabs or the tonsils themselves (Collier, 1956b; Elliott, Alexander & Thomas, 1966; Boehm, 1969; Armstrong & Ellis, 1971).

Persistence of bacterial infection in the tonsils was found following oral and intravenous exposure to Erysipelothrix insidiosa during an investigation of the immunopathological and bacteriological aspects of arthritis in swine (Timoney & Berman, 1970). There was, however, little evidence to suggest that such a tonsillar infection resulted in a significant bacterial excretion in the faeces to contaminate the environment; nor did the authors favour the hypothesis that the tonsils act as a focus releasing antigen to maintain a chronic arthritogenic stimulus. After oral exposure to Escherichia coli the tonsillar crypts of piglets were packed with the bacteria, specifically demonstrated by immunofluorescent microscopy, but organisms were never observed to lie within or beneath the mucosa (Drees & Waxler, 1970a).

#### Streptococci in Pigs

The association between certain streptococci and disease states of the pig has been recognised and these streptococci characterised, mainly within the last three decades. Organisms of the genus Streptococcus have now been isolated from a wide range of conditions in both young and adult animals and in some cases established as

aetiological agents. 14's Groups A, B, C, D, K and N have all been

isolated. The earliest report of streptococci in swine (Hutyra & Marek, 1898) described their isolation from cervical lymph node abscesses, the condition being referred to as "strangles of hogs" since it was not dissimilar to Strep. equi in horses. In a group of 6 month old pigs, Newsom (1937) noted an identical pattern of cervical lymph node abscesses from which he cultured haemolytic streptococci.

With the introduction of a serological method of grouping haemolytic streptococci (Lancefield, 1933), the classification of these organisms of porcine origin commenced in the 1940's: Group E streptococci were isolated from cervical abscesses in a 7 month old pig (Stafseth & Clinton, 1941) and Groups C, E and L from catarrhal, puerperal, septicaemic syndromes and dermatitis (Hare, Fry & Orr, 1942). In the catarrhal form characterised by a mucopurulent conjunctivitis and catarrhal rhinitis, Groups C and L were recovered from tonsillar swabs and it was assumed from this that bacterial multiplication occurred in the tonsillar mucus with lymphoid tissue invasion as a sequel. Similarly, positive Group C streptococcal cultures were obtained from the septicaemic cases (Hare et al., 1942). These workers therefore suggested that transmission of organisms might occur via droplets, contaminated food or water. They considered the various syndromes observed to be different manifestations of a single disease entity caused by Group C streptococci. In a survey of beta-haemolytic streptococci isolated from swine in Iowa (Collier, 1951), these were recovered from a wide range of disease states - bacteraemia, arthritis, abortion, abscesses, vegetative endocarditis, pneumonia and periostitis. Groups C, E and L were identified and the remainder, though biochemically they closely resembled Group E, were not

classified. Lancefield's Groups A, B, C, G, K and N have all been isolated from pigs (Simons, 1963) - the majority of the organisms belonged to Group C and were present in liver, lung and joints. Reviewing streptococci found in domestic animals, Krantz and Dunne (1965) also included Group H organisms isolated from cases of abortion in sows. Intra-venous inoculation of a Group C streptococcus, Strep. equisimilis, cultured from naturally occurring arthritic conditions was found to reproduce the clinical disease (Roberts, Ramsey, Switzer & Layton, 1968).

Streptococcal diseases of the pig of particular relevance to the tonsils are the conditions of cervical (jowl) abscesses caused by Group E streptococci and of meningitis and arthritis in piglets by Group D organisms. The pathogenesis of the latter condition is investigated in this thesis. Both these groups have been recovered from the pharyngeal and nasal regions in cases of natural disease, have been shown to be pathogenic following their inoculation into this area, and are therefore considered in greater detail.

Group E streptococci and swine cervical abscesses (streptococcal lymphadenitis), responsible for a significant economic loss through condemnation of meat at slaughter, have been extensively studied by American workers. In endemic areas a morbidity rate approaching 100% was noted by Collier (1954). The abscesses were most frequently observed in pigs between weaning and marketing age, involving the submandibular lymph nodes, the cervical chain of lymph nodes, and the retro-pharyngeal and parotid lymph nodes occasionally. Group E were consistently cultured from these abscesses, in the pus of which small Gram positive cocci were observed. In a herd with a history of cervical abscesses over a three year period, Group E streptococci

were isolated from clinical cases (Snoeyenbos, Bachman & Wilson, 1952). Cultural examination of abscesses, frequently inapparent clinically but detected at the abattoir, yielded Group E streptococci from the majority of such lesions (Collier, 1956a), suggesting that organisms of this group merited consideration in the aetiology of this condition in swine. Typical cervical abscesses were experimentally established through contamination of food and water with Group E streptococci, and by intra-nasal or intra-pharyngeal instillation of these organisms (Collier, 1956b; Gouge, Brown & Elliott, 1957). The pharyngeal mucus and tonsillar tissue frequently yielded the Group E streptococci as occasionally also did the nasal mucus (Collier, 1956b). Although he observed congestion of the submandibular lymph nodes by 48 hours post-infection, Collier was unable to recover the Group E streptococci until 7 days post-infection when the lymph nodes were turgid, oedematous and presented miliary abscesses. A further study of the Group E organisms from streptococcal lymphadenitis showed that the majority of strains isolated belonged to one type within the group - Type IV, that they were highly host-specific for pigs and that only rarely did they affect sites other than the cervical region (Diebel, Yao, Jacobs & Niven, 1964; Yao, Jacobs, Diebel & Niven, 1964).

Investigating clinical diagnostic methods, Schuman and Wood (1966) isolated from tonsillar cultures strains of Group E streptococci identical to those fed. Swine experimentally infected developed abscesses mainly in the submandibular and retro-pharyngeal nodes, with a lesser number occurring in the cervical nodes, and occasionally in the tonsils (Boehm, 1969; Armstrong, Boehm & Ellis, 1970). These latter workers did not observe any significant difference between the nasal and oral routes of infection, obtaining

approximately 100% morbidity with each. The results also indicated that peak susceptibility occurred in animals between the ages of 9 weeks and 5 - 6 months, correlating with Collier's observations (1954). Macroscopic and cultural examinations on lymph nodes, tonsils and spleens from experimentally infected pigs firmly established the relationship of Group E streptococci to the induced abscesses: the organisms were recovered from every lesion while approximately half the grossly normal lymph nodes, spleens and tonsils also yielded streptococci serologically and morphologically identical to those administered (Armstrong & Ellis, 1971).

As a disease entity, streptococcal cervical abscesses are less important in Europe, whereas arthritis and meningitis in baby pigs infected with Group D streptococci is of greater interest. In the acute phase of the disease piglets, usually between the ages of 2 - 6 weeks, become pyrexia with accompanying anorexia and depression. If this phase is survived, either endometritis and meningitis with ataxia and muscular tremors, or arthritis with tenderness and swelling of the joints, or both, may develop. During bacteraemia, positive cultures of Group D streptococci are obtained from the visceral organs but in the later stages of the disease only from the brain and joints. The incidence is very variable between herds, and between individual litters within any particular herd; no seasonal, housing or managemental factors are thought to be involved (Blood & Henderson, 1968).

The first report from Britain of streptococcal meningitis and arthritis was that of Field, Buntain and Done (1954), based on observations made during a number of outbreaks in East Anglia over the previous years. Streptococcal infections of the central nervous

system had however already been reported from the continent of Europe and North America; meningitis had been experimentally reproduced by intra-venous or intra-cerebral inoculation of streptococci isolated from the natural disease (Jansen & van Dorseen, 1951). Field et al (1954) likewise found streptococci from clinical cases to be pathogenic for piglets if administered intra-venously or sub-durally but completely non-pathogenic for laboratory animals. They were unable to assign these streptococci to any of the then recognised Lancefield's groups. Morphologically they were Gram-positive organisms, frequently occurring in pairs or else singly; beta-haemolysis was evident when they were grown on horse blood agar; and growth in nutrient broth was enhanced by the addition of horse serum, producing an even turbidity.

New Lancefield's Groups, R, S and T, were created when streptococci from sporadic outbreaks of disease in pigs and piglets in Holland could not be fitted into the existing group classification system (de Moor, 1963a & b). The Group S organisms were found to closely resemble biochemically the streptococci isolated by Field et al (1954), to be recoverable in disease of baby pigs only and to occur in association with a clinical disease corresponding to that described in the English outbreaks.

Studies of the streptococci from arthritis and meningitis were undertaken by Elliott (1966) whose work identified the organisms with Lancefield's Group D, revealed those streptococci isolated by Field et al (1954) and by de Moor (1963a & b) to be serologically identical and to belong also to Group D. Elliott considered that the isolates he examined belonged to a single serotype within the group, that the type specific antigen was present in the capsule and that it was of a

polysaccharide nature. He suggested that the organism be designated Streptococcus suis - Capsular Type I, a new subgroup of Group D. Epidemiological studies and experimental production of streptococcal disease in baby pigs followed: throat swabs from clinically affected piglets and their healthy littermates frequently yielded Strep. suis; intra-nasal or intra-pharyngeal aerosol inoculation of these organisms produced clinical disease in piglets (Elliott, Alexander & Thomas, 1966). Two 21 day old animals failed to succumb although the natural condition may occur up to 6 weeks of age. Nasal swabs from 7% of sows examined in one herd, and one throat swab were positive for Strep. suis, cultures of which proved pathogenic for baby pigs. Serum from convalescent animals was shown to passively confer protection against Strep. suis within the susceptible age group. These workers observed the early onset of bacteraemia in the experimental disease and in surviving animals, the secondary involvement of joints and meninges following within a few days.

The observations of Field et al. (1954) and the data of Elliott et al. (1966) show that the disease is confined to pigs younger than 6 weeks of age and infection can be induced only in the early post-natal period - a stage when piglets are not immunologically competent to all antigens (Binns, 1968). Adult pig serum was shown to completely inhibit the in vitro growth of Strep. suis and the organisms were phagocytosed by the polymorphonuclear leucocytes in the serum; the addition of such serum to that of piglets conferred the ability to inhibit in vitro growth of Strep. suis; and transfer of adult serum passively protected piglets from experimental infection (Agarwal, Elliott & Lachmann, 1969). No growth inhibition of the pathogenic capsulated strain of Strep. suis was demonstrable with

serum from approximately 40% of conventionally reared piglets and from a higher percentage of gnotobiotic animals. The properties of growth inhibition and leucocyte phagocytosis of Strep. suis develop within 5 - 8 weeks of age, and piglets reared in a normal environment become resistant to infection by 5 - 6 weeks of age.

The component in serum conferring the bacteriocidal property is a class M immunoglobulin acting specifically against the capsular polysaccharide of Strep. suis, although this could not be demonstrated by usual serological techniques (Agarwal et al. 1969). Agarwal et al. (1969) suggested that this IgM might arise from inapparent infections in early life since Elliott et al. (1966) isolated Strep. suis from the throats of many apparently healthy piglets within infected litters.

Employing Strep. suis to study the pathogenesis of infection in the respiratory tract of young pigs, Alexander (1969) was unable to demonstrate that the alveoli of the lung formed a major site of entry of bacteria leading to systemic infection. Delayed onset of illness was observed in those piglets infected with a fine aerosol spray of organisms and housed in a dry atmosphere, compared with a rapidly-developed septicaemia in those infected using a syringe and maintained under very humid conditions. No firm conclusions can be drawn from this experiment as appropriate controls were omitted.

Strep. suis and Group D streptococci in general differ from other Lancefield's groups in that the type antigen or type-specific substances, polysaccharides, are located in the cell wall and the group-specific substance is dextran-like in its chemical composition (Elliott, 1960). The group specificity is associated in part at least with the presence of technoic acid (Elliott, 1962; Wicken, Elliott & Baddiley, 1963) which is not so superficially situated in

the cell wall as are the carbohydrate group-specific antigens of other Lancefield's groups (Slade & Slamp, 1962; Wicken et al., 1963).

That clinical disease is reproducible by oral exposure to Group D and E organisms suggests that the oro-pharyngeal area is a major site of bacterial lodgement and invasion. It is against this background that the role of the palatine tonsils of the pig in the pathogenesis of Group D streptococcal infection in baby pigs has been studied.

#### Plan of work

The work to be described falls into four main sections. The first (Chapter III) deals with the ontogeny of the porcine palatine tonsils, a study designed to provide a concept of normality against which to interpret later experimental observations. Next, vital dyes were employed to study lymphatic pathways associated with the tonsils, with particular emphasis on the question of an afferent route to the tonsillar lymphoid tissue (Chapters IV & V). The third area of study was concerned with the pathogenesis of disease following oral administration of Streptococcus suis, in an attempt to define the role of tonsillar involvement (Chapters VI, VII & VIII). Finally, cellular and serological responses to infection with Strept. suis were briefly investigated (Chapter IX).

General CHAPTER II Methods

Source and Management of Pigs

PLAN OF WORK

The sources of pigs and tonsils for the ontogenetic study are detailed in Chapter III.

AND

GENERAL MATERIALS AND METHODS

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Anesthesia was performed by intravenous injection of pentobarbitone sodium (40mg per ml) and exsanguination via the anterior vena cava, except where otherwise stated.

Autopsy Procedure

To permit macroscopic examination of the palatine tonsils, the

## General Materials and Methods

### Source and Management of Pigs

The sources of pigs and tonsils for the ontogenetic study are detailed in Chapter III.

Experimental animals were from two sources. For the non-infective studies and for the Strep. suis studies of Chapter IX the animals used were discards from a research institute and were housed together in loose boxes, bedded with straw and fed a conventional pelleted diet with water ad lib. For the remainder of the infective studies, piglets were obtained from a closed minimal disease herd (Betts, Lamont & Littlewort, 1960) maintained at the Veterinary Field Station\*. These were kept in individual straw bale pens which for the very young were provided with an infra-red bulb heater (Fig.II.1). Infected and non-infected control piglets were housed within separate loose boxes. The piglets of ten days of age were fed a pelleted diet (Sowlac 21 - BOCM) and water ad lib; the older piglets of six weeks of age similarly received a pelleted diet (Piglet Creep Feed Pellets - BOCM) and water.

### Euthanasia of Pigs

Euthanasia was performed by intravenous injection of pentobarbitone sodium (200mg per ml) and exsanguination via the anterior vena cava, except where otherwise stated.

### Autopsy Procedure

To permit macroscopic examination of the palatine tonsils, the

\*Royal (Dick) School of Veterinary Studies, Edinburgh University.

submandibular, parotid and retropharyngeal lymph nodes, each carcass was placed in dorsal recumbency, a midline incision made from the symphysis of the mandible to the manubrium of the sternum and the skin reflected laterally. The submandibular lymph nodes (Fig.II.2) were readily located at the caudoventral border of the mandible in relation to the border of the mandibular salivary glands. Partially covered by the parotid salivary glands, the parotid lymph nodes were situated at the dorsal posterior border of the masseter muscles, ventral to the tempero-mandibular joint. The retropharyngeal nodes (Fig.II.3) were located on the dorso-lateral wall of the pharynx. Following exposure of these nodes by dissection, the larynx was incised between the arytenoid and epiglottic cartilages and the trachea reflected caudally. After eversion of the epiglottis from the isthmus faucium, incisions through the hyoid bone, root of the tongue and the frenulum permitted excision of the tongue. This exposed the soft palate in the oral surface of which the palatine tonsils are situated (Fig.II.4).

The palatine tonsils and the lymph nodes were first examined macroscopically in situ, then excised. The tonsils were readily dissected in their entirety from the underlying musculature of the soft palate. Following macroscopic examination of the surface and substance of the tonsils and nodes, blocks of these tissues were placed in fixative for processing for histological examination.

#### Fixation and Processing

All blocks were fixed in 10% formol saline, processed routinely, embedded in paraffin wax and sections cut at 5 $\mu$ .

## Histological Stains

Sections from all blocks were routinely stained with alum haematoxylin (Cole, 1943) and eosin (H & E) for initial histological examination. Special stains were employed to study specific features: silver impregnation (Slidders, Frazer & Lendrum, 1958), counterstained with neutral red, to illustrate reticulin; Masson's trichrome (1929) for connective tissue; Gram's stain (1884) for bacteria, modified for paraffin sections (Kopeloff & Beerman); and a methyl-green/pyronin stain (Jordan & Baker, 1955) for pyroninophilic cells, particularly plasma cells, modified to using 2% solutions of methyl-green and pyronin with formol-saline-fixed sections.

## Fixation, Processing and Staining for Electron-Microscopy

Blocks, 2 x 1 x 1mm approximately, were selected for processing for electron-microscopic examination. Fixation was carried out in phosphate-buffered 10% formalin pH 7.2 for  $\frac{1}{2}$  hour, followed by  $1\frac{1}{2}$  hours in phosphate-buffered 1% osmium tetroxide pH 7.2, the blocks processed through graded ethanols and propylene oxide and embedded in Araldite.

Thin sections were cut at  $1\mu$  on a Porter-Blum ultramicrotome using glass knives, stained with 2% thionine/0.5% toluidine blue in 1% borax at 60°C and examined by light microscopy to select areas for electron-microscopic study. Ultrathin sections (500 - 1000Å), silver to pale gold in colour, were cut, mounted on copper grids and stained as follows:

lead citrate (Reynolds, 1963)

2 minutes

0.02N sodium hydroxide	20 seconds
distilled water	10 seconds
uranyl acetate (sat. soln. in 50% ethanol)	10 minutes
50% ethanol	20 seconds

All grids were examined at 50KV on an AEI-EM6B electron-microscope.

Electron-micrographs were taken on Ilford EM4 photographic plates.

### Bacteriological Sampling Procedures

Tonsillar swabs and blood samples were taken from the live animal during the course of the streptococcal experiments. Sterile cotton swabs were rotated over the mucosal surface of the soft palate of restrained unanaesthetised pigs. Blood was sampled from the anterior vena cava (Carle & Dewhurst, 1942), employing sterile evacuated 5ml tubes (Vacutainer - Becton, Dickinson). Following filtration (0.3µ filter - Millipore), heparin (Pularin) was added at 250 units per tube for a 2ml sample.

At autopsy, tissues and body fluids were culturally examined. Surfaces were seared with a hot spatula, the parenchyma and fluids sampled with a sterile pipette and inoculated on to media for incubation. The sera were harvested on day 15 from the

### Bacteriological Media

- Horse blood agar (HBA): Blood Agar Base No. 2 (Oxoid CM271) with 10% horse blood.
- Edwards medium: Edwards (1933) thallos acetate medium modified (Oxoid CM27).

Horse serum broth (HSB): 5% horse serum, inactivated at 56°C for 30 minutes, in Hartley's ox heart digest broth (Cruickshank, 1965).

#### Stock Cultures of Streptococcus suis

A dried culture of a recently isolated strain of Strep. suis (PM34)\*, from a 2-week-old piglet with meningitis, was reconstituted and plated on HBA. After aerobic incubation for 18 hours at 37°C, this plate culture was used to inoculate a number of HS broths which in turn were incubated at 37°C for 18 hours; 17% glycerol was added to the cultures and these were stored in 5ml aliquots at -70°C.

#### Production of Antisera to Strep. suis

Antiserum to Strep. suis was produced according to Elliott's method (1960). By the same regime a second antiserum was obtained in response to administration of live antigen: a 24 hour 10ml HSB culture of Strep. suis was deposited by centrifugation and resuspended in 1ml phosphate-buffered saline (PBS) pH7.2, 0.01M for immediate injection. The sera were harvested on day 16 from the initial dose and the "live" and "formalised" antisera designated PM34/A and PM34/B respectively.

The sera were tested for streptococcal Group D activity and cross-reactivity with Groups C and L streptococci, employing the hydrochloric acid extracted antigens (see below) of Strep. faecalis (Group D), Strep. zooepidemicus (Group C) and a Group L streptococcus in a capillary precipitin test, modified after Lancefield (1938). Group D activity was present in these sera but reactions were less

\*Kindly supplied by Dr. S. D. Elliott, Department of Pathology, University of Cambridge.

than with the homologous antigen; no cross-reactions were obtained with the Group C and L organisms. When tested against the homologous antigen, Strep. suis, the PM34/A serum gave a stronger precipitin reaction than the PM34/B and was therefore used for all subsequent precipitin tests. Although these tests indicated that the antisera to Strep. suis were mainly type-specific, absorption of the sera with Strep. faecalis and Strep. zooepidemicus was undertaken to eliminate any possible non-specificity in their reactions.

Absorption of the PM34/A antiserum was carried out as follows: 150ml and 50ml of 18 hour glucose broth cultures of Strep. faecalis and Strep. zooepidemicus respectively were deposited by centrifugation, formalised in PBS pH 7.2, 0.1M, with 0.2% formalin for  $\frac{1}{2}$  hour at room temperature and washed twice in PBS pH 7.2, 0.1M. 2ml PM34/A serum was added to the combined deposit of the cultures and shaken for 1 hour at room temperature. Following centrifugation, the resultant serum gave no reaction when tested against the hydrochloric acid extracted antigen of the Group D streptococcus, Strep. faecalis, which had reacted previously with the unabsorbed serum.

#### Isolation of Strep. suis

All samples were first inoculated on to HBA plates, and, where gross contamination of the sample was unavoidable, e. g. tonsillar swabs, also plates of Edwards medium, selective for streptococci. Strep. suis is not readily identifiable from other porcine tonsillar streptococci on the basis of colonial morphological characteristics or haemolytic properties. Following incubation at 37°C for 18 - 24 hours, colonies resembling Strep. suis (Field et al., 1954) were

subcultured to HBA and incubated for a further 24 hours. These isolates were inoculated into HSB and the resultant overnight growth used as a source of antigen in the precipitin tests. Blood samples were initially inoculated on to HBA and also into HSB: 0.2ml were spread over the surface of a well-dried HBA plate and 1ml added to 10ml HSB. The broths, examined daily for turbidity, were subcultured to HBA when turbid and when negative were discarded after incubation for 7 days. Suspect colonies on HBA were subcultured as above.

#### Identification of Strep. suis

Strep. suis produces an even turbidity of the medium when incubated in HSB (Field et al., 1954) and this feature was employed to select isolates for serological testing from amongst those colonies originally subcultured. Antigen was prepared by a modification of Lancefield's hot hydrochloric acid extraction method (1933). The deposits from 5ml 18 - 24 hour HSB cultures were resuspended in 0.5ml N/20 hydrochloric acid in 0.85% saline, boiled for 15 minutes in a water bath, and then rapidly cooled. Following neutralisation with N/1 sodium hydroxide using a few drops of 0.02% phenol red as an indicator, the suspensions were centrifuged and the supernatant fluids tested for Strep. suis antigen. A modified method of Lancefield's microprecipitin technique (1938) was employed to test the suspect antigens: the absorbed MM34/A serum was taken up into a capillary tube and the test antigen layered above. A positive reaction, specifically indicative of Strep. suis in the original sample, was demonstrated by the rapid formation (within 30 seconds) of a dense white precipitate at the interface between the

serum and antigen (Fig.II.5). The reaction was clear cut and during the experiments only a very occasional culture gave a slow trace reaction (Appendix I). Further examination, however, revealed these isolates to be unrelated to Strep. suis as absorption of the PM34/A serum with them removed cross-reacting antibody whilst specific activity against Strep. suis was retained.

#### Fixation and Processing for Immunofluorescent Microscopy

Blocks of tissue 5 x 5 x 3mm were snap-frozen at  $-160^{\circ}\text{C}$  in a freezing mixture of isopentane and liquid nitrogen (Maxwell, Ward & Nairn, 1966) and stored in liquid nitrogen. Sections were cut at  $8\mu$  on a Pearse-Slee cryostat operating at  $-20^{\circ}\text{C}$ , transferred by thawing to chemically clean microscope slides and air-dried.

Fixation was carried out in cold acetone at  $4^{\circ}\text{C}$  for 15 minutes and the sections allowed to dry overnight at  $4^{\circ}\text{C}$ . Parallel sections were cut for histological examination and stained with H & E, Gram and where required methyl-green/pyronin. These were fixed respectively in acetic alcohol formaldehyde (Bancroft, 1967) for  $\frac{1}{2}$  minute, cold acetone at  $4^{\circ}\text{C}$  for 15 minutes and absolute alcohol for 2 minutes.

#### Antisera for Immunofluorescent Microscopy

The PM34/B antiserum produced the brighter fluorescence when the two antisera PM34/A and PM34/B were tested in an indirect "sandwich" stain on an acetone-fixed smear of Strep. suis. It was therefore employed in all immunofluorescent procedures. The gamma-globulin fraction of the PM34/B serum, precipitated with two-thirds volume of saturated ammonium sulphate (Nairn, 1969), was absorbed

with the formalised deposits from 100ml glucose broth cultures of Strep. faecalis and Strep. zooepidemicus as for PM34/A above. The resultant supernatant was filtered through a 0.3 $\mu$  filter (Millipore) and stored at -20°C.

Goat anti-rabbit gamma-globulin (GAR)\*, conjugated with lissamine rhodamine B 200 (RB 200) and fluorescein iso-thiocyanate (FITC), were similarly absorbed with the formalised deposits from 50ml glucose broth cultures of Strep. faecalis and Strep. zooepidemicus and with acetone-dried pig's liver (100mg per ml). Thiomersal was added at 1 in 10,000 and the globulin stored at -20°C.

Staining Technique for Immunofluorescent Microscopy

All staining was carried out at room temperature and, to maintain a moist atmosphere, the slides were contained within a shallow covered tray. An indirect "sandwich" staining technique (Nairn, 1969) was employed as follows:

Section or smear	+ PM34/B	30 minutes
"	+ PBS pH7.2, 0.01M	2 x 5 minutes
"	+ GAR - RB 200 or - FITC	30 minutes
"	+ PBS pH7.2, 0.01M	2 x 5 minutes.

Sections were mounted immediately in 10% glycerol/90% PBS with a thin coverslip or alternatively rinsed briefly with distilled water and allowed to dry prior to mounting.

Acetone-fixed smears of Strep. faecalis and Strep. zooepidemicus stained by the absorbed reagents failed to demonstrate fluorescence, indicating the specificity of the method and removal of cross-reacting Group D activity which was present in the original serum. Initially, a number of control slides were set up in parallel with the positive

\*Kindly supplied by Dr. G. H. K. Lawson, Department of Veterinary Pathology, Edinburgh University.

staining procedure detailed above: III

Positive control: acetone-fixed smear of Strep. suis

SMIERS ON THE stained as above. PALATINE TONSILS

Negative control: non-infected tissue stained as above.

Negative control: non-immune rabbit serum substituted  
for PM34/B.

A wealth of documented information exists on anatomical and  
Negative control: tissue stained with GAR conjugate  
developmental aspects of the palatine tonsils of the pig, a  
only.

deficiency pointed out by the Russian worker Kutilev (1956) and by  
Routinely it was found sufficient to employ only the negative control  
Hans (1968). Post-natal development in particular has been a  
substituting non-immune serum for PM34/B gamma-globulin.

regarded field of study. The histology of the adult porcine  
Sections were examined by blue light on a Wild fluorescent  
tonsil has been described (Levin, 1950) and the foetal development  
microscope fitted with an HB200 mercury bulb. Photomicrographs were  
studied by Levin (1950) and Hans (1968). Age changes in the  
taken on Ektachrome High Speed Daylight film, ASA160, and Ilford HP4,  
palatine tonsils of man are recorded and it is generally agreed  
ASA400, to give colour transparencies and black and white prints  
that they undergo an initial period of hypertrophy during childhood  
respectively. Following examination, sections were washed overnight  
and adolescence, followed by involution during adulthood (see  
in water and stained with Gram for histological examination.  
Chapter I).

The palatine tonsils are classified as part of the lympho-  
reticular system (Trautmann & Flebiger, 1952) within which they  
belong, together with the thymus, other alimentary lymphoid tissue  
and the avian Bursa of Fabricius, to the group of lympho-epithelial  
organs. If these, the mammalian thymus is considered to perform a  
"select" role, seeding immunologically competent lymphocytes to the  
spleen and spleen, while the function of the other lympho-  
epithelial tissues remains to be defined. The character of the  
palatine tonsils therefore is still an open question (Hansal & Ada,  
1971) although one school now believes that these organs will prove  
to belong to the "peripheral" compartment of the lymphoid system  
(Good, 1967). Morphological ontogenetic studies alone cannot

STUDIES ON THE ONTOGENY OF THE PALATINE TONSILSIntroduction

A dearth of documented information exists on anatomical and developmental aspects of the palatine tonsils of the pig, a deficiency pointed out by the Russian worker Kutilov (1956) and by Binns (1968). Post-natal development in particular has been a neglected field of study. The histology of the adult porcine tonsil has been described (Levin, 1930) and the foetal development studied by Levin (1930) and Binns (1968). Age changes in the palatine tonsils of man are recorded and it is generally agreed that they undergo an initial period of hypertrophy during childhood and adolescence, followed by involution during adulthood (see Chapter I).

The palatine tonsils are classified as part of the lympho-reticular system (Trautmann & Fiebiger, 1952) within which they belong, together with the thymus, other alimentary lymphoid tissue and the avian Bursa of Fabricius, to the group of lympho-epithelial organs. Of these, the mammalian thymus is considered to perform a "central" role, seeding immunologically competent lymphocytes to the lymph nodes and spleen, while the function of the other lympho-epithelial tissues remains to be defined. The character of the palatine tonsils therefore is still an open question (Nossal & Ada, 1971) although one school now believes that these organs will prove to belong to the "peripheral" compartment of the lymphoid system (Good, 1967). Morphological ontogenetic studies alone cannot

supply the answer to this question, but comparisons made on this basis may provide useful pointers to the place occupied by these structures. In the foetal pig, the thymus becomes lymphoid in character well in advance of all the other lympho-reticular tissues (Pestana, Hallenbeck & Shorter, 1965; Binns, 1968) while lymphocytic populations, although sparse, are present in the spleen and lymph nodes before the palatine tonsils and alimentary lymphoid tissue (Binns, 1968).

The tonsils of the mature foetus present a light diffuse lymphocytic population together with distinct primary follicle development (Levin, 1930; Binns, 1968). Germinal centres and plasma cells, features of the adult tonsil (Bloom & Fawcett, 1968), therefore appear in post-natal life, a period in which tonsillar development in the pig has not been recorded. Since function is presumably inherent in the normal morphological components of the tissue, knowledge of ontogenetic events is of signal importance to any investigation into the tonsillar role, particularly involving young animals. To this end, therefore, the following histological observations on the development of the porcine tonsils were made.

A number of local herds, the minimal disease pigs from the herd maintained at the Veterinary Field Station, the autopsy material in the Department of Veterinary Pathology, and the germ-free and dibiotic material from the Unilever Research Laboratory\* (A pathogenic strain of *Escherichia coli* had been administered producing a dibiotic state three days prior to the collection of tonsillar material at autopsy following euthanasia).

The tonsils were fixed, processed and sectioned as described in Chapter II. Sections from all tonsils were stained with H & E.

\* Kindly made available by Mr. R. Kenworthy, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford

Materials and Methods

Palatine tonsils from pigs of different ages were examined. They ranged from late foetal to aged and were from five different source categories: conventional, minimal disease, germ-free, dibiotic and autopsied animals (Table III.1). The usage of terms is as follows:

- "conventional" : healthy animals from ordinary commercial-type herds.
- "minimal disease" : animals in herds established from pathogen-free, colostrum-deprived pigs, and kept away from possible infection as far as practicable (Betts et al., 1960).
- "dibiotic" : initially germ-free animals infected with a single known species of micro-organism (Kenworthy, 1970).

The foetuses were collected from the Edinburgh Corporation Abattoir, Gorgie, and their ages estimated on crown-rump measurements (Pestana et al., 1965). The tonsils of conventional pigs were obtained from a number of local herds, the minimal disease pigs from the herd maintained at the Veterinary Field Station, the autopsy material in the Department of Veterinary Pathology, and the germ-free and dibiotic material from the Unilever Research Laboratory\*. (A pathogenic strain of Escherichia coli had been administered producing a dibiotic state three days prior to the collection of tonsillar material at autopsy following euthanasia).

The tonsils were fixed, processed and sectioned as described in Chapter II. Sections from all tonsils were stained with H & E ,

\* Kindly made available by Mr. R. Kenworthy, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

Masson's trichrome, methyl-green/pyronin and impregnated with silver for examination by light microscopy.

Histological observations on the ontogeny of the palatine tonsils from late gestation up to twelve years of age are recorded below under the various age groups.

### Age 12-18

The simple unbranched tonsillar crypts were lined by deep stratified epithelium; reteculatation and infiltration with mononuclear cells were apparent on a limited scale, never reaching the basal epithelial border. Distinct follicular development was noted (Fig. III.1), particularly in relation to the tips of the short crypts, creating lymphocytic cups around them (Fig. III.2). The remainder of the tonsillar area, the interfollicular areas, were only very lightly populated (Fig. III.3) with cells of the lymphocytic series and large, pale-stained cells, probably undifferentiated mesenchymal cells, supported on a well-developed reticulum network. No plasma cells were present.

### Age 19-25

Branching of occasional tonsillar crypts was evident. Reteculatation of the stratified crypt epithelium noted was very limited in extent. Occasional mononuclear cells were noted in these areas. Lymphocytic follicles had developed and were generally similar in appearance, cellular density and distribution to those described above. The interfollicular areas showed a sparse population of lymphocytes and the larger pale-stained mesenchymal cells noted above. Although no plasma cells

Observations

Histological observations on the ontogeny of the palatine tonsils from late gestation up to twelve years of age are recorded below under the various age groups.

Late Foetal

The simple unbranched tonsillar crypts were lined by deep stratified epithelium; reticulation and infiltration with mononuclear cells were apparent on a limited scale, never reaching the luminal epithelial border. Distinct follicular development was noted (Fig.III.1), particularly in relation to the tips of the short crypts, creating lymphocytic cups around these (Fig.III.2). The remainder of the tonsillar area, the interfollicular areas, were only very lightly populated (Fig.III.3) with cells of the lymphocytic series and large, pale-nucleated cells, probably undifferentiated mesenchymal cells, supported on a well-developed reticulin network. No plasma cells were present.

Full-term Foetal

Rudimentary branching of occasional tonsillar crypts was evident (Fig.III.4). Reticulation of the stratified crypt epithelium noted at this age was very limited in extent. Occasional mononuclear cells infiltrated these areas. Lymphocytic follicles had developed and were essentially similar in appearance, cellular density and distribution to those described above. The interfollicular areas presented a dense population of lymphocytes and the larger paler-staining mesenchymal cells noted above. Although no plasma cells

were observed, occasional large pyroninophilic cells were scattered throughout the interfollicular tissue. The connective tissue stroma of the tonsillar parenchyma was light. The vascular elements were situated within relatively acellular connective tissue areas on the aboral aspect, often occurring as indentations into the lymphoid tissue between crypts.

#### Neo-nate (<24 Hours Old)

Isolated desquamated epithelial cells were a feature of some crypt lumina. Rudimentary branching of the crypts was noted but no reticulation or cellular infiltration of the deep stratified epithelium was observed. Lymphocytic follicular development was variable, observed only in one of the three tonsils examined. Where present, they were located around the crypts as noted previously. The population of the interfollicular areas showed a slight increase in density compared with the foetal tonsils (Fig.III.5). The reticulin framework was well-developed and when stained specifically follicle formation was confirmed. Plasma cells or other pyroninophilic cells were absent.

#### 1 Week Old

Increased numbers of desquamated epithelial cells were contained in the lumina of the crypts which again showed primitive branching. Very limited areas of reticulation of the stratified epithelium with only an occasional infiltrating mononuclear cell were observed. Lymphocytic follicles were rare in the tonsils of the three piglets examined and there was no increase in density of the lymphocytic and

mesenchymal cell populations of the interfollicular areas (Fig.III.6). Early formation of trabeculae within the tonsillar substance was indicated by narrow bands of connective tissue originating from the aboral aspect, where the capsule had not yet developed.

#### 1 Month Old

Luminal cellular detritus was an extremely variable feature throughout any individual tonsil, sometimes abundant but often negligible: this comprised desquamated epithelial cells, mononuclear cells and frequently nuclear and cellular fragments of undetermined origin. Similarly, the epithelium presented marked variation regarding the degree of reticulation and cellular infiltration. Amongst the mononuclear cells infiltrating the epithelium, occasional plasma cells were noted (Fig.III.7) and also in the immediate subepithelial zone of the crypts; polymorphonuclear leucocytes were rarely observed although the luminal debris on occasion could resemble them in a degenerate form.

Follicular development was marked in all tonsils at this age, the majority being germinal centres (Fig.III.8) rather than primary lymphocytic follicles. These centres frequently exhibited a distinct pyroninophilia, sometimes over-all, sometimes peripheral (Fig.III.9), containing in the main large pale cells morphologically resembling macrophages, with vesicular nuclei and abundant cytoplasm, large lymphocytes, lymphoblasts and variable amounts of cellular fragments. The lymphocytes were generally situated more peripherally with the macrophages in the central zone but this was not an invariable feature. A few mitoses were regularly encountered. The majority of germinal centres were large and where

in close proximity to crypts, the crypt-orientated pole sometimes possessed a less well defined border, seeming to merge with the sub-epithelial zone (Fig.III.8). The interfollicular tissue was moderately to heavily populated with small lymphocytes admixed with macrophages. Plasma cells were rare while large pyroninophilic cells occurred more commonly. Trabeculae were distinct, particularly when stained with Masson's trichrome and for reticulin fibres, subdividing the interfollicular tissue from the aboral aspect into crypt units.

1 Month Old: Germ-free/Dibiotic

The tonsils of the germ-free pig and the three dibiotes are described together since the 3-day infection of the dibiotes with E. coli had not perceptibly altered their histological appearance. Histologically, the tonsils were essentially the same as those of conventionally-reared piglets of a similar age, described above.

Variable amounts of luminal detritus, mononuclear cells as well as desquamated epithelial cells, were present. Reticulation and cellular infiltration of the deep stratified epithelium occurred (Fig.III.10), occasionally extensive within an individual crypt. Plasma cells were to be found amongst the infiltrating mononuclear cells (Fig.III.11). Germinal centres were numerous and frequently large (Fig.III.12), but few primary lymphocytic follicles were observed. Within the germinal centres, which were densely cellular in appearance, numerous macrophages, large lymphocytes, lymphoblasts and cellular fragments were present (Fig.III.13). Mitotic figures were rarely more numerous than two or three per centre. The inter-follicular areas were less heavily populated than for the

conventional piglets but contained a similar population of epithelium, lymphocytes, macrophages and occasional large pyroninophilic cells scattered throughout the interfollicular tissue. A distinct

## 2 Months

of the periphery of a variable number of germinal centres was apparent, usually associated with a dense population of luminal detritus, comprising epithelial and mononuclear cells, including plasma cells, was contained within a variable number of crypts. Distinct branching of the crypts was apparent, as at one month of age. Although the degree of reticulation of the epithelium varied, it was frequently extensive with infiltration by lymphocytes and plasma cells (Fig.III.14). In areas where cellular infiltration was heavy, the basal layer of the epithelium was often obscured.

Primary lymphocytic follicles were rarely seen; germinal centres on the other hand were abundant, occasionally manifesting a crypt-orientated lymphocytic cap. The central zones where the majority of macrophages were found, were more eosinophilic in appearance than the peripheral areas with large and medium lymphocytes. Mitotic figures were a regular feature although the number per centre was variable (Fig.III.15). Cellular, often nuclear, fragments were frequently scattered throughout the centres, mainly in the central zone. The germinal centres were clearly outlined by the well-developed reticulin framework of the tonsillar parenchyma (Fig.III.16).

The interfollicular tissue was not extensive compared with the area of germinal centre activity. It was a moderately populated zone, showing lymphocytes, large pale-nucleated cells with abundant cytoplasm, morphologically resembling macrophages. Occasional mitotic figures were observed. Eosinophils were a variable feature of this area and on occasion could be numerous.

Specific staining confirmed plasma cells in the crypt epithelium, more commonly in the subepithelial zone (Fig.III.17), others scattered throughout the interfollicular tissue. A distinct pyroninophilia of the periphery of a variable number of germinal centres was apparent, usually associated with a dense population of large lymphocytes.

The trabeculae, composed of a heavy connective tissue, were well formed and carried blood vessels to and from the aboral aspect where the connective tissue elements were condensing into a distinct capsule.

The histological features of the tonsils of these three age groups did not differ significantly from that detailed above for 2 months of age.

### 3, 4 and 6 Months

The histological features of the tonsils of these three age groups did not differ significantly from that detailed above for 2 months of age.

Neutrophil polymorphonuclear leucocytes were sometimes noted within the luminal detritus, in reticulated areas of the epithelium and in the interfollicular areas. The connective tissue stroma increased steadily with age and the trabeculae developed a branched structure within the tonsillar substance.

### 10 Months

Extensive reticulation of the epithelium was apparent with in many areas heavy mononuclear cell infiltration including numerous plasma cells. Germinal centres were abundant as before but greater size variation occurred. Frequently they showed a marked decrease in cellularity, with few mitoses or cellular fragments. Pyroninophilia

of the peripheral zones of the centres was related to the degree of activity and was generally absent where the centres were depleted. Plasma cells were often noted in the subepithelial area but were scarce elsewhere.

The centres were markedly decreased in size (Fig.III.19) and the

1 Year follicular areas were only lightly populated. The connective

tissue elements were greatly increased, readily appreciated when

Epithelial reticulation and infiltration were prominent specifically stained, and the trabeculae and capsule markedly features. Germinal centres varied in size, being often small to

medium-sized in comparison with those at 2 - 6 months of age.

Peripheral lymphocytic cuffing was sometimes noted. The inter-

follicular areas were more extensive relative to that occupied by

centres (Fig.III.18). Pyroninophilia of the centres was not marked.

Plasma cells were most numerous in the subepithelial zone although a

number were scattered through the interfollicular tissue, together

with other large pyroninophilic cells. The lamina propria was less thick, in some areas

being as little as one or two cells deep, and not uncommonly the basal

2 Years layer presented a flattened "pavement" appearance (Fig.III.20).

Reticulation and mononuclear cellular infiltration of the epithelium

The crypt epithelium exhibited variable but frequently extensive

degrees of reticulation and cellular infiltration. Crypt-orientated

lymphocytic caps on the germinal centres occurred moderately

frequently. In other respects the tonsils were essentially similar

to those at one year of age.

The majority of

crypts possessed a distinct lamina propria, relatively free of or only

4 Years lightly populated with cells, which were mainly plasma cells. The

A generalised reduction in the thickness of the crypt stratified

epithelium was apparent. Reticulation was still extensive, while

cellular infiltration was often decreased. Commonly, a distinct

connective tissue layer, a crypt lamina propria, was apparent. The germinal centres were on the whole inactive, non-pyroninophilic and relatively acellular in appearance: mitotic figures, nuclear fragments and macrophages were reduced leaving mainly lymphocytes. The centres were markedly decreased in size (Fig.III.19) and the interfollicular areas were only lightly populated. The connective tissue elements were greatly increased, readily appreciated when specifically stained, and the trabeculae and capsule markedly thickened.

10 - 12 Years: Aged

In aged tonsils, a distinct atrophy of the crypt system was apparent: in one, the crypts were reduced to simple unbranched structures and the others exhibited decreased ramification. The stratified crypt epithelium in the main was less thick, in some areas being as little as one or two cells deep, and not uncommonly the basal germinal layer presented a flattened "pavement" appearance (Fig.III.20). Reticulation and mononuclear cellular infiltration of the epithelium were variable, sometimes moderately extensive, but were generally confined to the thicker areas of the epithelium (Fig.III.21). Within the tonsillar substance, the connective tissue stroma was markedly increased, often creating wide bands isolating individual crypts with their associated lymphoid tissue (Fig.III.22). The majority of crypts possessed a distinct lamina propria, relatively free of or only lightly populated with cells, which were mainly plasma cells. The tonsillar lymphoid parenchyma was as a whole reduced in amount with the interfollicular tissue greater than that of the germinal centres. These latter structures were markedly fewer in number, small, inactive

and sparsely populated, mainly with lymphocytes (Fig.III.21). The

cellular population of the interfollicular areas was light to

moderate, comprising in the main lymphocytes, with macrophages and plasma cells.

From these observations it is apparent that the palatine tonsils become lymphoid in foetal life, possessing primary lymphocytic follicles but no germinal centres before birth. During the first month, the population of lymphocytes and macrophage-like cells within the tonsillar parenchyma steadily increases; numerous active germinal centres develop; and plasma cells make their appearance.

Reticulation of the tonsillar crypt epithelium becomes more extensive, increasingly infiltrated with mononuclear cells, including plasma cells. Germinal centre activity is the predominant feature from this stage until around ten months of age when signs of involution have appeared. The histological pattern from then on is extremely variable but in general there is a reduction in size and activity of germinal centres, a marked increase in the connective tissue stroma, variable reduction in thickness, reticulation and infiltration of the crypt epithelium but plasma cells may remain relatively numerous.

In the aged tonsil, much of the lymphoid parenchyma has been replaced by connective tissue, the germinal centres are mainly inactive, small and depleted of cells and the interfollicular cellular population reduced. The tonsils of the germ-free and diphtheric piglets were essentially similar to those of conventionally-reared animals of the same age.

Levin (1950) and Rimmé (1968) both observed follicle formation and a light lymphocytic population in the tonsillar parenchyma before birth but no germinal centres. Their findings are confirmed here where the palatine tonsils were studied late in gestation; follicular development would seem however to have a variable time scale, follicles being inconsistently observed up to one week of age.

Discussion

Kutlief (1956) commented that these cells were absent in the neo-nate,

making their appearance within the first month. All tonsils, including those from germ-free and dibiotic piglets were found here to become lymphoid in foetal life, possessing primary lymphocytic follicles but no germinal centres before birth. During the first month, the population of lymphocytes and macrophage-like cells within the tonsillar parenchyma steadily increases; numerous active germinal centres develop; and plasma cells make their appearance.

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Kutilov (1956) commented that follicles were absent in the neo-nate, making their appearance within the first month. All tonsils, including those from germ-free and dibiocotic piglets, were found here to contain numerous well-developed and active germinal centres at one month and also a detectable plasma cell component. Parkinson (1951) stated that in the human tonsil plasma cells appear within three weeks of birth although germinal centres develop rather later at three to six months.

Although no branching of the tonsillar crypts was noted, three foetuses only being examined, branches may be formed from around 60 days gestation (Levin, 1930). Reticulation and lymphocytic infiltration of the crypt epithelium, both on a very limited scale, were featured in the foetal tonsils. Binns (1968) made no mention of this but lymphocytic migration into the tonsillar epithelium prior to birth has been noted in the pig by Levin (1930) and Kruml et al., (1970), in the bovine by James (1956) and in man (Parkinson, 1951; James, 1956). Migration of lymphocytes into the crypt lumina was not observed by James (1956) nor in this study.

Although direct comparisons cannot be made between the palatine tonsils of the pig and man at the same age, similarities in post-natal development are apparent. Numerous large active germinal centres were present in these porcine tonsils by one to two months of age, showing little change during the next few months. An analogy may be drawn between this period in the pig and the tonsillar hypertrophy produced by germinal centre development and activity observed in childhood and early adolescence (Minear et al., 1937; Parkinson, 1951; Brown, 1966; Tajima, 1967). Tajima (1967) also noted that the number of lymphocytes infiltrating the tonsillar

epithelium was at a maximum during this time. Correspondingly, in this study, the infiltrating cellular population became more variable from the age of ten months, having been fairly constant at a high level in the younger animals. Involution of the tonsillar lymphoid tissue, both of the germinal centres and the interfollicular tissue, followed on adolescence and puberty (Parkinson, 1951; Tajima, 1967), accompanied by gradual atrophy of the tonsillar crypt system and increased formation of fibrous tissue (Minear et al., 1937; Parkinson, 1951). These age changes were paralleled in the pig by decreased germinal centre size and activity, reduction in the population of the interfollicular tissue, marked development of the connective tissue stroma and atrophy of the crypts, and were often advanced in the very aged animal. As in man, therefore, the palatine tonsils of the pig exhibit a distinct pattern of development from birth to around the time of puberty, followed by a gradual atrophy of the tissues as a whole with advancing age.

Similar ontogenetic studies on other areas of the alimentary lympho-epithelial tissues have not been recorded although in the bovine there occurs a marked reduction in total mass of the tonsils and of the mesenteric lymph nodes in older animals (Lawson). No information is available on the development and maturation of the tonsils in the germ-free animal and since tonsillar function has yet to be explicitly defined, it is impossible to assess the significance of these changes in lymphoid tissues closely associated with innumerable diverse antigens carried in the alimentary tract.

It was exceedingly interesting to note here that at one month of age there was essentially no difference at a histological level between the palatine tonsils of germ-free and dibiocotic animals and

conventionally-reared piglets. This observation is open to two interpretations: the first that germinal centre development in the tonsils is independent of antigenic stimulation as with follicle formation in the Bursa of Fabricius (Nossal & Ada, 1971); or secondly that dietary antigens alone can stimulate development of the tonsillar lymphoid tissue in a manner similar to that occurring in conventional animals. This latter postulate is supported by the observations of Kim and Watson (1971) on the reactivity of the submaxillary and mesenteric lymph nodes of germ-free pigs to dietary antigens which they consider to provide a chronic stimulus.

It may be criticised that some autopsy material was included in this study. In no case, however, was this unsupported by examination of tonsils from healthy pigs and since histologically the two groups of material were indistinguishable, the inclusion of such material was considered valid.

The primary objective of this ontogenetic study was to determine the normal histology of the palatine tonsils for age groups likely to be used in experimental studies. It is therefore apparent from the observations recorded above that from an early age the tonsils of piglets normally present a marked degree of activity of the lymphoid tissue, assessed on development of the lymphocytic, macrophage and plasma cell populations and of the germinal centres.

It is generally held that the palatine tonsils are composed of lympho-epithelial tissue completely lacking afferent vessels as those described for lymph nodes (Wright, 1950; Schack, 1951; Parkinson, 1951; Trautman & Steigler, 1953; Lums, 1964). Kobayashi (1967) states that it has been repeatedly confirmed that the tonsil has no afferent, only afferent, lymphatic vessels.



CHAPTER IV

THE LYMPHATIC AND BLOOD VASCULAR SYSTEMS  
OF THE PALATINE TONSILS

Introduction

The palatine tonsils are part of the extensive lymphoid system which is basically concerned with the development and elaboration of the body defence mechanisms at both a humoral and cellular level.

These responses are stimulated when antigen is presented to the lymphoid tissue by an afferent route. On present evidence (see Chapter I) the mammalian thymus exerts a powerful influence on the lymphocyte-mediated (cell-bound) component of the system, but appears itself to be excluded from active participation in the response to antigenic stimuli and it lacks an afferent lymphatic system. In general terms, the spleen is the major filtering organ for disseminated antigen with the splenic artery as its afferent limb. The peripheral lymph nodes on the other hand have afferent lymphatic vessels which carry tissue fluid to the filter bed of the lymph node sinuses prior to being returned to the blood via efferent lymphatics and the thoracic duct.

It is generally held that the palatine tonsils and other lympho-epithelial tissues completely lack afferent vessels such as those described for lymph nodes (Wright, 1950; Schenck, 1950; Parkinson, 1951; Trautmann & Fiebiger 1952; Kraus, 1964). Koburg (1967) states that it has been "repeatedly confirmed that the tonsil has no afferent, only efferent, lymphatic vessels."

To my knowledge no specific experimental investigation of the afferent pathway to the palatine tonsils has been made, although certain observations have been noted. Griffiths (1937), investigating the possibility of an anatomical link between the nasal sinuses and the tonsils since sinusitis occurred more frequently in tonsillectomised children than in normal subjects, found that dyes injected into the sinuses drained only to the submandibular and cervical lymph nodes but not to the tonsils. Dyes injected intra-nasally are carried to the cervical lymph nodes but not into the tonsils; therefore no lymphatics exist between the nose and tonsils (Schlemmer, 1921). Examining the inter-relationship of the lymphatics of the lymph nodes of the head and neck of the pig, Saar and Getty (1964) traced the vessels to and from the head lymph nodes but omit any mention of drainage to the palatine tonsils - perhaps this resulted from the general assumption that these lack afferent vessels.

It has been said that the palatine tonsils act as a filtering site for the blood (Meyer, 1950) and this has been one of the theories discussed relating to tonsillar function (Koburg, 1966). There exists then the possibility that the afferent route to the palatine tonsils is primarily haematogenous, although no specialisation of the terminal vascular bed in the tonsil has been recorded or even suggested.

The situation regarding the efferent route from the palatine tonsils is much more clearly defined. Efferent lymphatic vessels originate in the tonsillar trabeculae whence they drain to the cervical nodes of the neck (Wood, 1920; Trautmann & Fiebiger, 1952). Since these efferent vessels do not take origin from

sinuses, a feature absent from tonsillar lymphoid tissue, Kraus (1964)

comments that they cannot be compared directly with the efferent

The intravital stains employed were 5% aqueous solution of lymphatics of lymph nodes. Saar and Getty (1964) demonstrated an Vital New Red (G. F. Carr Ltd., London), 1% aqueous solution of efferent route to the submandibular lymph nodes in the pig.

Trypan Blue (G. F. Carr Ltd., London) and India ink (Pelikan Black 17 - Gunther-Vagner, Germany). It was decided, since experimental evidence relating to the existence of a tonsillar afferent route is not abundant, to

investigate likely afferent pathways to the palatine tonsils in the Afferent Route Study classical manner, i.e. by the injection of vital dyes at local sites and establishment of their drainage routes, and similarly to confirm the reported tonsillar efferent drainage route. Since a functional haematogenous route to the palatine tonsils has been postulated, the tonsillar vascular network was visualised by perfusion with India ink to illustrate the absence of a vascular sinusoidal filter bed analogous to that of the spleen.

0.5ml Trypan Blue in the mucous membrane of the right nostril.

1ml Trypan Blue sprayed into the left nostril.

Fig 12: 0.5ml Vital New Red into the rostrum (right side).

0.5ml Vital New Red into the anterior hard palate (left side).

0.5ml India ink into the rostrum (left side).

0.5ml India ink into the anterior hard palate (right side).

0.5ml India ink into the buccal mucosa (right side).

Figs 11 and 12 were destroyed at 30 minutes and 4 hours

respectively after dye injection. The sites of deposition of these

Materials and Methods

The intravital stains employed were 5% aqueous solution of Vital New Red (G. T. Gurr Ltd., London), 1% aqueous solution of Trypan Blue (G. T. Gurr Ltd., London) and India ink (Pelikan Black 17 - Gunther-Wagner, Germany).

Afferent Route Study

Two pigs, both approximately 16 weeks old, were used in this experiment. Surgical anaesthesia was induced in each pig with intravenous administration of pentobarbitone sodium. Through a 2ml syringe and 23 gauge hypodermic needle, intravital dyes were deposited as follows:

- Fig A1: 1ml Vital New Red at base of right ear.  
0.5ml Trypan Blue in the mucous membrane of the right nostril.
- Fig A1: 1ml Trypan Blue sprayed into the left nostril.
- Fig A2: 0.5ml Vital New Red into the rostrum (right side).  
0.5ml Vital New Red into the anterior hard palate (left side).
- Fig A3: 0.5ml India ink into the rostrum (left side).  
0.5ml India ink into the anterior hard palate (right side).  
0.5ml India ink into the buccal mucosa (right side).

Figs A1 and A2 were destroyed at 30 minutes and 4 hours respectively after dye injection. The sites of deposition of these

Blood Vascular Study

A single pig, 14 weeks of age, was employed in this study.

intravital dyes were revealed for macroscopic examination by reflecting the skin from a midline submandibular incision to the base of the ears. The regional lymph nodes of the auricular, nasal, pharyngeal and oral areas, namely the parotid group, the dorsal superficial cervical, cervical, ventral retropharyngeal and submandibular lymph nodes and the palatine tonsils were exposed by dissection as described previously.

### Efferent Route Study

Three pigs, approximately 16 weeks old, were anaesthetised with intravenous pentobarbitone sodium and a metal gag inserted between the jaws to facilitate access to the palatine tonsils. Intravital dyes were injected into the tonsillar parenchyma through a 23 gauge hypodermic needle implanted with forceps and connected by catheter tubing to a 2ml syringe. The dyes were injected as follows:-

Fig E1: 1ml Trypan Blue deep to the oral epithelium of the right tonsil.

Fig E2: 0.25ml India ink into two sites in both the right and left tonsils.

Fig E3: 0.5ml India ink into one site in both the right and left tonsils.

Figs E1, E2 and E3 were destroyed after intervals of 15 minutes, 30 minutes and 4 hours respectively, when the palatine tonsils and regional lymph nodes were exposed routinely for macroscopic examination.

### Blood Vascular Study

A single pig, 14 weeks of age, was employed in this study. A

method of perfusion with India ink to illustrate the vascular system (Jenkinson) was adapted for the pig. General anaesthesia was induced with intravenous pentobarbitone sodium and both carotid arteries surgically exposed. These were selected for accessibility, the tonsils themselves receiving their blood supply from branches of the lingual arteries (Sisson, 1953). Following intra-arterial injection of amyl nitrate to produce vaso-dilation, a lethal dose of pentobarbitone sodium was administered and the carotid arteries were cannulated to connect with the pressure perfusion apparatus set to operate at 140mm Hg, 10mm Hg above the average porcine systolic blood pressure (Scheunert & Trautmann, 1965). Perfusion with India ink was continued until ink issued freely from the sectioned jugular veins and the skin was deeply stained. The palatine tonsils were dissected out, blocks of  $1\text{cm}^2$  in cross-section fixed in 10% formal saline with a few drops of glacial acetic acid to aid precipitation of the India ink and processed routinely to paraffin wax. Sections were stained with H & E, and impregnated with silver to demonstrate reticulin.

floor of the nasal cavity resulting in traces entering the posterior aspect of the right nasal and the oral cavities from the naso-pharynx which was diffusely light blue in colour. The palatine tonsils were completely free from Trypan Blue.

In this pig the right submandibular and retropharyngeal lymph nodes showed a faint blue colouration. Since no afferent vessels were defined, the origin could not be ascertained, although corresponding unilaterally with the right nasal mucosa into which Trypan Blue had been injected.

Fig 12:

From the rostrum, lymphatics, stained red on the right side

Results

and black on the left corresponding to Vital New Red and India ink

Afferent Route Study

Fig A1: nodes of the parotid group after crossing the dorsal border

of A stained area of irregular shape two to three centimetres in diameter was observed at the base of the right ear, corresponding to the site of injection of Vital New Red. Lymphatics containing a slim red thread of dye illustrated an afferent route to the dorsal nodes of the parotid group, these two being coloured with the dye. Although afferent lymphatics were not apparent, the dorsal superficial cervical node also received dye from this site. In addition, the first nodes of the ventral cervical chain were lightly stained.

Originating from the mucosa of the right nostril, a large blue-stained lymphatic traversed the masseter muscle to drain into the ventral node of the parotid group. No afferent lymphatics to any lymph nodes or stained lymph nodes were discerned relating to the Trypan Blue sprayed into the left nostril. Dye had spread along the floor of the nasal cavity resulting in traces entering the posterior aspect of the right nasal and the oral cavities from the naso-pharynx which was diffusely light blue in colour. The palatine tonsils were completely free from Trypan Blue.

In this pig the right submandibular and retropharyngeal lymph nodes showed a faint blue colouration. Since no afferent vessels were defined, the origin could not be ascertained, although

The right tonsil was deep blue in colour at the site of corresponding unilaterally with the right nasal mucosa into which injection. On the aboral capsule a single blue-stained lymphatic Trypan Blue had been injected.

Fig A2:

Further. Although the left tonsil presented a light blue From the rostrum, lymphatics, stained red on the right side (discolouration of the oral surface, no dye was present within its

and black on the left corresponding to Vital New Red and India ink injections respectively, demonstrated an afferent route to the ventral nodes of the parotid group after crossing the dorsal border of the masseter muscles (Fig.IV.1). (Fig.IV.3). Stained afferent

A black afferent vessel to the right submandibular lymph node (anterior portion) was evident taking origin from the India ink deposited in the buccal mucosa. On both sides, drainage from the anterior hard palate occurred to the ventral parotid nodes and to the submandibular lymph nodes, the anterior portions of the latter being stained black and red on the right and left respectively. No involvement of the palatine tonsils (Fig.IV.2) or retropharyngeal lymph nodes was visible macroscopically, nor any lymphatics observed draining to these tissues.

Examination of the palatine tonsils of both Pigs A1 and A2 therefore failed to reveal these intravital dyes in their lymphoid substances nor was there any evidence of afferent vessels to them from the areas where dyes were deposited.

These observations on the lymphatic drainage from specific areas of the head in relation to the palatine tonsils and regional lymph nodes are summarised in Table IV.1.

#### Efferent Route Study

##### Fig E1:

The right tonsil was deep blue in colour at the site of injection. On the aboral capsule a single blue-stained lymphatic could be traced to the postero-lateral margin of the tonsil but no further. Although the left tonsil presented a light blue discolouration of the oral surface, no dye was present within its

lymphoid substance or on the aboral aspect.

The posterior area of the right submandibular lymph node was densely blue in colour and the area of node immediately adjacent presented a stippled blue appearance (Fig.IV.3). Stained afferent lymphatics were not found, although the unilateral presence of dye corresponded to the deposition of Trypan Blue. The anterior portion of the retropharyngeal node contained blue dye. Blue staining of the parotid group and the first two cervical nodes was also noted.

Fig E2:

The surface of the palatine tonsils presented four irregular black foci corresponding to the injection sites of India ink and on section these extended locally through the lymphoid substance. No lymphatics were apparent on the aboral capsule although the areas of ink deposition were clearly visible.

The submandibular lymph nodes both presented a black mottling of their substance in the posterior portions of the nodes. As in Fig E1, afferent lymphatics were not stained. In the lymphoid parenchyma of the left retropharyngeal node, a light black mottled pattern was apparent. The right node, however, presented a few black foci only in the main node while a smaller accessory node was deeply stained. No India ink was present in the parotid lymph nodes.

Fig E3:

One black injection site on each tonsil was apparent and in these areas the lymphoid parenchyma was deeply stained with ink. No tonsillar lymphatics were noted on the aboral capsule nor any efferent vessels emerging beyond the capsule. The submandibular

nodes presented diffusely stained black posterior portions; a light stippled pattern of tiny black foci was observed in the substance of both retropharyngeal nodes; but as in Fig E2 no ink was contained in the parotid group.

### Blood Vascular Study

Macroscopic examination of the palatine tonsils revealed these to be diffusely stained dark grey-black in colour as were the regional lymph nodes and all other tissues of the head. No particular features relating to blood vessel distribution therefore could be discerned grossly.

Histologically the majority of vessels in the tonsillar aboral capsule were observed to be completely filled with ink. From these, branches entered into the trabeculae from which smaller vessels penetrated into and ramified within the lymphoid parenchyma, at the same time supplying the crypt epithelium (Fig.IV.4).

Terminal trabecular branches extended into and along the lamina propria of the oral mucosa, sending capillaries into the rete pegs of the epithelium. Within the tonsillar interfollicular lymphoid tissue, numerous arterioles, capillaries and venules were noted, sometimes partially encircling germinal centres into which capillaries penetrated (Fig.IV.5). Nowhere in the lymphoid tissue of the tonsils did these vascular branches terminate in sinusoidal structures or merge with the reticular framework, features which are characteristic of the splenic red pulp. This negative observation was confirmed by examination of sections silver impregnated to illustrate the reticular framework of the

tonsillar lymphoid parenchyma (Fig.IV.6).

Discussion

The observations detailed above, following the deposition of intravital dyes at a variety of head sites, failed to define an afferent system to the tonsils. The nasal region drained to the ventral parotid lymph nodes and to the submandibular and retropharyngeal nodes, the hard palate to the ventral parotid and submandibular, the buccal mucosa to the submandibular, and the auricular area to the dorsal parotid, dorsal superficial cervical and ventral cervical chain nodes. Intratonsillar injections of dye confirmed consistently the tonsillar efferent drainage to the submandibular lymph nodes, thence to the retropharyngeal, and variably to the parotid nodes. Pressure perfusion of the vascular system with India ink enabled the distribution of blood vessels within the palatine tonsils to be visualised and verified the absence of a tonsillar equivalent of the splenic red pulp with its sinusoidal blood filtering and macrophage systems.

Histologists are agreed that there are no afferent lymphatic vessels to the palatine tonsils (Trautmann & Fiebiger, 1952; Bloom & Fawcett, 1968) and this apparently has been accepted as fact by those reviewing or currently engaged in research into tonsillar function (Wright, 1950; Parkinson, 1951; Kraus, 1964; Eigler, 1965; Koburg, 1967; Surjan & Surjan, 1970). Both Schlemmer (1921) and Griffiths (1937) found that drainage did not occur to the palatine tonsils from the nasal cavity or from the nasal sinuses, but that their injected dyes drained instead into the cervical chain or to the submandibular and cervical nodes.

In this experiment, by extending the investigation into the question of tonsillar afferent lymphatics to include other sites

anatomically likely to give origin to vessels draining to the nodes. palatine tonsils, the negative observations recorded provide additional evidence against their existence. The sites of single intravital dye injection were selected on the basis of anatomical proximity to, and therefore possible lymphatic connection with, the tonsils. It appears exceedingly improbable therefore that a tonsillar afferent system, should one be present, takes the form of lymphatic vessels draining local tissues as for peripheral lymph nodes. Additionally, Saar and Getty (1964) did not comment, following their extensive study of the interrelationship of the lymphatic connections of the head lymph nodes of the pig, on drainage from any of their injection sites to the palatine tonsils. The drainage lymph nodes for the injection sites in Pigs A1 and A2 however corresponded with the findings of the above workers.

Considering next the tonsillar efferent system, this was not described by Sisson (1953) for any of the domestic animals but has been established for the pig by Saar and Getty (1964) who found that the submandibular lymph nodes received lymphatics from the tonsils following intra-tonsillar injections. These nodes drained to the cervical chain and variably to the retropharyngeal nodes. They comment that other workers have described deviations in the pattern of lymph drainage in the head and neck of the pig because of its foreshortened skeleton. In man, it has been established that the palatine (faucial) tonsils drain to the cervical lymph nodes, particularly to the large anterior node just below the angle of the jaw (Wood, 1920). In addition to the submandibular lymph nodes, in this experiment the retropharyngeal nodes of all three pigs were stained.

suggesting therefore a regular connection between these two nodes. This relationship is mentioned by Sisson (1953). Only in one pig was the parotid node involved, laterally corresponding to the single intra-tonsillar deposition of dye. This may illustrate one of the variations of lymphatic drainage from the submandibular lymph node commented on by Saar and Getty (1964) although they themselves did not confirm this particular route. Since the parotid lymph nodes drain the superficial and deep structures of the head cranial to a line drawn horizontally through the commissures of the lips (Saar & Getty, 1964), there also exists the possibility that the dye in the parotid node of the one pig drained directly from the tonsils. Such a route was neither demonstrated nor discussed by these workers.

As commented earlier, histologically there is no evidence to suggest a specialised sinusoidal filter or macrophage system associated with the tonsillar vascular bed. This has been borne out by the pressure perfusion of the dilated vascular supply to the tonsils, a procedure which illustrated a simple distribution of blood vessels to the interfollicular lymphoid tissue, the follicles and germinal centres, and the epithelium. With the exception of the vessels to the epithelial element of the tonsils, the vascular distribution otherwise closely resembled that within the cortical area of the porcine lymph node (personal observation). These observations do not, however, preclude a haematogenous tonsillar afferent route in states of widely disseminated antigen, e.g. septicaemia, when antigen may be recovered from a wide range of peripheral lymphoid tissues, but do suggest that tonsillar involvement via the haematogenous route is uncommon.

Not all workers, however, consider an afferent antigen pathway a component of tonsils. THE AFFERENT ROUTE TO in two schools of

thought regarding the THE PALATINE TONSILS of the tonsils

(see Chapter 1). If, like the thymus, the tonsils belong to that category of the lymphoid Introduction (i) "central", then the

absence of afferent lymphatics is not remarkable; but if, on the other hand, the tonsils act as peripheral lymph nodes, some form of afferent system is presumably essential to their function.

It has been frequently commented that the tonsils and other intestinal lympho-epithelial tissues are ideally situated to be readily penetrable by antigenic material from the surface and from the lumen of the digestive tract (Wright, 1950; Parkinson, 1951; Kocsard-Varo, 1964). That the palatine tonsils do not possess the

afferent lymphatic system characteristic of lymph nodes has been supported by the work described in the previous chapter. In the

absence therefore of afferent vessels, a generally held thesis is that the tonsillar crypts constitute a direct afferent route from the oral cavity to the subepithelial lymphoid tissue. The

location of the tonsils permits close contact with digesta during deglutition (Wright, 1950); the tonsillar crypts penetrate deeply into the lymphoid parenchyma (Ham, 1969); and the reticulation of

the crypt epithelium creates a spatial connection between the environment and the lymphoid tissue (Kraus, 1964) and may allow easy percolation of antigens through it from the crypt lumina

(Meyer zum Gottesberge & Koburg, 1967). Koburg (1967) remakes this point when he states that the tonsils must be regarded as undergoing constant antigenic exposure "due to the highly penetrable

epithelium". On this assumption, he applied antigens to the tonsillar oral surface in order to study antigen-induced cellular proliferation and differentiation in the tonsils of rabbits

(Koburg, 1970).

Materials and Methods

Not all workers, however, consider an afferent antigen pathway a component of tonsillar function and there remain two schools of thought regarding the possible physiological role of the tonsils (see Chapter I). If, like the thymus, the tonsils belong to that category of the lymphoid system designated "central", then the absence of afferent lymphatics is not remarkable; but if, on the other hand, the tonsils act as peripheral lymph nodes, some form of afferent system is presumably essential to their function.

The present situation regarding the postulated afferent route to the tonsillar lymphoid tissue has recently been summed up in the statement that the "tonsillar crypts seem to be suitable for antigen reception but we have no direct proof of it" (Surjan & Surjan, 1970). The experiment described here was therefore designed to examine the hypothesis of a tonsillar crypt afferent system: India ink was applied topically to, i.e. to the oral surface of, the palatine tonsils and its fate studied. The palatine and parotid lymph nodes, including any areas macroscopically positive for ink, were fixed and processed for histological examination. Sections were stained with H & E, and hematoxylin (unblued) and tartrazine, the latter having been selected to enhance perception of ink particles.

Materials and Methods

Sixteen pigs, aged 10 to 16 weeks, were used in this experiment. One of these was untreated and was killed to provide normal material for a controlled study. India ink (Pelikan Black 17 - Gunther-Wagner, Germany) was applied topically to the oral surface of the palatine tonsils of restrained unanaesthetised pigs for approximately one minute employing a fine hair brush saturated with ink. Each pig was released back into the pen but food and water were withheld for 4 hours in an attempt to reduce loss of ink during ingestion and deglutition. Pigs were destroyed post-application of ink at 1½, 4, 8, 16 and 24 hours, thereafter at 24-hourly intervals up to 168 hours (7 days).

Post-mortem, a macroscopic examination of the head and neck was undertaken with particular reference to the palatine tonsils and the regional lymph nodes to ascertain the gross distribution of ink.

Blocks from the palatine tonsils, submandibular, retropharyngeal and parotid lymph nodes, including any areas macroscopically positive for ink, were fixed and processed for histological examination.

Sections were stained with H & E, and haematoxylin (unblued) and tartrazine, the latter having been selected to enhance perception of ink particles.

The tonsils of the untreated control pig were completely negative for any of the features described above. Macroscopic examination of all pigs failed to reveal any India ink in the submandibular, retropharyngeal, parotid or cervical lymph nodes when viewed either intact or on section.

Results

Macroscopic Examination

The palatine tonsils were all superficially free from ink with the exception of pig 652 in which the tonsils, at  $1\frac{1}{2}$  hours post-application of ink, presented a grey oral surface resulting from a thin film of ink over the mucosa. In the tonsils of the four pigs examined at 4, 8, 16 and 24 hours, no ink was apparent grossly except on section when occasional black streaks or foci were noted in the tonsillar substance. Close macroscopic examination through the tonsillar oral mucosa revealed faint grey shadows, defined but variable in extent and distribution, within the tonsils of the two pigs at 48 and 72 hours post-application and of the two pigs both examined at 96 hours. At 120, 144 and 168 hours, two pigs being examined at each stage, occasional isolated black foci were discernible through the oral surface of the tonsils. On section all tonsils from 48 to 168 hours presented from few to numerous black foci or black streaks running at right angles to the oral surface, apparently related to tonsillar crypts. The distribution of these throughout the tonsillar parenchyma was irregular. To sum up, ink was evident at macroscopic examination in all tonsils from 4 to 168 hours post-application.

The tonsils of the untreated control pig were completely negative for any of the features described above. Macroscopic examination of all pigs failed to reveal any India ink in the submandibular, retropharyngeal, parotid or cervical lymph nodes when viewed either intact or on section.

Histological Examination

While histological examination confirmed the presence of ink in those tonsillar areas positive macroscopically, it also revealed ink in areas of tonsillar tissue grossly negative. The distribution of ink determined histologically is set out in Table V.1.

Up to 24 hours post-application of ink to the palatine tonsils, the presence of ink was confined to the crypt lumina and areas of crypt epithelium, with only a sparse amount on the oral surface.

The ink was either mixed with luminal cellular detritus (Fig.V.1) or occurred in small particle aggregations. Within the epithelium, the ink particles occurred in intercellular spaces but more frequently they were apparently contained in the cell cytoplasm.

Where the structure of the epithelium was reticulated and particularly where cellular infiltration of the epithelium was a feature, it was not possible to determine the cell type associated with intracellular ink. In many other instances, however, the cells involved were indistinguishable from epithelial cells (Fig.V.2).

From 48 hours, ink, almost invariably intracellular, was located in increasing amounts in the subepithelial zone of the crypts and extended into the interfollicular lymphoid tissue in those areas adjacent to the epithelial ink deposits described above (Fig.V.3). In addition, throughout random areas of the interfollicular tissue, scattered cells or loose aggregations of cells were noted containing intracellular ink. Associated with the presence of ink in the interfollicular lymphoid tissue, occasional germinal centres were observed to contain intracellular ink within one or two cells which were randomly distributed, mainly in the

peripheral zone (Fig.V.4). Such ink-positive centres were relatively abundant in the tonsils of pig 74, examined after an interval of 120 hours post-application.

Ink particles, again intracellular, were observed in the trabeculae, usually towards the aboral capsule, at 48 hours and from 96 to 168 hours. This location was however infrequent and rarely involved more than a few particles. Minute intracellular traces were also noted occasionally in the capsular tissue, adjacent to, but not within lymphatics. Ink in these trabecular and capsular sites was always associated with its presence in adjacent interfollicular lymphoid tissue (Fig.V.5).

In no one tonsillar section, and a number were examined per pig, was there any consistency in the distribution or quantity of ink when the various areas where it was identified were compared so that a quantitative evaluation of the uptake of ink was not attempted.

Histological study of the lymph nodes failed to illustrate any India ink particles within their substance, either in the sinus macrophages or the cortical lymphoid tissue, confirming the absence of ink at macroscopic examination.

Electron-microscopic examination was therefore performed post-application of ink topically to the tonsils.

Materials and Methods  
Introduction (ii)

Three pigs, each approximately 4 months old, were used in this experiment. India ink was applied topically, as previously, to the epithelium apparently within the cytoplasm of epithelial cells. That phagocytosis may be a function of the epithelial zone was therefore suggested by these observations with the light microscope.

The concept of phagocytosis by epithelial cells is not original. Kraehenbuhl and Campiche (1969) observed endocytosis of antibodies by intestinal epithelial cells in neonates. Epidermal cell engulfment of material has been illustrated by intra-epidermal injection (Platt, 1963). Human epidermis in tissue culture showed active phagocytosis (Blois, 1968) and Nordquist, Olsen and Everett (1966) noted rapid access of circulating material to epidermal cells. Additionally, Nosaka (1966) expresses the opinion that "the normal tonsil has an active capacity for absorption", having observed foreign bodies within epithelial intercellular spaces.

To further study the question of phagocytosis by epithelial cells, perhaps of significance in the tonsillar afferent route, an ultra-structural examination of the tonsillar crypt epithelium was indicated. An electron-microscopic examination was therefore undertaken post-application of ink topically to the tonsils.

Thin 1/2 sections from the tonsillar tissue embedded in Araldite were examined with the light microscope to select areas of crypt epithelium from which to cut ultra-thin sections. These latter were stained with lead citrate and uranyl acetate. A drop of India ink, diluted 1 in 100 in water, was placed on a collodion membrane supported on a copper grid and allowed to dry prior to electron-microscopical examination.

### Materials and Methods

Three pigs, each approximately 4 months old, were used in this experiment. India ink was applied topically, as previously, to the palatine tonsils of two pigs (PT1 & PT2) which were destroyed at 48 hours post-application. The third (PT3) was left to provide untreated control material.

Primary fixation of the tonsils in situ was undertaken to ensure adequate fixation for electron-microscopy. The method of vascular pressure perfusion described in Chapter IV was adapted for this purpose. Vaso-dilation with amyl nitrate was omitted. Initially, the vascular system was flushed through with physiological saline to prevent intra-vascular blood coagulation, followed by perfusion with phosphate-buffered 10% formalin pH 7.2 until it issued freely and clear from the sectioned jugular vessels.

Areas positive for ink at macroscopic examination of the tonsils were selected and 2 x 1 x 1mm blocks were processed for electron-microscopic study. Other positive areas and the remainder of the tonsillar tissue were further fixed in 10% formol saline and processed routinely to paraffin wax. Sections were stained with H & E and haematoxylin (unblued) and tartrazine for histological examination.

Thin 1 $\mu$  sections from the tonsillar tissue embedded in Araldite were examined with the light microscope to select areas of crypt epithelium from which to cut ultra-thin sections. These latter were stained with lead citrate and uranyl acetate. A drop of India ink, diluted 1 in 100 in water, was placed on a collodion membrane supported on a copper grid and allowed to dry prior to electron-microscopical examination.

## Results

During the electron-microscopical study, the following features, based on the general description given by Rhodin (1963) were used to identify epithelial cells, lamellipodia and tonofibrils.

### Histological Examination

Examination of 5µ sections from the paraffin-embedded blocks confirmed an essentially similar distribution of India ink in PT1 and PT2 to that described above for the pig examined at 48 hours.

Electron-microscopical examination of ultrathin sections showed that although ink was infrequently observed within the crypt lumina, areas of crypt epithelium, subepithelial and lymphoid tissues were positive for its presence. Within the epithelium, the ink was for the greater part contained intra-cellularly, and a proportion of the cells at least were apparently epithelial cells. All sections examined from the control pig PT3 were completely negative for India ink.

These observations therefore established that the specimens from PT1 and PT2 were suitable for a more detailed electron-microscopical study of the distribution of the ink within the crypt epithelium.

### Electron-Microscopical Examination

The dried droplet of diluted India ink was first examined at varying magnifications to establish familiarity with its appearance at an ultrastructural level. The particles are approximately of uniform size, 28nm, round and are markedly electron-dense (Fig.V.6).

In the thin lu sections examined by light microscopy to select crypt epithelial areas no evidence of ink was apparent, despite the fact that the blocks had been selected from tonsillar tissue positive for ink at a gross level. This was attributed to lack of contrast between the ink and the blue staining of thionine/toluidine blue. These were infiltrating mesenchymal cells was not further studied.

During the electron-microscopic study, the following features, based on the general description given by Rhodin (1963) were used to identify epithelial cells: desmosomes and tonofilaments, large pale nucleus, lack of specialised cell organelles and microvilli on the free luminal surface (Figs. V.7 & 8).

Electron-microscopical examination of ultrathin sections showed ink particles free within the crypt lumina (Fig.V.9) and intracytoplasmic aggregations within cells of the epithelium (Fig.V.10) in all areas except the basal germinal layer. Examination of the crypt epithelium for the distribution of ink revealed that a number of the cells with intracytoplasmic ink could positively be classified as epithelial cells (Fig.V.11). Although frequently the carbon particles were enclosed by a distinct membrane, constituting a phagosome, this was not a constant feature and was independent of the number of particles or the size of the aggregation. The appearance of the phagosomes was extremely variable. Some, large and small, were homogeneous in character, containing solely ink particles, while others presented a markedly heterogeneous content of ink particles amongst a mass of unidentifiable material (Fig.V.12). Although these membrane-bound intracellular structures may be termed phagosomes since the ink had acquired an intracellular location post-topical application, at no time was active phagocytosis of particles into the cells observed.

Situated within the crypt epithelium, the remainder of the cells with intracytoplasmic ink lacked desmosomes and tonofilaments and could not therefore be termed epithelial cells. The possibility that these were infiltrating mesenchymal cells was not further studied.

Examination of all sections from the control pig PT3 failed to illustrate the presence of any material resembling the carbon

The results of the first experiment conclusively demonstrate an afferent route from the oral cavity to the subepithelial lymphoid tissue via the tonsillar crypts: India ink applied to the oral cells and other non-classified cells as above.

surface of the palatine tonsils entered into the crypt lumina and epithelium whence it gained access to macrophages present in the interfollicular lymphoid tissue, occasional germinal centres, and the trabeculae and capsule. No efferent drainage to the regional lymph nodes was observed. An interesting feature of the afferent route was that at light-microscopical examination ink particles were noted apparently within epithelial cells. The electron-microscopic study undertaken subsequent to this observation confirmed an intracellular location of ink in these cells.

The extensively ramifying tonsillar crypt system undoubtedly enhances this afferent route from the oral cavity to the lymphoid tissue. Structurally the crypts produce an enormous increase in the epithelial surface area in contact with the tonsillar parenchyma compared with the overlying oral epithelium alone (Loburg, 1966). Ingested antigens may therefore be carried, by penetration of the crypt system, into closer proximity with a substantially larger volume of the tonsillar subepithelial lymphoid tissue than would otherwise be possible.

These features suggest that there exists a specific functional relationship between the environment of the oral cavity and the tonsillar lympho-epithelial tissue. The observation that ink applied topically to the palatine tonsils penetrated the epithelium of the crypts but not that of the oral surface defines this

relationship, in part at least Discussion of absorption. The fact that

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The results of the first experiment conclusively demonstrate an afferent route from the oral cavity to the subepithelial lymphoid tissue via the tonsillar crypts: India ink applied to the oral surface of the palatine tonsils entered into the crypt lumina and epithelium whence it gained access to macrophages present in the interfollicular lymphoid tissue, occasional germinal centres, and the trabeculae and capsule. No efferent drainage to the regional lymph nodes was observed. An interesting feature of the afferent route was that at light-microscopical examination ink particles were noted apparently within epithelial cells. The electron-microscopic study undertaken subsequent to this observation confirmed an intracellular location of ink in these cells.

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These features suggest that there exists a specific functional relationship between the environment of the oral cavity and the tonsillar lympho-epithelial tissue. The observation that ink applied topically to the palatine tonsils penetrates the epithelium of the crypts but not that of the oral surface defines this relationship. It may be that this results in elimination of substantial quantities of ingested antigen, leaving a small sample only to reach the lymphoid tissue. Although ink was observed within the luminal debris concurrent with its presence within the epithelium up to 7 days post-application, it is pure conjecture to suggest that some may have already been ingested and returned

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relationship, in part at least, as one of absorption. The fact that Koburg (1970) was able to study, within the tonsils and distant areas of the body, cellular events related specifically to antigens which he had applied topically to rabbit tonsils additionally indicates that absorption or percolation takes place through the tonsillar epithelium. (1965). In his electron-microscopic studies of the human tonsil phagocytosis of ink by epithelial cells was confirmed by the examination of the crypt epithelium at an ultrastructural level. Although epithelial phagocytosis in the epidermis has been described (Nordquist, Olsen & Everett, 1966; Mottaz & Zelickson, 1970; Wolff & Schreiner, 1970), none of these reports illustrates uptake of foreign material from the external environment. Human epidermal cells grown in tissue culture were shown to phagocytose melanin and carbon particles added to the culture medium (Blois, 1968). In these various studies it has been postulated that epithelial phagocytosis performs a storage role (Nordquist, et al., 1966) or a macrophage role after epidermal injury (Mottaz & Zelickson, 1970; Wolff & Schreiner, 1970). Following intra-epidermal injection, ink is ingested and retained for life by epithelial cells, eventually being eliminated from the epidermis at desquamation (Platt, 1963). A feature of the tonsils is desquamation of epithelial cells and loss of migratory mesenchymal cells into the crypt lumina (Trautmann & Fiebiger, 1952). It may be that this results in elimination of substantial quantities of ingested antigen, leaving a small sample only to reach the lymphoid tissue. Although ink was observed within the luminal detritus concurrent with its presence within the epithelium up to 7 days post-application, it is pure conjecture to suggest that some may have already been ingested and returned

through desquamation to the crypts. Epithelial phagocytosis in the intestine, on the other hand, serves a very definite absorptive function as in the uptake in the neonate of antibody macromolecules from the colostrum and subsequent transfer of these to the circulation (Kraehenbuhl & Campiche, 1969).

Nosaka (1966), in his electron-microscopic studies of the human tonsils, observed foreign bodies in epithelial intercellular spaces. He suggested that these may be phagocytosed but did not further investigate this aspect. In the present study, a number of the cells within the epithelial zone which contained ink could definitely be classified by their ultrastructural features as epithelial cells, thereby confirming this postulate. The significance of the phenomenon, however, escaped elucidation since the object of the study was solely to confirm or refute the process of epithelial cell phagocytosis suggested by the initial experiment. One may postulate that perhaps these cells play an important role in the afferent pathway to the tonsillar lymphoid tissue by ingesting foreign matter from the crypt lumina, thus penetrating the epithelial barrier. This process may be more significant in areas of non-reticulated epithelium where it is difficult to envisage passage by simple percolation but no evidence was obtained to suggest any difference in ink uptake between reticulated and non-reticulated areas. At an ultrastructural level, however, it is apparent that numerous intercellular spaces exist throughout the tonsillar epithelium: One cannot therefore entirely dismiss the possibility that access to the lymphoid tissue is gained by filtration.

An electron-microscopic examination of the phagocytic process in the splenic sinusoids revealed that intravenously administered carbon

was ingested by macrophage pseudopodia insinuated through apertures in the basement membrane and between the sinus endothelial cells into the lumina (Burke & Simon, 1970). It has been suggested (Kawaguchi, 1967) that the basement membrane of the tonsillar epithelium may readily allow migration of cells from the lymphoid tissue into the epithelium. Although no passage across the basement membrane was noted, phagocytosis and transfer of foreign matter from the crypt lumina to the lymphoid tissue may perhaps be a function of migratory macrophages, either as a sequel to or independent of primary ingestion by epithelial cells.

Macrophages are a regular component of lymphoid tissue, being best illustrated in the reticulin meshwork of the cortical and medullary sinuses of lymph nodes and of the splenic red pulp. In lymph nodes the afferent lymph is discharged into the cortical sinuses whence it filters through the node to the medullary sinuses and efferent lymphatics (Ham, 1969) which take origin from the hilus in the majority of species and from the capsule of the porcine node (Trautmann & Fiebiger, 1952). Should foreign material be present in the afferent lymph, it is phagocytosed and effectively eliminated from the lymphatic circulation by the macrophages of the sinuses and those scattered in the lymphoid substance. A proportion of antigen trapped by macrophages is instrumental in initiating the immune response.

In this study of the tonsillar afferent system, ink which penetrated the crypt epithelium was contained within macrophages of the lymphoid tissue. The information yielded by serial examination indicates that transfer takes place from the immediate subepithelial zone into the interfollicular tissue and that ingested foreign material ultimately reaches the trabeculae and capsule where the tonsillar efferent lymphatics take origin.

Collating the results of the experiments in this and the previous chapter, which demonstrate afferent and efferent tonsillar pathways, one may be permitted to draw an analogy between the palatine tonsils and lymph nodes. Epithelial desquamation and mesenchymal cell migration to the exterior is however unique to the tonsils, perhaps, as already discussed, resulting in elimination of antigenic material. Further, in the absence of afferent vessels, there is no substantial lymph flow through the tonsils. This may enhance macrophage efficiency, an important point where, unlike lymph nodes, constant exposure to antigens is inevitable.

The ultrastructural examination revealed a heterogeneity of phagosome content within the cells of the epithelium, and histologically minute amounts of ink in active germinal centres were noted. It is therefore probable that the ink particles observed in the centres were only a visible example of a wide range of foreign matter stimulating germinal centre proliferation.

Cervical lymphadenitis in swine, of which the causative agent is a Group B streptococcus, was similarly experimentally reproduced by oral or nasal administration of the organism (Collier, 1956b; Gouge *et al.*, 1957; Armstrong *et al.*, 1970). The streptococci were recovered from the palatine tonsils as well as from abscessed lymph nodes (Armstrong *et al.*, 1970; Armstrong & Ellis, 1971). Collier (1956b) expressed the opinion that the site of entry was the pharyngeal area, either the mucosa or the tonsils.

Payne and Derbyshire (1963) suggested that in calves and piglets the tonsils act as portals of bacterial entry. Lawson (1965) postulated that penetration of *Salmonella cholerae swia* might occur through the mucous membrane of the pharyngeal area. Following

CHAPTER VI

STREPTOCOCCUS SUIS INFECTION IN THE YOUNG PIG

Introduction

Streptococcal meningitis and arthritis occur in baby pigs, is most frequently considered a human ailment. There are generally between two and six weeks of age (Field et al., 1954). The aetiological agent was classified by Elliott (1966) as a distinct (Lancefield's Group A) with the faucial (palatine) tonsils of man. serological type, Streptococcus suis, within Lancefield's Group D. Bacteria in the tonsils are not however exclusively pathogens. The epidemiology and experimental production of the disease has been described by Elliott et al. (1966) whose work strongly suggested that Strep. suis gained entry through either the upper digestive or respiratory tract. Alexander (1969) showed it to be improbable that the lung alveoli were significant sites of entry for Strep. suis, although earlier he had considered them to be a more likely route of entry than the palatine tonsils.

Cervical lymphadenitis in swine, of which the causative agent is a Group E streptococcus, was similarly experimentally reproduced by oral or nasal administration of the organism (Collier, 1956b; Gouge et al., 1957; Armstrong et al., 1970). The streptococci were recovered from the palatine tonsils as well as from abscessed lymph nodes (Armstrong et al., 1970; Armstrong & Ellis, 1971). Collier (1956b) expressed the opinion that the site of entry was the pharyngeal area, either the mucosa or the tonsils.

Payne and Derbyshire (1963) suggested that in calves and piglets the tonsils act as portals of bacterial entry. Lawson (1965) postulated that penetration of Salmonella cholerae suis might occur through the mucous membrane of the pharyngeal area. Following

oral administration of bacteria in experimental situations,

Escherichia coli organisms have been identified within the pharyngeal tonsillar crypts (Drees & Waxler, 1970a) and Erysipelothrix insidiosa recovered from the palatine tonsils (Timoney & Berman, 1970).

Although streptococci and other pathogens are recoverable from the tonsils of the pig and other species, streptococcal tonsillitis is most frequently considered a human ailment. There are innumerable reports of the association of Strep. pyogenes (Lancefield's Group A) with the faucial (palatine) tonsils of man.

Bacteria in the tonsils are not however exclusively pathogens. Numerous streptococci, from a variety of Lancefield's groups,

including Group D, naturally inhabit the porcine palatine tonsils (Kohler & Mochmann, 1958). Observations on the bacterial flora of

the bovine tonsil revealed the predominant organisms to be streptococci, micrococci and Gram-negative bacilli (Daleel & Frost, 1967).

In Chapter V an afferent route from the oral cavity to the tonsillar lymphoid tissue via the crypt system was clearly demonstrated following topical application of India ink. The above-mentioned reports indicate that bacteria may frequently be associated with the palatine tonsils and that Strep. suis may effect entry in the throat area. To investigate the possibility that the palatine tonsils therefore form a portal of entry for Strep. suis, piglets of a susceptible age were experimentally infected with Strep. suis, and the involvement of the tonsils studied.

#### Daily procedure

Materials and Methods

Six weaned piglets, 10 days of age, from the minimal disease herd (see Chapter II) were used in this experiment, four in the group were infected and two non-infected negative controls housed in a separate loose box. The infected piglets were numbered PM34/1, PM34/2, PM34/3 and PM34/4 and the controls PM34/5 and PM34/6, hereafter referred to as 1, 2, 3, 4, 5 and 6. A milk substitute (SMA - John Wyeth & Brother Ltd., Havant, Hants.) was made available to piglet 3 from day 12 (day 1 = day of infection); the others were fed a pelleted diet throughout (Chapter II).

Infection Procedure

From a stock culture thawed and inoculated on to HBA, single colonies of Strep. suis were subcultured into 10ml HSB and incubated for 18 hours. After heat-fixed, Gram-stained smears of these HSB cultures were examined to check purity of growth, a culture was selected to provide the infective doses and shaken for 10 minutes to break up any long chain growth. 4 x 1ml aliquots were withdrawn for the infective inocula and a further 1ml taken to estimate the number of viable organisms. From log<sub>10</sub> serial dilutions in nutrient broth, 0.02ml amounts were plated on HBA, incubated 18 hours at 37°C and colony-forming units counted: 1.7 x 10<sup>6</sup> present per ml.

A small sterile brush was used to apply the inocula topically to the palatine tonsils of piglets 1 and 2. The inocula were sprayed intra-orally to piglets 3 and 4. Piglets 5 and 6 were retained as uninfected controls.

Daily Procedure

All piglets were examined clinically and rectal temperatures both noted prior to infection, on the evening of day 1, morning and the evening for the next four days and thereafter every morning. Tonsillar swabs and blood samples were taken daily for bacteriological examination for Strep. suis, with the exception of days 10, 17 and 24.

#### Post-mortem Examinations

Ante-mortem blood samples were taken and piglets 2, 4 and 6 were killed on day 25, piglets 1 and 3 on day 26, and 5 on day 27. A full macroscopic examination was carried out, paying particular attention to tonsils and head regional lymph nodes, the meninges, cerebro-spinal fluid (CSF), brain and spinal cord, the limb joints, joint capsules and synovial fluid, the regional lymph nodes for these tissues, and the spleen.

The palatine tonsils, submandibular, retropharyngeal and parotid lymph nodes, brain and spinal cord, carpal and tarsal synovial membranes, popliteal and pre-scapular lymph nodes, fixed and processed routinely for paraffin sections, were stained with H & E. Additionally, the palatine tonsils were stained with Gram's, methyl-green/pyronin, Masson's trichrome and impregnated with silver.

The palatine tonsils, the submandibular, retropharyngeal and parotid lymph nodes, the spleen, CSF, carpal and tarsal synovial fluid, tonsillar, pharyngeal and nasal swabs and blood and an abscess from piglet 3 were culturally examined for Strep. suis.

For immunofluorescent microscopy, the palatine tonsils, submandibular lymph nodes and spleen were frozen for cryostat sections and also processed by Sainte-Marie's method (1962) to be

embedded in paraffin wax. Cryostat sections were stained with both RB 200 and FITC, paraffin sections with RB 200. Sections of the palatine tonsils, submandibular lymph nodes and the spleens were stained with methyl-green/pyronin.

The recovery of *Staph. aureus* from the tonsillar swabs and blood samples is set out in table VI.2.

All piglets remained clinically normal until day 12 when piglet 3 became pyrexia ( $105.8^{\circ}\text{F}$ ), with an increased respiratory rate, congested mucous membranes and cutaneous hyperaemia along the abdomen. Although it was reluctant to stand, there was no evidence of joint involvement at this stage. Later in the day shivering commenced, its temperature rose to  $106.5^{\circ}\text{F}$  and the piglet developed intermittent nystagmus, spasticity of the fore limbs at examination and cyanosis of the tip of the left ear. That day, it was pyrexia, ataxic and reluctant to move, and resisted handling. Little or no food was consumed until day 17 when the piglet commenced to drink milk substitute unaided. On this day the first joint involvement was noted: both the carpal and tarsal joints were swollen and tender, particularly the left tarsus. The degree of pyrexia and clinical condition fluctuated from day 15 to 23 with the dominant clinical signs involving the nervous system and joints. Some recovery was then observed when it became able to move on its hocks and knees and drank milk substitute freely. The piglet showed a marked loss of bodily condition, with a dry skin and rough staring coat, weighing only 3.4kg on day 26 at 36 days of age (Fig.VI.1). Sampling blood from the anterior vena cava became progressively more difficult throughout the experiment.

The other infected piglets, 1, 2 and 4, and the controls,

Results

Daily Examinations

The record of rectal temperatures is contained in Table VI.1; the recovery of Strep. suis from the tonsillar swabs and blood samples is set out in Table VI.2.

All piglets remained clinically normal until day 12 when piglet 3 became pyrexia (105.8°F), with an increased respiratory rate, congested mucous membranes and cutaneous hyperaemia along the abdomen. Although it was reluctant to stand, there was no evidence of joint involvement at this stage. Later in the day shivering commenced, its temperature rose to 106.5°F and the piglet developed intermittent nystagmus, spasticity of the fore limbs at examination and cyanosis of the tip of the left ear. Next day, it was pyrexia, ataxic and reluctant to move, and resented handling. Little or no food was consumed until day 17 when the piglet commenced to drink milk substitute unaided. On this day the first joint involvement was noted: both the carpal and tarsal joints were swollen and tender, particularly the left tarsus. The degree of pyrexia and clinical condition fluctuated from day 15 to 23 with the dominant clinical signs involving the nervous system and joints. Some recovery was then observed when it became able to move on its hocks and knees and drank milk substitute freely. The piglet showed a marked loss of bodily condition, with a dry skin and rough staring coat, weighing only 3.4kg on day 26 at 36 days of age (Fig.VI.1). Sampling blood from the anterior vena cava became progressively more difficult throughout the experiment.

The other infected piglets, 1, 2 and 4, and the controls,

5 and 6, remained clinically healthy throughout the whole period of the experiment and showed no check in growth rate.

Cultural examination of the tonsillar swabs yielded Strep. suis from piglets 3 and 4 on day 4 and from 1 and 2 on day 12. The swabs gave intermittently positive results from the first recovery of the organism until the piglets were killed. Strep. suis was never isolated from the tonsillar swabs of the non-infected controls, 5 and 6.

Strep. suis bacteraemia was detected only in piglet 3. The organism was first isolated on day 11, the day prior to the onset of clinical symptoms; bacteraemia persisted until day 20 following which recovery of Strep. suis was intermittent.

#### Post-mortem Macroscopic Examination

Macroscopic examination of the carcasses of the infected piglets, 1, 2 and 4, and the controls, 5 and 6, failed to reveal any significant abnormalities and are therefore described together. The carcasses were well nourished and in excellent bodily condition. Haemorrhage from the ante-mortem blood sampling had occurred at the entrance to the thorax of pig 1 and around the trachea in the posterior cervical region of 2. Peri-vascular fibrosis at the thoracic inlet was noted to varying degrees in all piglets. The medullae of the pre- and supra-sternal lymph nodes were haemorrhagic. The palatine tonsils, grey-white in colour, both superficially and on section as a result of exsanguination, did not present any abnormalities and were firm to palpate and cellular in appearance. The submandibular, retropharyngeal and parotid lymph nodes were firm and cellular with marked but not abnormal development of the

lymphoid tissue. No hyperplasia of the splenic white or red pulp was apparent. The joints of both the fore and hind limbs were without abnormality, presenting clear synovial fluid, moist glistening articular surfaces and normal synovial membranes. The popliteal and iliac lymph nodes contained brown pigment, otherwise the drainage lymph nodes of the limbs were unremarkable. No abnormalities of the meninges, brains or spinal cords were noted.

Piglet 3, on the other hand, presented an extremely impoverished carcass with marked loss of bodily condition, and depletion of fat deposits. The coat was very rough. Oedema of many tissues was apparent, including the subcutis, the carcass lymph nodes, lungs and bronchial lymph nodes. Excess fluid was present in the serous cavities. The thoracic cavity contained fresh blood following exsanguination. Marked fibrosis was apparent in the caudal cervical region where the blood samples had been withdrawn, with a small encapsulated lesion of inspissated yellow material on the right side.

The palatine tonsils were grey/white in colour, oedematous, lacked depth of the lymphoid tissue when compared with all the other piglets, and were not markedly cellular. The submandibular, retropharyngeal and parotid lymph nodes were oedematous, small and relatively non-reactive in appearance, lacking the firm cellular development seen in the other piglets.

All joints contained excess synovial fluid and presented smooth glistening articular surfaces. The synovial membrane of the carpal and tarsal joints exhibited slight thickening, most marked in the left tarsus. Macroscopically no abnormality of the meninges, brain or spinal cord was apparent.

### Bacteriological Examination

Recovery of Strep. suis from the tissues and fluids sampled at post-mortem examination are presented in Table VI.3.

The organism was recovered from the tonsillar swabs of piglets 1, 2 and 3 only, but not at all from the nasal and pharyngeal swabs. The palatine tonsils of 1, 3 and 4 yielded Strep. suis, the tonsillar cultures from 2 and 6 unfortunately were overgrown by other organisms and those from 5 were negative. Strep. suis was cultured from the submandibular lymph nodes of the four infected piglets, 1, 2, 3 and 4, from the retropharyngeal and the parotid nodes of number 2. All four blood samples failed to yield Strep. suis. Piglet 3, which developed clinical disease, additionally yielded the organism from the spleen, CSF, and left tarsus and the inspissated contents of the abscess anterior to the thoracic inlet. All tissues sampled from the two control piglets were negative for the presence of Strep. suis.

### Histological Examination

The brains and meninges, the carpal and tarsal synovial membranes, the pre-scapular and popliteal lymph nodes, and spleens from the infected piglets 1, 2 and 4, and the controls, 5 and 6, were without abnormality. The palatine tonsils, submandibular, retropharyngeal and parotid lymph nodes of both infected and controls were essentially normal in appearance for this age of pig and did not present any significant differences between the two groups.

The tonsillar lymphoid parenchyma was well developed, presenting

numerous moderate to large germinal centres (Fig.VI.2) in which mitotic figures, nuclear debris and large tingible-body macrophages were common features. Perifollicular accumulations or follicular crypt-orientated lymphocytic caps were generally absent. The interfollicular tissue was not extensive. Although crypt luminal debris, desquamated epithelial cells, polymorphonuclear leucocytes and mononuclear cells, was of variable quantity, it was negligible in many crypts. Reticulation and cellular infiltration of the crypt epithelium was also a variable feature within individual tonsils but was noted in all piglets. Silver impregnation to illustrate reticulin fibres confirmed germinal centre development and the connective tissue elements were defined by Masson's trichrome stain: no abnormalities were noted. Pyroninophilia of the germinal centres, deeper in the peripheral zone, large pyroninophilic cells in the interfollicular lymphoid tissue and immediate subepithelial area where plasma cells were also identified in small numbers. Similar cells were noted in areas of infiltrated epithelium. Gram's stain revealed Gram-positive organisms in the crypt lumina, in the epithelium and occasionally in the subepithelial lymphoid tissue, in both the infected and control groups (Fig.VI.3).

The submandibular and retropharyngeal lymph nodes of these piglets presented numerous germinal centres within well-developed cortical tissue. Sinus macrophage proliferation was a marked feature. The parotid nodes were less active by comparison, with fewer centres and reduced lymphocytic and macrophage proliferation. An exception were the nodes of piglet 2 from which Strep. suis was recovered: these were essentially similar in their degree of activity and development to the submandibular and retropharyngeal

lymph nodes.

Piglet 3 only of the infected group presented pathological lesions at a histological level. Although the meninges were relatively normal, containing only occasional mononuclear cells, areas of mononuclear cell vascular cuffing were noted in the brain substance around the fourth ventricle (Fig.VI.4) and in the spinal cord. Isolated polymorphonuclear cells were present, and some at least of the mononuclears were plasma cells (Fig.VI.5). The synovial membranes of the carpi and tarsi were oedematous, thickened by deposition of fibrous tissue and presented a light mononuclear cell reaction featuring a few plasma cells. The drainage lymph nodes were oedematous and non-hyperplastic. Plasma cells were observed in the red pulp of the spleen (Fig.VI.6); the lymphoid white pulp on the other hand was almost totally inactive, presenting only small peri-articular aggregations of lymphocytes and virtual absence of germinal centres (Fig.VI.7).

Oedema was also a feature of the palatine tonsils, submandibular, retropharyngeal and parotid lymph nodes. The tonsillar parenchyma comprised mainly interfollicular tissue with a light to moderate population of lymphocytes and macrophages; germinal centres, although a small number were present, showed marked cellular depletion (Fig.VI.8) and lacked evidence of activity such as mitotic figures and nuclear debris. Epithelial reticulation and infiltration were noted on a small scale. Crypt luminal detritus, although variable in amount, was present in many crypts in small quantities. Gram-positive organisms were observed in the crypt lumina, the epithelium and lymphoid tissue, as in the other piglets. A moderate number of large pyroninophilic cells and occasional

plasma cells were present in the interfollicular tissue, immediate subepithelial zones, and the epithelium itself (Fig.VI.9).

Pyroninophilia was not a feature of the inactive or depleted germinal centres. Silver impregnation confirmed the poor follicular and germinal centre development. The oedema of the tonsils was demonstrated by the wide separation of the reticulin fibres and of the connective tissue stroma when stained by Masson's trichrome.

Although oedema of the cortical and medullary sinuses was marked in the submandibular, retropharyngeal and parotid lymph nodes, a moderate degree of sinus macrophage hyperplasia was also apparent. The cortical lymphoid tissue was not extensive nor densely cellular. In comparison with the other piglets, germinal centres were less numerous, frequently inactive or showing some degree of cellular depletion.

#### Immunofluorescent Examination

The findings of immunofluorescent examination of the palatine tonsils are summarised in Table VI.4.

The Sainte-Marie method of fixation and processing gave superior sections for architectural interpretation; frozen sections gave brighter fluorescence, with adequate morphological definition. The observations are however described together since the method of preparation did not influence the recognition and distribution of fluorescing bacteria or antigen. Similarly, although the FITC conjugate fluoresced much more brightly and discretely than the RB 200 conjugate, allowing differentiation of individual bacteria, the findings in no other way differed so that they also are described as one.

Tonsillar sections stained with immune globulin and control sections (Chapter II) from the two uninfected piglets, 5 and 6, demonstrated green autofluorescence of the luminal cellular detritus and the connective tissue of the capsule and blood vessels, irrespective of the fluorescent conjugate employed. Bright yellow/orange fluorescing granules were present randomly distributed throughout the tonsillar lymphoid parenchyma. Cells with strongly-fluorescing cytoplasmic granules, green when stained with FITC, red with RB 200, were frequently observed. In H & E sections demonstrated in similar sites eosinophils, which morphologically resembled these fluorescing cells. These were also noted in the submandibular lymph nodes and spleens. That this feature was part auto- and part non-specific fluorescence was shown by its absence when examining the sections by ultra-violet light, and by the presence of the granules when looked at by dark-ground microscopy. Specific fluorescence denoting the presence of Strep. suis or its antigenic products was completely absent from all tissues examined from piglets 5 and 6 (Fig.VI.10). In the infected group both auto- and non-specific fluorescence were observed as described above. The location and distribution of Strep. suis or its antigenic products was essentially the same in the palatine tonsils of all four piglets. Whole bacteria were frequently present within the crypt lumina and were sometimes very numerous, particularly in diseased piglet 3 (Fig.VI.11). They were also observed within the epithelium and immediate subepithelial zone. In addition, a similar distribution of specifically fluorescing antigenic material, both particulate and amorphous, was noted (Fig.VI.12). Within the lymphoid parenchyma, this material

occurred in large and small aggregations and generally, but not invariably, it was associated with antigen in adjacent crypts. Antigen present in the interfollicular tissue sometimes closely invested cell nuclei. Small amounts of specific particulate fluorescence were observed within a minority of the tonsillar germinal centres, distributed peri-cellularly in the central area (Fig.VI.13). All control sections completely failed to demonstrate Strep. suis or its products in the crypts, epithelium and subepithelial zone (Fig.VI.14) or within the interfollicular lymphoid tissue and germinal centres, confirming the "in vivo" specificity of the fluorescent system for this streptococcal antigen.

Histological examination of the serial sections stained with H & E was employed merely to confirm tonsillar architecture and therefore location of antigen. Gram's stain on serial and washed fluorescent sections revealed Gram-positive organisms within crypt lumina, areas of epithelium and adjacent subepithelial lymphoid zones (Fig.VI.15) correlating with the distribution of specific fluorescence. Additionally, Gram-positive bacteria were noted in similar but non-fluorescent sites. They were not, however, identified deeper in the lymphoid parenchyma, neither in the interfollicular tissue nor in germinal centres. Observations made on sections stained methyl-green/pyronin corresponded to those described earlier during the histological examination.

Specific fluorescence within the submandibular lymph nodes was negligible in comparison with the palatine tonsils. Occasionally, however, small quantities of antigen, particulate and amorphous, were observed, mainly within the sinuses but also in the cortical

lymphoid tissue. Intact bacteria were absent and germinal centre fluorescence was not convincingly demonstrated. Gram-stained serial or washed fluorescent sections failed in all cases to reveal Gram-positive organisms where antigen had been identified.

The spleens of piglets 1, 2 and 4 were completely negative for Strep. suis and its antigenic products. On examination of the spleen of the clinically diseased piglet, 3, isolated bacteria and occasional small clumps of particulate or amorphous antigen were observed fluorescing in the red pulp. No Gram-positive organisms were demonstrated in the Gram-stained sections.

Examination of the infected and diseased piglet, on the other hand, revealed an abscess, lymphadenitis and renal disease with central nervous system and joint lesions and non-reactive, partially-depleted lymphoid tissue, including the palatine tonsils. All four infected piglets yielded Strep. suis from the palatine tonsils, either from the surface swabs or from the parenchyma, from the submandibular lymph nodes but not from the pharyngeal or nasal swabs. The organism was also recovered from the parotid and retropharyngeal nodes of one of the non-diseased infected animals, and from the CNS, tarsus, spleen and an abscess from the piglet which became ill. By immunofluorescent microscopy, Strep. suis antigen was identified within the tonsillar crypt lumina, the crypt epithelium and lymphoid parenchyma, and in the submandibular lymph nodes of all infected animals. In addition, it was noted in the spleen of the diseased piglet. The two uninfected control piglets remained clinically healthy throughout the duration of the experiment; Strep. suis was not recovered at any stage; no gross or histological lesions were observed; and immunofluorescent examination was completely negative.

for Strep. suis antigen. Discussion

From these results, it is apparent that Strep. suis administered orally, either topically to the soft palate or by intra-oral spray, established in all four piglets infected orally with Strep. suis although only one developed clinical disease with symptoms of meningitis and arthritis. In the diseased piglet, Strep. suis bacteraemia followed on the first recovery of the organism from a tonsillar swab, whereas circulating bacteria were not detected in the other three. At post-mortem examination of these infected but clinically normal piglets, no gross or microscopic lesions of the palatine tonsils or other tissues were observed. Examination of the infected and diseased piglet, on the other hand, revealed an oedematous, impoverished and runted carcass with central nervous system and joint lesions and non-reactive, partially-depleted lymphoid tissue, including the palatine tonsils. All four infected piglets yielded Strep. suis from the palatine tonsils, either from the surface swabs or from the parenchyma, from the submandibular lymph nodes but not from the pharyngeal or nasal swabs. The organism was also recovered from the parotid and retropharyngeal nodes of one of the non-diseased infected animals, and from the CSF, tarsus, spleen and an abscess from the piglet which became ill. By immunofluorescent microscopy, Strep. suis antigen was identified within the tonsillar crypt lumina, the crypt epithelium and lymphoid parenchyma, and in the submandibular lymph nodes of all infected animals. In addition, it was noted in the spleen of the diseased piglet. The two uninfected control piglets remained clinically healthy throughout the duration of the experiment; Strep. suis was not recovered at any stage; no gross or histological lesions were observed; and immunofluorescent examination was completely negative

for Strep. suis antigen. *as noted earlier (Chapter IV).*

From these results, it is apparent that Strep. suis administered orally, either topically to the soft palate or by intra-oral spray, becomes established in the palatine tonsils and may produce clinical disease. Elliott *et al.* (1966) reproduced this streptococcal disease by a variety of oral and nasal routes but not by the intragastric route, obtaining a higher incidence of clinical disease than was the case in the experiment reported here. In both cases, however, the numbers of animals employed were small. Although Strep. suis was recovered from the throats of piglets infected naturally and experimentally (Elliott *et al.*, 1966), the area sampled was not defined more specifically, leaving the question of tonsillar involvement unanswered. Despite the employment in this experiment of two methods of infection, the distribution of the organism in the oro-pharyngeal area was similar. Later observations (Chapter VII & Appendix I) revealed that the organism may be pathogenic following topical application to the tonsils, suggesting that the particular method of administration is unimportant and that natural infection may be aerogenous or ingested. Interestingly, the nasal and pharyngeal swabs culturally examined post-mortem failed to yield Strep. suis, unlike the tonsillar swabs taken throughout the experiment and at autopsy. Although it would therefore appear unlikely in this instance, penetration of bacteria through the pharyngeal mucosa, as postulated by Lawson (1965), is not precluded: contamination of the pharyngeal area probably occurred at infection and the isolation of the organism from the parotid and retropharyngeal lymph nodes in one piglet may reflect penetration from this site. More possibly, however, it represents a variation in the tonsillar

efferent lymphatic drainage noted earlier (Chapter IV).

The results of the immunofluorescent examination demonstrated that Strep. suis or its antigenic products were not confined to the tonsillar crypts. This contrasts with the observations of Drees and Waxler (1970a) working with E. coli which was noted solely in the crypts of the pharyngeal tonsils. Other reports on the association of bacteria with the palatine tonsils (Kohler & Mochmann, 1958; Daleel & Frost, 1967) were based on cultural examination so that they contribute little to defining the degree of tonsillar involvement. Quinn and Lowry (1970) recently commented on the lack of information on the location of streptococci within the oro-nasopharynx of man but similarly their investigations into this aspect were limited to bacterial recovery.

That the isolation of Strep. suis from the tonsillar swabs preceded the onset of bacteraemia in the diseased piglet suggests the possibility that a critical degree of bacterial multiplication, perhaps in conjunction with immaturity of local defences, may have precipitated systemic infection. The data presented by Elliott *et al.* (1966) is insufficiently comprehensive to permit a comparison on this point. Such a lengthy lag phase between infection and recovery of the organism from either tonsillar swabs or blood as was noted in this experiment is not constant; but it appears that bacteraemia is secondary to the establishment of the bacterium in the palatine tonsils (Appendix I). Perhaps tonsillar infection is a prerequisite for bacteraemia in this disease - the tonsils would then be unequivocally a significant portal of entry for Strep. suis.

Strep. suis antigen was demonstrated in the tonsillar epithelium and lymphoid parenchyma by immunofluorescent staining.

Although Gram-positive bacteria were noted in those areas where intact Strep. suis organisms were identified, additionally they were present in similar but non-fluorescent sites. This indicates, as might be expected, that the phenomenon of bacterial penetration of the palatine tonsillar epithelium and parenchyma is not unique to Strep. suis but is shared by some at least of the naturally acquired bacterial flora.

That at least part of the antigenic material present in the lymphoid tissue exists in the form of viable organisms is supported by the isolation of Strep. suis from the submandibular lymph nodes to which the tonsillar lymphatics drain (Saar & Getty, 1964; Chapter IV). It is an intriguing phenomenon that in three of the infected piglets the organisms had penetrated as far as these nodes and yet were still recoverable 25 days post-infection in the absence of clinical disease. The epithelium of the small intestine of the neo-natal pig, E. coli organisms have been observed by immunofluorescent examination (Drees & Waxler, 1970a) and by electron-microscopy (Staley, Vynn-Jones & Corley, 1969; Drees & Waxler, 1970b). Drees and Waxler (1970c) comment that no E. coli were present in the epithelial cells of the pharyngeal mucosa, which in their work included the pharyngeal tonsils. Unfortunately, the morphological relationship between bacteria and the host tissue appears to be a recent field of investigation and little information on this aspect is available. Although viral material has been demonstrated within the palatine tonsillar epithelium (Cheville & Mengeling, 1969), the situation is not strictly analogous to bacterial infection since viruses are by definition intra-cellular parasites, frequently epitheliotropic.

Bacterial phagocytosis in the palatine tonsils of the rabbit

## CHAPTER VII

### STREPTOCOCCUS SUIS IN THE TONSILLAR CRYPT EPITHELIUM

#### Introduction

Earlier, in delineating the afferent route to the palatine tonsils, it was ascertained that the tonsillar crypt epithelial cells phagocytose particulate material from the crypt lumina (Chapter V). In the experiment described in Chapter VI, Strep. suis was specifically identified by immunofluorescent microscopy within the crypt epithelium, suggesting that phagocytosis by the crypt epithelial cells might be a more generalised phenomenon, involving bacteria as well as non-infectious material.

Phagocytosis of bacteria by epithelial cells is not unknown: in the epithelium of the small intestines of the neo-natal pig, E. coli organisms have been observed by immunofluorescent examination (Drees & Waxler, 1970a) and by electron-microscopy (Staley, Wynn-Jones & Corley, 1969; Drees & Waxler, 1970b). Drees and Waxler (1970a) comment that no E. coli were present in the epithelial cells of the pharyngeal mucosa, which in their work included the pharyngeal tonsils. Unfortunately, the morphological relationship between bacteria and the host tissues appears to be a recent field of investigation and little information on this aspect is available. Although viral material has been demonstrated within the palatine tonsillar epithelium (Cheville & Mengeling, 1969), the situation is not strictly analogous to bacterial infection since viruses are by definition intra-cellular parasites, frequently epitheliotropic.

Bacterial phagocytosis in the palatine tonsils of the rabbit

has been studied by electron-microscopy (Kato, 1963). The results, however, do not have any significance in relation to the tonsillar epithelium since the organisms, Staphylococcus albus, were injected into the lymphoid parenchyma where phagocytosis by "reticulum" cells took place. The effect of haemolytic streptococci on human tonsillar "epithelial-like" cells in tissue culture was described by Lowry and Quinn (1964). Only occasionally, when using a large inoculum, did they note organisms intra-cellularly. These workers comment on the lack of information on the host-parasite relationship in tonsillitis and pharyngitis.

To investigate the relationship between Strep. suis and the tonsillar crypt epithelium, particularly in the light of earlier results in this work, piglets were infected orally with the organism, the establishment of infection confirmed and the epithelium studied by light and electron-microscopy.

#### Clinical Observations

Piglets were examined clinically daily throughout the experiment and rectal temperatures noted.

Tonsillar swabs were taken daily for cultural examination from day 4 (day 1 = day of infection), based on the first recovery of the organism in the previous experiment. A blood sample from piglet 8 was obtained on day 5.

#### Post-mortem Bacteriological Examination

All three piglets were destroyed on day 6 and a full macroscopic examination carried out.

#### Histological Examination

Blocks from the Materials and Methods and processed

Block routinely for histological examination. Sections were stained with  
Three weaned, minimal disease piglets, littermates aged 10 days,  
were employed in this experiment. They were numbered PM34/7, PM34/8  
and PM34/9, hereafter referred to as 7, 8 and 9. One piglet, 9, was  
housed separately as a non-infected control animal; 7 and 8 formed  
the infected group.

Infection Procedure

1ml infective doses from an 18 hour HSB culture, prepared,  
checked and counted as described in Chapter VI, were applied  
topically, using a small sterile brush, to the soft palates of  
piglets 7 and 8.  $2 \times 10^9$  colony-forming units were present per ml  
of broth.

Clinical Procedures

Piglets were examined clinically daily throughout the experiment  
and rectal temperatures noted.

Tonsillar swabs were taken daily for cultural examination from  
day 4 (day 1 = day of infection), based on the first recovery of the  
organism in the previous experiment. A blood sample from piglet 8  
was obtained on day 5.

Post-mortem Macroscopic Examination

All three piglets were destroyed on day 6 and a full macroscopic  
examination carried out.

Histological Examination

Blocks from the palatine tonsils were fixed and processed routinely for histological examination. Sections were stained with H & E and with Gram's stain. Once set, the agar was cut into cubes, 1 x 1 x 1mm, fixed for 10 minutes in 10% buffered formalin.

#### Bacteriological Examination

Post-mortem, cultural examination for Strep. suis was carried out on the blood, on tonsillar, nasal and pharyngeal swabs, the tonsillar substance, the submandibular, retropharyngeal and parotid lymph nodes, the spleen, CSF, elbow and tarsal synovial fluid of all three piglets and on the deep cervical, right and left prescapular lymph nodes of number 8.

#### Immunofluorescent Microscopy

The palatine tonsils were frozen for immunofluorescent microscopical examination. Cryostat sections were stained with immune globulin or non-immune serum and FITC. Parallel sections were stained with H & E and Gram, washed fluorescent sections with Gram.

#### Electron-Microscopy

Blocks from the palatine tonsils were immersion fixed for electron-microscopical examination as detailed in Chapter II.

To prepare Strep. suis for examination, a modification of the method of Kellenberger, Ryter and Sechaud (1958) was employed. 10ml of an 18 hour HSB culture of the organism, checked by Gram's stain on a heat-fixed smear for purity of growth, was deposited by centrifugation and the supernatant discarded. Still in the tube, the organisms were warmed to 45°C in a water bath prior to the

addition of a small drop (approximately 0.05ml) of a 2% solution of agar in PBS pH7.2, 0.1M, at 45°C. The tube was immediately tilted to run the agar on to a cool slide. Once set, the agar was cut into cubes, 1 x 1 x 1mm, fixed for 10 minutes in 10% buffered formal, 30 minutes in buffered osmium tetroxide, processed, embedded in Araldite, sectioned for electron-microscopical examination as for tissue blocks (Chapter II) and stained with uranyl acetate only.

Piglet 8 yielded Strept. suis from the tonsillar swabs on days 4 and 5 and from the blood sample on day 5, while the swabs from pigs 7 and 9 were negative on both days.

Post-mortem Macroscopic Examination

Examination of the carcasses of piglets 7 and 9, infected and non-infected, revealed them to be in similar good bodily condition and without gross abnormality. The palatine tonsils were grey/white in colour and finely cellular in appearance.

By comparison, however, the clinically diseased piglet, 8, showed a slight loss in bodily condition; the stomach and intestines were devoid of digesta. The brain substance was grossly normal; the meninges presented a trace opacity, increased in the sulci and fissures of the cerebrum and cerebellum; and the abundant CSF was cloudy. Excess turbid synovial fluid was present in the tarsal joints and the right elbow and slight thickening of their synovial membranes was apparent. The articular surfaces were smooth and glistening. All other joints were without gross abnormality. The palatine tonsils were macroscopically no

Results

Clinical Examinations

All three piglets remained clinically normal until day 5 when piglet 8 developed anorexia and pyrexia (104° F in the morning; 106.8° F by evening), manifesting a jerky, ataxic gait and constant trembling. Both hocks and the right elbow were swollen and markedly tender and the right fore limb was held retracted on day 6; when disturbed from lateral recumbency, the piglet showed extreme ataxia and movement was very painful.

Piglet 8 yielded Strep. suis from the tonsillar swabs on days 4 and 5 and from the blood sample on day 5, while the swabs from pigs 7 and 9 were negative on both days.

Post-mortem Macroscopic Examination

Examination of the carcasses of piglets 7 and 9, infected and non-infected, revealed them to be in similar good bodily condition and without gross abnormality. The palatine tonsils were grey/white in colour and firmly cellular in appearance.

By comparison, however, the clinically diseased piglet, 8, showed a slight loss in bodily condition; the stomach and intestines were devoid of digesta. The brain substance was grossly normal; the meninges presented a trace opacity, increased in the sulci and fissures of the cerebrum and cerebellum; and the abundant CSF was cloudy. Excess turbid synovial fluid was present in the tarsal joints and the right elbow and slight thickening of their synovial membranes was apparent. The articular surfaces were smooth and glistening. All other joints were without gross abnormality. The palatine tonsils were macroscopically no

different from those of 7 and 9, being grey/white in colour and firmly cellular.

### Bacteriological Examination

The recovery of Strep. suis from tissues and fluids sampled at autopsy are summarised in Table VII.1.

All tissues examined from the non-infected piglet, 9, were completely negative for the organism. The palatine tonsils, submandibular and retropharyngeal lymph nodes of both infected piglets, 7 and 8, yielded Strep. suis which was recovered also from the tonsillar swab, parotid, deep cervical, right and left prescapular lymph nodes, the spleen, blood, CSF, elbow and tarsal synovial fluid of number 8.

### Histological Examination

The palatine tonsils of piglets 7, which failed to develop clinical disease, and 9, the control animal, presented a normal histological pattern. Crypt luminal debris was very variable in amount from crypt to crypt, mononuclear and occasionally polymorphonuclear cell infiltration of the epithelium was apparent in many areas, the interfollicular lymphoid tissue was well populated with lymphocytes and macrophages and a number of small to moderately-sized germinal centres were present. The tonsils of the clinically-ill piglet, 8, were essentially similar histologically to those of 7 and 9 described above. Plasma cells were observed in the subepithelial lymphoid tissue and the crypt epithelium (Fig.VII.1).

Examination of Gram-stained sections revealed Gram-positive organisms in the crypt lumina and epithelium of all three piglets. Marked variation in number was noted between crypts, many of which in piglets 7 and 9 were devoid of organisms. The tonsils of piglet 8 contained a greater Gram-positive bacterial population than those of the other two. No organisms were observed in the depths of the lymphoid parenchyma.

#### Immunofluorescent Microscopical Examination

Sections from piglets 7 and 8, stained with immune globulin, contained numerous specifically fluorescing bacteria, particulate and amorphous Strep. suis antigen within the tonsillar crypt lumina and, to a lesser degree, in the epithelium (Fig.VII.2). Antigen within the lymphoid tissue was negligible and none was observed in follicles or germinal centres. Sections from these two infected animals, stained with non-immune rabbit serum, and all sections from the non-infected control piglet proved completely negative for specific fluorescence.

Gram's stain on parallel cryostat and washed fluorescent sections demonstrated Gram-positive organisms in luminal and epithelial areas corresponding to those where specific fluorescence had been noted (Fig.VII.3) and also in similar but non-fluorescent sites. Such bacteria were also observed in the tonsillar crypts and epithelium of the control piglet, 9.

#### Electron-Microscopical Examination

Strep. suis organisms, when sectioned, stained, and examined ultramicroscopically, frequently occurred in pairs when a distinct

partition zone was usually apparent (Fig.VII.4). The cell wall and capsule were formed by a double membrane with outer short filamentous projections (Fig.VII.5).

An area of tonsillar crypt epithelium typical of those areas selected by light microscopy (Chapter II) for ultramicroscopical examination is illustrated in Fig.VII.6. Ultrathin sections from all three piglets revealed a marked heterogeneity of luminal detritus but other than noting that a variety of bacteria were present, no attempt was made to define its nature. Some at least of the bacteria in sections from 7 and 8 morphologically resembled Strep. suis (Fig.VII.7) but such a striking similarity could not be ascribed to those bacteria in the crypt lumina of the non-infected control piglet, 9.

Within the crypt epithelium of all three piglets, numerous cells contained a variety of foreign intra-cellular material (Fig.VII.8), frequently similar in character to that found in the adjacent lumen. This was sometimes free within the cytoplasm, sometimes contained in a phagosomal structure bounded by a double membrane. As in the lumen, bacterial cells (Fig.VII.9) or cellular fragments (Fig.VII.10) were observed. At no time were bacteria apparent in the intercellular areas. Although certain of the cells of the epithelium involved could not be defined morphologically as epithelial cells since desmosomes and tonofilaments were not apparent, a proportion at least were classical epithelial cells (Fig.VII.11) demonstrating the phenomenon of phagocytosis. In no case was the actual phagocytic process observed, although often the extraneous cellular content lay immediately below the luminal epithelial border.

The observations described above were essentially similar for the

three piglets, the infected two and the control. In piglets 7 and 8,

however, a number of the bacteria contained intra-cellularly bore a marked resemblance to Strep. suis, confirmed by cultural and fluorescent examinations, was established in the tonsillar epithelium of the control, piglet 9.

infected piglets. Histologically, Gram's stain identified organisms within the crypt epithelium, while Strep. suis was specifically

demonstrated in this site by immunofluorescent staining. Bacteria and bacterial products were readily observed intra-cellularly in the tonsillar crypt epithelium of all three piglets when examined at an ultramicroscopical level. Although not all the cells involved were classified, some at least were typical epithelial cells.

Morphologically, a proportion of the bacteria in the tonsillar epithelium of the infected pair markedly resembled Strep. suis. This, in conjunction with specific identification by immunofluorescence, permits the statement that Strep. suis was phagocytosed by crypt epithelial cells following introduction by the oral route.

Other reports of bacterial phagocytosis by epithelial cells (Staley et al., 1969; Dross & Varley, 1970b; Kenworthy, 1970) relate to the simple epithelium of the small intestine. Although the tonsillar crypts are lined by stratified epithelium, albeit sometimes reticulated and infiltrated by non-epithelial cells, the observations described here illustrate that the epithelial cells are capable of a similar phagocytic process. In the neo-natal intestinal epithelium no intra-cellular digestion of phagocytosed E. coli was observed (Staley et al., 1969). Within the tonsillar epithelium, both whole bacteria and bacterial fragments were to be found, raising the question of digestion taking place. It might be, however, that this observation was an example of the apparently non-selective nature of phagocytosis in the crypt lining since both bacteria and

Discussion

Tonsillar infection with Strep. suis, confirmed by cultural and fluorescent examinations, was established in the two experimentally-infected piglets. Histologically, Gram's stain identified organisms within the crypt epithelium, while Strep. suis was specifically demonstrated in this site by immunofluorescent staining. Bacteria and bacterial products were readily observed intra-cellularly in the tonsillar crypt epithelium of all three piglets when examined at an ultramicroscopical level. Although not all the cells involved were classified, some at least were typical epithelial cells. Morphologically, a proportion of the bacteria in the tonsillar epithelium of the infected pair markedly resembled Strep. suis. This, in conjunction with specific identification by immunofluorescence, permits the statement that Strep. suis was phagocytosed by crypt epithelial cells following introduction by the oral route.

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their breakdown products were also contained in the crypt luminal detritus.

In the work of Staley et al. (1969), the intra-cellular bacteria, irrespective of the number per cell, were invariably membrane-enveloped. Drees and Waxler (1970b), on the other hand, noted that the E. coli were always free within the cytoplasm of the intestinal epithelial cells. The intra-cellular location of bacteria and other material in the present study was however inconsistent, sometimes membrane-bound, at other times free. Enclosure of ingested material within a membrane-bound vesicle, a phagosome, is a feature of phagocytic cells (Carr, 1970; North, 1970) and may be followed by lysosomal fusion and digestion. The apparently free intra-cellular matter observed here may be sectioning or fixation artefact; otherwise its significance is obscure. As with Drees and Waxler (1970b), active phagocytosis was not observed, and although Staley et al. (1969) noted attachment of organisms to epithelial cells, they were unable to observe the process of ingestion beyond the primary invagination of the cell membrane.

In the present study, bacteria were not seen in epithelial intercellular spaces, for which a number of explanations are possible. The blocks and sections examined were perhaps too few; the infection-examination interval might not have been optimal; the selection for examination of mainly non-reticulated stratified areas could have precluded this observation; or intercellular bacterial traffic did not take place. Similarly, Staley et al. (1969) commented that E. coli were also absent from the intercellular areas. Although organisms were found in the lamina propria, they failed to witness any transfer of intra-cellular bacteria from the epithelial cells to the

lamina propria, correlating with the observations on antibody absorption by Kraehenbuhl and Campiche (1969).

Kenworthy (1970) on the other hand, studying the effect of pathogenic E. coli in germ-free and gnotobiotic piglets, categorically stated that no penetration of organisms to the lamina propria took place. A probably significant point of contrast here to the work of Staley et al. (1969) and Drees and Waxler (1970b) was that the piglets used by Kenworthy had been allowed colostrum and were 21 days of age, well outside the neo-natal absorptive phase. He determined the presence of bacteria within the epithelium by specific immunofluorescence staining, no mention of intra-cellular bacteria being made in his ultramicroscopical observations.

The intriguing aspect of the phenomenon of phagocytosis by the tonsillar epithelium is its function. With the stratified epithelium continuously maturing and desquamating into the crypt lumina, there must inevitably be loss of ingested material, irrespective of its nature. This, together with the earlier results (Chapters V & VI) which demonstrated penetration to the lymphoid parenchyma from the crypt lumina of both particulate and bacterial matter, serves to further enhance the postulate that epithelial phagocytosis might create a mechanism for sampling crypt contents, particularly antigens (Chapter V). The handling of antigens and the induction of immune responses are still incompletely understood but in many instances the macrophage is thought to be intimately involved (Unanue & Cerottini, 1970; Nossal & Ada, 1971). The inter-relationship between the tonsillar crypt epithelial cells and macrophages infiltrating from or within the lymphoid parenchyma has not been elucidated, limiting further hypotheses on the phagocytic function of the epithelium.

Stratified epithelial surfaces are normally considered a barrier mechanism by histologists; phagocytosis by the tonsillar crypt epithelium therefore creates a situation of considerable interest for further study.

#### Introduction

Studying the experimental transmission and epidemiology of the disease, Elliott et al. (1966) found that convalescent piglets and healthy littermates could yield the organism from throat swabs, although they did not attempt to isolate the organism from adult swine. Field et al. (1954) suggested that the source of infection was probably the sow. Within infected herds, a small proportion of adult animals were shown to harbour Strep. suis in the upper respiratory tract (Elliott et al., 1966). These data indicate that Strep. suis may exist in the oro-pharyngeal area of both young and adult pigs for a non-defined period, thereby creating inapparent infection, the carrier state. In addition, Strep. suis was recovered from the palatine tonsils of the three clinically healthy piglets described in Chapter VI almost a month following oral infection with the organism.

Gellier (1956b) found that Lancefield's Group B streptococci, the aetiological agents of cervical lymphadenitis of swine, could become established and maintain themselves in the tonsils and nasal cavity for an undetermined period. Apparently healthy carriers might be responsible for transmitting these Group B organisms to other susceptible pigs (Suits, 1959). Erysipelothrix insidiosa was found to persist in the palatine tonsils of swine for periods up to four months at least (Timoney & Barman, 1976), despite the fact that affected joints had become sterile. These observations further

## CHAPTER VIII

### STREPTOCOCCUS SUIS INFECTION IN THE OLDER PIG

#### Introduction

Studying the experimental transmission and epidemiology of the disease, Elliott et al. (1966) found that convalescent piglets and healthy littermates could yield the organism from throat swabs. Although they did not attempt to isolate the organism from adult animals, Field et al. (1954) suggested that the source of infection was probably the sow. Within infected herds, a small proportion of adult animals were shown to harbour Strep. suis in the upper respiratory tract (Elliott et al., 1966). These data indicate that Strep. suis may exist in the oro-pharyngeal area of both young and adult pigs for a non-defined period, thereby creating inapparent infection, the carrier state. In addition, Strep. suis was recovered from the palatine tonsils of the three clinically healthy piglets described in Chapter VI almost a month following oral infection with the organism. Collier (1956b) found that Lancefield's Group E streptococci, the aetiological agents of cervical lymphadenitis of swine, could become established and maintain themselves in the tonsils and nasal cavity for an undetermined period. Apparently healthy carriers might be responsible for transmitting these Group E organisms to other susceptible pigs (Switzer, 1959). Erysipelothrix insidiosa was found to persist in the palatine tonsils of swine for periods up to four months at least (Timoney & Berman, 1970), despite the fact that affected joints had become sterile. These observations further

demonstrate that pathogens may be carried or harboured in the oropharyngeal region of pigs.

In man, a carrier state in the oro-naso-pharynx is well recognised in relation to Lancefield's Group A streptococci and various aspects of this phenomenon have been investigated by a number of workers (Harvey & Dunlap, 1964; Mozziconacci, Gerbeaux, Baculard, Gerbeaux & Caravano, 1966; Quinn & Lowry, 1970). Although Quinn and Lowry (1970) set out specifically to define the anatomical areas involved in the carrier state, the investigation was limited of necessity to a swabbing sampling technique and cultural examination. The tonsils, oro- and naso-pharynx were found to be the most rewarding of the sites sampled. These workers commented that "there is incomplete information concerning which histologic structures are involved...or the microscopic location of streptococci during the carrier state or infection", summing up the present situation in the medical field.

As noted in the Strep. suis infection described earlier, a healthy asymptomatic carrier state developed in three piglets. Immunofluorescent microscopy demonstrated the organism not only in the tonsillar crypts but also in the subepithelial lymphoid tissue, with antigenic breakdown products in these same areas, the interfollicular tissue and infrequently in germinal centres. It is not legitimate, however, to infer that such involvement of the tonsillar tissues is representative of the Strep. suis carrier state in all pigs since the three piglets were within the age group clinically susceptible to the organism.

Agarwal et al. (1969) demonstrated in the serum of adult pigs a bacteriostatic factor effective against Strep. suis. They suggested

Materials and Methods

that this component, probably IgM, might be a factor in adult immunity to infection with Strep. suis and could be a manifestation of specific immunity subsequent upon subclinical infection early in life. In adult animals, therefore, the localisation of the organism in the carrier state might be substantially influenced by this phenomenon.

The experiment detailed below was carried out to establish Strep. suis in the older pig and to examine the carrier state in relation to the palatine tonsils.

A 20 hour HSB culture of Strep. suis was grown, checked for purity, prepared and counted as described in Chapter VI.  $9.25 \times 10^6$  colony-forming units were present per ml. The pigs were restrained and the infective doses, 1ml each of the HSB culture, applied with a small sterile brush topically to the palatine tonsils.

Clinical Procedures

Clinical examination of the pigs was carried out daily. Rectal temperatures were noted from day 0 to day 9 (day 0 = day of infection). Tonsillar swabs were taken daily for cultural examination for Strep. suis from day 1 to 9, and again on days 20 and 21.

On days 4, 6 and 7, faecal swabs from pigs 24, 21 and 22, and 23 respectively were examined for enteric pathogens.\*

Post-mortem Bacteriologic Examination

Post-mortem examination was carried out on pig 24 when it died on day 6 and on 21, 22 and 23 following euthanasia on day 24.

\*Carried out by Dr. G. H. K. Lawson, Dept. of Veterinary Pathology, Edinburgh University.

Materials and Methods

Four weaned pigs, littermates of six weeks of age from the minimal disease herd, were employed in this experiment. The four were penned individually within a single loose box. All four pigs were infected as further control material was not considered necessary for this particular experiment. They were numbered PM34/21, PM34/22, PM34/23 and PM34/24, hereafter referred to as 21, 22, 23 and 24.

Infection Procedure

A 20 hour HSB culture of Strep. suis was grown, checked for purity, prepared and counted as described in Chapter VI.  $9.25 \times 10^6$  colony-forming units were present per ml. The pigs were restrained and the infective doses, 1ml each of the HSB culture, applied with a small sterile brush topically to the palatine tonsils.

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On days 4, 6 and 7, faecal swabs from pigs 24, 21 and 22, and 23 respectively were examined for enteric pathogens.\*

Post-mortem Macroscopic Examination

Post-mortem examination was carried out on pig 24 when it died on day 6 and on 21, 22 and 23 following euthanasia on day 34.

\*Carried out by Dr. G. H. K. Lawson, Dept. of Veterinary Pathology, Edinburgh University.

Histological Examination

Tissues from pig 24 were not examined histologically. Blocks from the palatine tonsils of pigs 21, 22 and 23 were fixed and processed routinely; sections were stained with H & E, Gram's stain, methyl-green/pyronin and impregnated with silver.

Bacteriological Examination

Post-mortem, the palatine tonsils, submandibular lymph nodes, spleen, CSF, carpal and tarsal synovial fluid of pig 24 were culturally examined for Strep. suis. From 21, 22 and 23 these tissues and fluids, together with the retropharyngeal and parotid lymph nodes and blood samples, were similarly examined.

Immunofluorescent Microscopical Examination

Blocks of the palatine tonsils of pigs 21, 22 and 23 were frozen for immunofluorescent microscopical examination. Cryostat sections were stained with immune and non-immune serum to examine for Strep. suis antigen. Parallel sections were stained with H & E, methyl-green/pyronin and Gram's stain; washed fluorescent sections were examined using Gram's stain.

Parallel cryostat sections were also directly stained with rabbit anti-pig serum globulin conjugated with FITC (RAP) to examine by immunofluorescent microscopy for the presence of globulin in the palatine tonsils. The staining procedure was as follows:

Section	+	RAP	30 minutes
"	+	PBS pH7.2, 0.01M	2 x 5 minutes

The sections were mounted immediately in 10% glycerol/90% PBS pH7.2, 0.01M.

\*0 group not examined.

in which all three pigs developed diarrhoea, symptoms of clinical disease were completely absent. No evidence of involvement of the central nervous system or the limb joints was apparent at any stage.

### Results

#### Clinical Procedures

Infection with Strep. suis was carried out immediately upon weaning the four pigs from their dam in the minimal disease unit and the weaning and separation into individual pens caused them considerable distress for the first few days. They failed to settle, continually sought to enter adjacent pens, sometimes succeeding, and were readily excited when approached.

Rectal temperatures are presented in Table VIII.1. Pig 24 developed diarrhoea on day 3, and a faecal swab examined on day 4 yielded a heavy growth of a pathogenic strain of E. coli, antigenic structure O149: K91, K88ac, which has previously been associated with cases of colibacillosis in the minimal disease herd. Diarrhoea became more marked on days 4 and 5 and the pig died on day 6. Pigs 21 and 23 developed diarrhoea for a few days from day 5, yielding a heavy growth of haemolytic E. coli, O149?: K91, K88ac, from faecal swabs on days 6 and 7 respectively. Pig 22 developed a much less marked diarrhoea which was not associated with the presence of

pathogenic E. coli serotypes on examination of a faecal swab taken on day 7. No antibiotic therapeutic measures were adopted to avoid influencing the course of the Strep. suis infection. The three remaining pigs recovered rapidly and thereafter thrived steadily.

The recovery of Strep. suis from the tonsillar swabs is summarised in Table VIII.2. Pig 23 yielded the organism for the first time on day 3, 21 on day 4, 24 on day 6 and 22 on one occasion only on day 8. Intermittent recovery of Strep. suis was made from the tonsillar swabs from 21 and 22. Apart from the initial period

in which all three pigs developed diarrhoea, symptoms of clinical disease were completely absent. No evidence of involvement of the

The palatine tonsils of pigs 21, 22 and 23 presented an essentially similar histological appearance. The majority of crypts throughout the duration of the experiment.

were relatively free of cellular debris. Infiltration and infiltration of the crypt epithelium was an extremely variable feature

Post-mortem Macroscopic Examination

in all three, being very extensive in some crypts, much less marked

in others. Examination of pig 24 revealed a markedly dehydrated carcass,

some loss of bodily condition and very flaccid fluid-filled intestines. These findings are compatible with a diagnosis of death from colibacillosis. No gross abnormalities of the palatine tonsils or head lymph nodes were observed, nor of any other body systems.

Macroscopic examination of pigs 21, 22 and 23 revealed the

carcasses to be well-nourished and well-grown. All body systems,

including the central nervous system and limb joints, were without

abnormality. The palatine tonsils were grey/white in colour both

superficially and on section, and firmly cellular; the submandibular,

retropharyngeal and parotid lymph nodes presented a moderate, but not abnormal, degree of reactivity.

abundant Strep. suis antigen. Autofluorescence and non-specific

fluorescence, as described in chapter VI, were noted in both these and

Bacteriological Examination

The sections stained with anti-immune serum. The specific fluorescence

of The isolation of Strep. suis from tissues and fluids sampled

post-mortem is detailed in Table VIII.3. Strep. suis was recovered

from the tonsils and submandibular lymph nodes of all four pigs, from

the retropharyngeal nodes of 22 but not from any other sites.

No further examinations were carried out on pig 24 since the

experiment was designed to investigate antigen distribution following

a prolonged infection. bacteria were identified singly, often in pairs

### Histological Examination

The palatine tonsils of pigs 21, 22 and 23 presented an essentially similar histological appearance. The majority of crypts were relatively free of cellular detritus. Reticulation and infiltration of the crypt epithelium was an extremely variable feature in all three, being very extensive in some crypts, much less marked in others. Infiltrating cells comprised cells of the lymphocytic series, plasma and other pyroninophilic cells and some neutrophil polymorphs. Germinal centre development was marked: the majority were active and were moderate to large in size. The interfollicular tissue was not extensive nor very cellular so that the connective tissue stroma was readily apparent. Large pyroninophilic cells were relatively numerous in this area.

### Immunofluorescent Microscopy

Examination of sections of the palatine tonsils of all three pigs, stained with immune globulin, demonstrated a widespread distribution of abundant Strep. suis antigen. Autofluorescence and non-specific fluorescence, as described in Chapter VI, were noted in both these and the sections stained with non-immune serum. The specific fluorescence of Strep. suis or its antigenic breakdown products was completely absent from these latter sections.

Specifically fluorescing bacteria were frequently observed within the tonsillar crypts (Fig.VIII.1). The numbers of organisms per crypt and of crypts involved per section, however, showed considerable variation, indicating a non-uniform pattern of distribution throughout the tonsils. Whole bacteria were identified singly, often in pairs

and sometimes in large loose aggregations; particulate and amorphous antigen was also present, similarly scattered and aggregated. The crypt epithelium contained bacteria and antigen (Fig.VIII.2), often in amounts proportional to that in the adjacent lumina. In the immediate subepithelial zone, Strep. suis antigen occurred more commonly in the particulate or amorphous form (Fig.VIII.3) although intact bacteria were also to be found. The distribution was extremely variable but was frequently related to abundant antigen in the crypt lumina and epithelium. Although, in many instances, antigen in epithelial and subepithelial areas appeared to closely invest or be contained within cells, this could not definitely be established. Where whole organisms were observed, confirmation was obtained by examining these and parallel sections using Gram's stain: Gram-positive bacteria were noted both extra- and intra-cellularly (Fig.VIII.4). In the majority of sections examined, little antigen was found deep in the interfollicular tissue. A few areas contained particulate antigen (Fig.VIII.5) in close association with cells and in some instances distinctly within the cells. Macrophages, exhibiting amorphous, diffuse, cytoplasmic fluorescence, were occasionally present, showing a scattered distribution (Fig.VIII.6). These occurred more often in the vicinity of or on the periphery of germinal centres.

The most striking feature of these tonsillar sections was the generalised occurrence of abundant particulate Strep. suis antigen in germinal centres. Although the numbers of centres involved varied from section to section, specific fluorescence was demonstrated in the majority. The antigen particles were rarely aggregated; rather

they were irregularly scattered, mainly throughout the inner zone of the centres (Fig.VIII.7). That some at least of this particulate antigen was located peripherally in relation to the germinal centre cells was illustrated by separation from the cell nuclei by non-fluorescent cytoplasm; the exact cellular relationship of the remainder was difficult to determine with any accuracy (Fig.VIII.8). Comparison with parallel sections stained H & E failed to specifically identify these centre cells amongst the macrophages, lymphocytes and intermediate blast-cell types present.

Ig globulin was demonstrated in sections of the palatine tonsils stained directly with RAP: the specific fluorescence was therefore less bright compared with that of Strep. suis antigen which was stained using an indirect "sandwich" technique. Uniformly amorphous in character, globulin was observed in crypt and vascular lumina, in cells of the interfollicular tissue and in germinal centres. Cells with diffusely-fluorescent cytoplasm, present in the interfollicular areas, were never numerous but generally were found in groups of two or three. These cells were irregular in shape and in the main were large with abundant cytoplasm. Their exact nature was not established.

As with the particulate Strep. suis antigen noted above in germinal centres, the globulin was distributed mainly in the inner zone (Fig.VIII.9) but differed in forming a ring of fluorescence around nuclei, in being less abundant and involving fewer centres.

Examination of parallel sections to those stained for antigen failed to illustrate a correlation between globulin and antigen presence in germinal centres: although both did occur in the same centres, other centres contained solely antigen or solely globulin.

Discussion

Strep. suis, administered orally to six-week-old pigs, became established in the palatine tonsils from which it was recoverable five weeks later. Of the four pigs on experiment, the three which survived a natural infection of colibacillosis did not manifest signs of clinical streptococcal infection nor was evidence of systemic disease detected post-mortem: a Strep. suis carrier state had therefore developed. In addition to the tonsils, the organism was cultured from the submandibular lymph nodes of all pigs and in one animal from the retropharyngeal nodes. Histologically the tonsils were unremarkable, presenting a normal pattern of reactivity for this age. Strep. suis antigen, either as whole bacteria or as breakdown products, was widely distributed throughout the tonsils, with marked involvement of the germinal centres. Similarly globulin was demonstrated in the centres, although to a much lesser degree, also in the crypt and vascular lumina and in cells of the interfollicular tissue.

In view of the age susceptibility to clinical disease with Strep. suis (Field et al., 1954; Elliott et al., 1966), it might have been anticipated that in these older pigs the organism would be confined to the crypt lumina on the periphery of the immune environment. It was assumed here that the bacteriostatic factor described by Agarwal et al. (1969) had developed in these animals which were eleven weeks of age at the termination of the experiment. Contrary to expectations, however, organisms were present not only in the lumina but also in the tonsillar parenchyma and, in addition, were recovered from the tonsillar drainage lymph nodes. Although bacteria were demonstrated in the lymphoid substance of the tonsils, Strep. suis antigen was more

commonly observed in a particulate or amorphous form and it seems reasonable to postulate that the crypt bacterial population served as an antigen reservoir.

Antigen-bearing macrophages were not numerous within the interfollicular lymphoid tissue, despite abundant antigen in related crypts. Interestingly, those observed were often in the vicinity of germinal centres, possibly transporting antigen towards them.

Such limited macrophage activity "in the midst of plenty" further supports the concept of a tonsillar antigenic sampling mechanism.

Macrophages are believed to perform a dual role of degradation and clearance of foreign material and the retention of immunogenic fractions for the initiation of some immune responses (Unanue & Cerottini, 1970). In the immediate subepithelial areas, where larger depots of antigen were to be found, perhaps much was degraded in and eliminated via macrophages infiltrating the epithelium and being lost into the crypt lumina, an hypothesis impossible to substantiate in a non-dynamic study.

Strep. suis antigen was abundant in the majority of tonsillar germinal centres. These structures, along with the medullary macrophages of lymph nodes, are the sites within lymphoid tissue of antigen retention (Nossal & Ada, 1971). Corresponding with the observations of others (White, French & Stark, 1970; Nossal & Ada, 1971) on particulate antigen retained in germinal centres on the surface membrane and cytoplasmic processes of the follicular dendritic cells, Strep. suis antigen in the tonsillar germinal centres existed in a particulate form, apparently distributed peri-cellularly. Whether or not the abundance of antigen resulted from accumulation over the period of the experiment or from long-term retention

following the original infection cannot be established. White et al. (1970) commented however that it became progressively more difficult to discern antigen in the chicken splenic germinal centres but it was still present six weeks post-injection. In this experiment, on the other hand, antigen fluorescence in the tonsillar germinal centres was strong, bright and readily visible at five weeks post-infection. It might therefore be inferred that the tonsillar germinal centres were able to draw on an antigen source, the crypt population of Strep. suis, employing macrophages to traffic from the subepithelial areas to the centres. Although germinal centre function is still incompletely understood, they are considered to be indicators of immunological reactivity. One can therefore postulate that the collection, degradation and localisation of Strep. suis in the palatine tonsils of the pigs in this experiment served an immunological role, and that the carrier state is not a purely passive phenomenon.

Follicular trapping of antigen is believed to be dependent on the presence of antibody although the nature of the antibody, specific or "natural", required for different antigens systems would seem to be variable (Nossal & Ada, 1971). Agarwal et al. (1969) demonstrated in the serum of older pigs a bacteriostatic factor, probably IgM in character, which they suggested might result from inapparent infection early in life. It is not clear, however, from their work if the conventionally-reared pigs employed originated from herds in which Strep. suis is endemic, suggesting the alternative possibility that it is a non-specific or "natural" antibody factor appearing with age. In this experiment the pigs used were encountering Strep. suis for the first time and all demonstrated immunity to overt disease. Interestingly, the distribution of antigen was essentially no different

to that observed earlier (Chapter VI) in the younger pigs. The sites involved were identical but quantitatively antigen was markedly more abundant in the germinal centres: perhaps this latter observation is related to the development of antibody and promotion of antigen trapping.

#### Introduction

As a macroglobulin, IgM is however largely confined to the circulation: unless local IgM production takes place, Strep. suis may be able to penetrate into the tonsils of any age of pig, regardless of the serum bacteriostatic factor. Studying tonsillar immunoglobulin production, Ricci and Russolo (1970) found that the hypertrophied tonsils of children produce mainly IgM, compared with IgA in the normal adult tonsil. Burtin and Buffe (1967) demonstrated that the majority of tonsillar germinal centres reacted only with anti-IgM, but unfortunately they failed to specify the age group from which their material was obtained. The palatine tonsils of the young animal may therefore respond to antigen by IgM production. In this experiment pig globulin was demonstrated in the crypts, cells of the inter-follicular areas and in germinal centres but neither the character of the globulin nor a relationship between Strep. suis antigen and the globulin was established.

In conclusion, the observations made here on these pigs in which a Strep. suis carrier state was established do not suggest that any real difference in tonsillar involvement exists between the two age groups of pigs examined.

In man, antibody formation is induced by streptococcal tonsillitis. The situation in the carrier state, however, appears to be variable. In one outbreak in young adults due to a specific

ATTEMPTS TO DEMONSTRATE HUMORAL ANTIBODY AFTER EXPOSURE  
TO STREP. SUIIS AND THE RELATED HISTOLOGICAL CHANGES

Introduction

Strep. suis infection as a clinical disease is confined to the piglet (Field et al., 1954). In experimental infections, however, Elliott and his co-workers (1966) were able to passively protect pigs within the susceptible age group by the transfer of sow serum. Adult immunity to overt disease has been associated with the presence of an IgM bacteriostatic component in the serum, effective specifically against capsulated pathogenic strains of Strep. suis (Agarwal et al., 1969). Its development, these workers suggested, might be stimulated by inapparent infection with the organism in early life as bacteriostatic activity is usually present in the serum by six to eight weeks of age in conventionally-reared animals. Under both natural and experimental conditions healthy piglets may be found to be infected with Strep. suis and a small percentage of adult carrier animals has also been demonstrated in infected herds (Elliott et al., 1966). In inapparent infection in the piglet, the organism is recoverable from the throat (Elliott et al., 1966) and is localised in the palatine tonsils and submandibular lymph nodes (see Chapter VI) so that if antibody production is stimulated, it is likely to be present in these tissues. In man, antibody formation is induced by streptococcal tonsillitis. The situation in the carrier state, however, appears to be variable. In one outbreak in young adults due to a specific

type within Group A, antibody responses were demonstrated in nearly half of the healthy carriers as well as in the convalescent patients but transient carriers did not exhibit serological activity (Commission on Acute Respiratory Diseases, 1945); and in an investigation on children only a small proportion of healthy individuals completely lacked serological evidence of their carrier state (Mozziconacci, Gerbeaux, Baculard, Gerbeaux & Caravano, 1966). Harvey and Dunlap (1964), studying the carrier state in relation to streptococcal disease, found that in general an inverse relationship existed between the carrier rate and disease and that where illness developed in the carrier group, it followed the acquisition of a Group A type different from those previously carried. These workers therefore suggested that children might acquire type-specific streptococcal immunity through the carrier state, enabling them to resist type-specific infection in adulthood, analogous to the theory advanced by Agarwal et al., (1969) in relation to Strep. suis.

The hypothesis that antibody to Strep. suis might be present in the palatine tonsils of infected and carrier animals would be substantiated if specific immunoglobulin could be observed using immunofluorescent techniques and detected serologically. There are however few reports in the literature (McGavran, White, Eigelsbach and Kerpsack, 1962; White, McGavran, Prickett, Tulis and Eigelsbach, 1962; Eveland, 1964; Nayebi & Eveland, 1971) of the detection of specific antibody using bacterial antigens in immunofluorescent systems, none involving streptococci and only one (Timoney & Berman, 1970) employing pig tissues. A prerequisite here in the study of responses to Strep. suis infection was the development of a technique for the demonstration of specific antibody in tissues. Agarwal et al.,

(1969) were unable to demonstrate the IgM factor bacteriostatic for Strep. suis in a wide range of other in vitro tests, having to rely on passive protection of piglets as an alternative assay of its presence. In clinical infection of the pig with Group E streptococci, however, precipitating and haemagglutinating antibodies have been detected (Schuman & Wood, 1966; Wessman, Schuman & Nord, 1970).

Investigation of antibody responses to Strep. suis was therefore indicated at both a serological and histological level. In the first instance, to evaluate the efficacy of the techniques employed, the study was carried out on animals hyperimmunised with Strep. suis.

## Materials and Methods

The work described in this chapter involved three separate groups of animals: the first consisting of a single discard pig, 1071, aged approximately four months, the second of three similar pigs, 323, 332 and 333 and the third of six adult mice, PM1 - 6.

### Preparation of Antigens

#### Hydrochloric acid extracted antigen (HCLA):

Strep. suis antigen was extracted from 30ml HSB overnight culture of the organism employing hydrochloric acid as described in Chapter II and was dialysed against 50ml PBS pH7.2, 0.01M at 40°C for 2 hours. The antigen thus prepared gave a strong precipitin reaction when tested with the PM34/A and B antisera to Strep. suis. It was stored at -20°C.

#### Freeze-thaw extracted antigen (FT):

The preparation of this antigen was based on the method described by Timoney and Berman (1970) for Erysipelothrix insidiosa. 24 hour HSB cultures (5 x 100ml) of Strep. suis were centrifuged at 777g for 30 minutes and the supernatant decanted for use in the preparation of culture filtrate antigen. The cells were washed twice with 0.15M sodium chloride and resuspended in distilled water. The suspension was frozen at -20°C, rapidly thawed and the process repeated eight times. Following centrifugation at 777g for 30 minutes, the supernatant was decanted, filtered through a 0.3µ filter (Millipore), checked for antigenic activity against the PM34/A and B antisera and stored at -20°C.

#### Culture filtrate antigen (CF):

The method of preparation of this antigen was adapted from that described by Schuman and Wood (1966) for Group E streptococci. 20ml of the supernatant HSB culture medium decanted above was filtered through a Seitz filter and tested for sterility by overnight incubation of a sample. The antigen was checked against the PM34/A and B antisera, dispensed in 5ml aliquots, thiomersal added at 1:10,000 and it was stored at  $-20^{\circ}\text{C}$ .

Concentrated culture filtrate antigen (CCF):

100ml of the HSB culture medium were concentrated overnight against polyethylene glycol and dialysed twice against PBS pH7.2, 0.01M, at  $4^{\circ}\text{C}$  producing a final volume of 20ml. Following filtration through a  $0.3\mu$  filter (Millipore), the antigen was checked against the PM34/A and B antisera, and stored with the addition of thiomersal at 1:10,000 at  $-20^{\circ}\text{C}$ . (Method adapted from Schuman & Wood, 1967).

Immunisation Procedures

Group 1. - Pig 1071:

The deposit from 10ml of a 20 hour HSB culture of Strep. suis was resuspended in 0.2% formalised PBS pH7.2, 0.01M, for half an hour at room temperature, washed twice and finally suspended in 1ml PBS pH7.2, 0.01M. On four successive days a suspension of killed cells prepared thus was injected intravenously (ear vein). On days 7 and 22 the pig received intravenously the live bacterial cell deposit from 4ml HSB culture of Strep. suis resuspended in 1ml PBS pH7.2, 0.01M. Euthanasia was performed on day 36. Serum from an ante-mortem blood sample was stored at  $-20^{\circ}\text{C}$ . For histological

examination, blocks from the tonsils and spleen were routinely fixed in formol saline and for immunofluorescent studies stored in liquid nitrogen. Paraffin sections were stained with H & E and methyl-green pyronin.

Group 2 - Pigs 323, 332 and 333:

The deposit from 10ml of an 18 hour culture of Strep. suis was resuspended in 5ml PBS pH7.2, 0.01M, and thoroughly emulsified with 5ml Freund's incomplete adjuvant. Each pig received on days 1 and 8 1ml at three sites: subcutaneously anterior to the spine of the left scapula and intra-muscularly into the semitendinosus area of both hind legs. On day 15 each pig was injected at the same sites with 1ml of the deposit from 20ml of a 20 hour HSB culture resuspended in 10ml PBS pH7.2, 0.01M.

Blood samples were taken from the anterior vena cava pre-vaccination, and weekly from day 8. Euthanasia was carried out on day 56. Blocks of the internal iliac and left prescapular lymph nodes and spleen were taken and sections stained as for 1071 above.

Group 3 - Mice PM1 - 6:

The procedure employed to hyperimmunise the mice was adapted from Timoney and Berman (1970). 4 x 5ml 20 hour HSB cultures of Strep. suis were checked for purity of growth by Gram's stain and pooled. The mice were injected intra-peritoneally with live bacteria on days 1, 6, 8 and 12 as follows: PM1, PM2, PM3, PM4 and PM5 received the deposits, resuspended in 0.5ml PBS pH7.2, 0.01M, from 0.5ml, 0.1ml, 0.05ml, 0.01ml and 0.005ml of the original HSB culture respectively; PM6 was given a control injection of 0.5ml PBS. The mice were killed on day 18. Blocks of spleen were

fixed in formol saline for histological examination and stored in liquid nitrogen for immunofluorescent microscopy and sections were stained as for Groups 1 and 2 above.

### Immunofluorescent Microscopy

#### Demonstration of antibody to Strep. suis:

Frozen sections from Groups 2 and 3 were cut and fixed as previously described (Chapter II). In an attempt to demonstrate antibody to Strep. suis by immunofluorescence, the following staining method was adopted:

Section	+	FT	30 minutes
"	+	PBS pH7.2, 0.01M	2 x 5 "
"	+	PM34/B	30 "
"	+	PBS pH7.2, 0.01M	2 x 5 "
"	+	GAR-FITC	30 "
"	+	PBS pH7.2, 0.01M	2 x 5 "

GAR-FITC used to stain mouse spleen was additionally absorbed for 1 hour with homogenised fresh mouse liver. A number of parallel sections were employed as controls as follows:

Negative antibody/positive antigen control

- stained as detailed in Chapter II

to demonstrate injected Strep. suis  
antigen

Negative control - unstained

Negative control - section stained with GAR-FITC only

Negative control - PM34/B replaced by non-immune rabbit  
serum

Further parallel sections were fixed and stained with H & E and methyl-green/pyronin for histological examination.

To test an individual serum, it was placed in a central sealed well of 15mm diameter and the antigens placed in four wells of 7mm diameter.

Demonstration of pig globulin:  
Frozen sections from pig 1071 were stained directly with RAP-FITC as in Chapter VIII; parallel sections were stained with H & E and methyl-green pyronin.

Demonstration of pig IgM and IgG:

Frozen sections from pig 1071 were stained indirectly with rabbit anti-pig IgM (RAPIgM)\* or rabbit anti-pig IgG (RAPIgG)\* followed by GAR-FITC using the procedure described in Chapter II. Parallel sections were stained with H & E and methyl-green/pyronin, and for pig globulin.

### Serological Methods

#### Capsular swelling reaction:

The terminal sera from Groups 1 and 2 were tested with Strep. suis organisms from an overnight HSB culture for capsular swelling activity, a specific test used for serotyping pneumococci (Cruickshank, 1965). PM34/A antiserum was employed to give a positive control reaction.

#### Modified capillary microprecipitin test:

The serum of pig 1071 was tested for specific Strep. suis antibody in the modified microprecipitin technique (Chapter II) with the HCLA, CF, CCF and FT antigens. PM34/A antiserum was employed as a positive control serum.

#### Agar-gel diffusion tests:

\*Kindly supplied by Dr. K. A. Linklater, Department of Animal Health, Edinburgh University.

1% agar-gel (Oxoid L 12), borate-buffered at pH8.6, was used to fill the petri dishes in which the tests were carried out. To test an individual serum, it was placed in a central sealed well of 15mm diameter and the antigens placed in four wells of 7mm diameter, equidistant at 7mm from the central well. To test individual antigens against more than one serum simultaneously, antigen was placed in a central well of 7mm diameter and the sera placed in outer wells of 10mm diameter, equidistant at 6mm from the central well. The plates were incubated in a moist atmosphere within a closed container at 32°C and examined at 24, 48 and 72 hours. The serum of pig 1071 was tested with the HCLA antigen and the sera of the pigs in Group 2 with the CF, CCF, FT and HCLA antigens. HSB was included as a control antigen when testing the sera of Group 2. PM34/A antiserum was employed as a positive control serum throughout.

the spleen. When stained with Results and Fluorescent specific fluorescence was not observed and therefore the G and H classes of Group 1 antibody were not detected.

Histological Examination:

Extensive reticulation with mononuclear cell infiltration, including plasma cells, of the crypt epithelium was apparent in the tonsils of pig 1071. Germinal centres were numerous, large, densely cellular and active, showing many mitotic figures. The interfollicular lymphoid areas were extensive and were filled with lymphocytes, macrophages and a moderate number of plasma cells, particularly in the subepithelial zones. Plasma cells were confirmed in sections stained with methyl-green/pyronin which also demonstrated deep pyroninophilia of the majority of germinal centres and large pyroninophilic cells in the interfollicular areas.

The peri-arteriolar lymphoid tissue of the spleen was well-developed but only a moderate number of germinal centres were present. Macrophage hyperplasia of the red pulp was very marked. Plasma cells were relatively abundant. Pyroninophilia of the peri-arteriolar lymphocytic aggregations, large pyroninophilic cells in the red pulp and plasma cells were demonstrated in sections stained with methyl-green/pyronin.

Immunofluorescent Examination:

In the tonsils, pig globulin was demonstrated in the germinal centres, as cytoplasmic fluorescence in cells of the interfollicular lymphoid tissue and subepithelial areas, and in the crypt lumina, corresponding with the observations made in the previous chapter, and similarly in scattered cells of the red pulp and germinal centres of

the spleen. When stained with RAPIgG and RAPIgM specific activity fluorescence was not observed and therefore the G and M classes of immunoglobulin were not detected.

Group 2

Histological Examination:

The tissues of the three pigs in this Group were essentially similar in histological appearance and are therefore described together. The spleens and lymph nodes all showed very marked macrophage hyperplasia and activity. The lymphoid tissue, on the other hand, presented a lack of uniform activity: in some areas of the nodes and spleen, it was moderately hyperplastic and presented a number, although not abundant, of germinal centres; in others, however, it appeared relatively depleted of lymphoid cells. Clustered and scattered plasma cells and pyroninophilia of the centres, which were densely cellular and active, were specifically demonstrated with methyl-green/pyronin.

Immunofluorescent Microscopy:

Attempts to demonstrate antibody to Strep. suis in the spleens and iliac lymph nodes were unsuccessful, no differences being detectable between the control slides to demonstrate Strep. suis antigen used for immunisation and the test slides. Antigen was abundantly distributed within the areas of macrophage hyperplasia.

Group 3

Histological Examination:

The spleens of the mice PM1 - 5 all showed marked macrophage

hyperplasia and a moderate degree of lymphoid white pulp activity with a small number of germinal centres. Plasma cells were scattered throughout the red pulp. Pyroninophilia of the centres was demonstrated and the presence and distribution of plasma cells confirmed. The control mouse spleen did not exhibit macrophage hyperplasia and the lymphoid tissue was less developed.

Immunofluorescent examination:

Strong autofluorescence and large amounts of widely distributed antigen in the immunised spleens created difficulty in interpretation but as in Group 2, no antibody to Strep. suis was demonstrable.

Capsular Swelling Reaction

The terminal sera of the pigs in both Groups 1 and 2 failed to produce any perceptible swelling of the capsules of Strep. suis organisms.

Microprecipitin Test

The serum of pig 1071 did not react with any of the four antigens, CF, CCF, FT or HCLA.

Agar-Gel Diffusion

The serum of pig 1071 failed to exhibit any precipitating activity with the HCLA antigen, which, in common with the other antigens, FT, CF and CCF, gave one or more distinct precipitation lines with the PM34/A antiserum in the control reaction.

The pre-vaccination sera of the Group 2 pigs were all negative for

precipitating activity with all four antigens. The terminal sera similarly failed to react with the FT and HCLA antigens. They all reacted positively with the CCF antigen, each producing three distinct precipitation lines by 24 hours and the 332 and 333 sera with the CF antigen in similar but weaker reactions. Since, however, these positive reactions were common to HSB, the control antigen, they were attributed to the inclusion of small amounts of HSB in the immunising doses and no further sera were tested.

The negative observations in respect of immunoglobulin are inexplicable, particularly as a representative sample and in the presence of plasma cells. It may be that the purified antigens were of poor titre, and the negative reactions obtained in were sensitive than the blood group was not to detect them. The determination of the nature of the immunoglobulin response to HSB. This was however, compared to the sera of the immunisation into specific antibody responses of the blood group in the area was considered.

The fact of immunoglobulin response to HSB, and not to HSB, accounted for the negative results of the blood group. The results were attributed to the fact that the antibodies employed for the blood group were not of the same nature. Failure to demonstrate antibody response to HSB, however, is in agreement with the fact that the blood group was unable to show antibody response to HSB. The results of the methods other than the blood group, however, are in agreement with the results obtained here in the blood group. The results of the blood group support their findings. The fact that the blood group was unable to show antibody response in this connection is in agreement with the results of the blood group.

## Discussion

The attempts to demonstrate antibody to Strep. suis following hyperimmunisation of pigs and mice proved unsuccessful, despite the observations of plasma cells and lymphoid activity in the tissues examined. Although in the one animal examined, pig globulin was demonstrated by immunofluorescence, neither the G nor M class of immunoglobulin was detected.

The negative observations on classes of immunoglobulin are inexplicable, particularly in a hyperimmunised state and in the presence of plasma cells. In addition, the specific antisera were of good titre, and the indirect "sandwich" technique is more sensitive than the direct which was used to detect globulin. The determination of the nature of the immunoglobulin response to Strep. suis was however peripheral to the main line of investigation into specific antibody production so that further research in the area was considered unjustified.

The lack of demonstrable antibody to Strep. suis may be accounted for in two ways: either no antibody of the type sought was stimulated by the immunisation procedures adopted or the methods employed for its detection were insufficiently sensitive. Failure to demonstrate antibody by these serological methods, however, is in agreement with the findings of Agarwal et al. (1969) who were unable to show the presence of antibody to Strep. suis by in vitro methods other than the bacteriocidal test. The negative results obtained here in the capsular swelling reaction further support their findings. A major difference, however, lies in the fact that in this investigation the animals tested had been hyperimmunised with Strep. suis while Agarwal et al. (1969) were dealing with a

serum component of uncertain development but one which they suggested might have been stimulated by natural infection. It was anticipated that some form of antibody would have been detectable even if not synonymous with the IgM component described by these workers.

Positive reactions were readily observed with the rabbit antiserum, confirming the activity of the antigens employed.

In pigs abscessed following experimental oral infection with Group E streptococci, Schuman and Wood (1966) were similarly unable to detect precipitating antibodies in agar-gel diffusion tests with hydrochloric acid extracted antigen. In contrast, culture filtrate and concentrated culture filtrate antigens reacted positively and specifically with the sera of clinically infected pigs (Schuman & Wood, 1966 & 1967; Schuman, Wood & Nord, 1967).

Timoney and Berman (1970) demonstrated specific antibody to E. insidiosa in pig tissues and in smears from positive control mice spleens, employing a freeze-thaw extracted antigen. In their work, however, the rabbit antiserum was conjugated with FITC, unlike here where an extra "sandwich" layer was added to obtain fluorescent staining. The technique is therefore open to the criticism of complexity which must be balanced against its increased potential sensitivity. Although there are a number of possible causes for the failure of the method, the initial process of fixation is probably the most critical since antibody specificity could be radically damaged at this stage by unsuitable fixatives, thereby precluding the combination of antigen with tissue antibody upon which the technique is dependent. Cold acetone fixation was however successfully used by Timoney and Berman (1970) with their freeze-thaw antigen.

The failure to detect a serological response in terms of circulating antibody by a number of techniques made further investigation by the immunofluorescent method inappropriate at the present time employing Strep. suis as antigen. It may have been that insufficient time was allowed for the development of a detectable antibody response, but, on the other hand, Schuman and Wood (1966) were able to detect precipitins to Group B streptococci with culture filtrate antigen by day 25 post-infection. Alternatively, antibody activity in the terminal sera may have waned: however, Agarwal et al. (1969) postulated the bacteriocidal activity of adult serum to be stimulated by infection early in life, implying a persistent antibody response.

In conclusion, the investigations described here, both serological and immunofluorescent, proved completely negative for the demonstration of antibody to Strep. suis in hyperimmunised animals; neither were specific immunoglobulin classes shown in tonsil or spleen. It was therefore not possible to link the IgM factor described by Agarwal et al. (1969) with a specific antibody stimulated by Strep. suis infection.

CHAPTER X

GENERAL DISCUSSION

The palatine tonsils have been shown in this thesis not to be involved in the lymphatic drainage of the regional tissues but there exists an afferent route to the tonsillar lymphoid tissue via the crypt system and its lining epithelium. The tonsils are therefore open to the vast diversity of foreign matter which enters the oral cavity. Exposure is enhanced by the location of the porcine tonsils in the soft palate, allowing direct passage of ingesta over the oral surface of the organs. In addition, a feature of the tonsils created by the numerous crypts ramifying within the lymphoid tissue is their vast surface area, providing for substantial intimate contact with material entering the crypts. It therefore seems logical that tonsillar function should be linked to the environment of the oral cavity. If not, then the specialised nature of the phagocytic tonsillar crypt epithelium with its structural reticulation permitting, and perhaps actively aiding, passage to the lymphoid substance becomes redundant.

Numerous diverse antigens are undoubtedly present within the oral cavity and therefore may gain access to the tonsils. In some respects, the tonsils may be compared to lymph nodes, the epithelial relationship playing a key role in the afferent route, although some other significance in view of the still undetermined function of the alimentary lympho-epithelial tissues cannot be dismissed. Throughout the body, however, the lympho-reticular tissues form an important part of the body defence mechanisms through the clearance of foreign

matter and by the elaboration of immune responses to antigens, especially where these are pathogenic. That the tonsils are intimately concerned in defensive and immunological activity is therefore an obvious inference since they are lymphoid organs constantly exposed to antigens. Evidence in favour of this may be drawn from the ontogenic study from which it is apparent that germinal centres and plasma cells appear in post-natal life. This can be readily interpreted to mean that activity of the lymphoid tissue is stimulated by oral antigens when exposure to these commences at birth. Since the tonsils are linked by efferent lymphatics to the lymphoid system as a whole, it seems improbable that they function independently, but that instead the tonsillar drainage lymph nodes supplement the tonsillar response to antigenic exposure.

The potential significance of the tonsillar afferent route lies in the evocation of immunological activity in the lymphoid parenchyma thus exposed to oral antigens. The possibility of antigen sampling and elimination by the tonsils has been previously discussed: controlled exposure to antigen may therefore occur. This may provide a mechanism whereby cellular and circulating antibodies and "committed" lymphocytes are continually produced, creating the basis of immunity to future encounters with the same antigens anywhere in the body. Some evidence to support this theory has already been produced (Koburg, 1970). The whole question, however, of tonsillar exposure and response demands considerable further detailed study before these aspects can be fully elucidated.

Having demonstrated the afferent route to the tonsils, thereby linking their function to the environment of the oral cavity, this

work was directed at investigating the role of the tonsils in the pathogenesis of Strep. suis infection of piglets. Oral infection leads to the establishment of the organisms in the tonsillar crypts, to penetration through the epithelium into the lymphoid parenchyma and passage to the drainage lymph nodes. The tonsils, therefore, act as a portal of bacterial entry. Invasion alone, however, is not the criterion for the expression of pathogenicity because these events may be independent of the development of clinical disease in piglets of a susceptible age. Neither is resistance to overt disease accounted for solely by the factor described by Agarwal et al. (1969) since the piglets in the first streptococcal experiment were shown to lack serum bacteriocidal activity\*. Although beyond this point it becomes a purely hypothetical exercise to attempt to explain the resistance of animals of a susceptible age, bacterial multiplication in the crypts and the total weight of tonsillar crypt infection may be critical factors in susceptibility. Certainly, the impression was gained that organisms were more numerous in the crypts of the diseased piglet PM34/3. Competition with the natural flora of the tonsils may also be an important factor in determining the degree of replication in the crypts.

The theory that the immunity of older pigs may develop through inapparent infection in early life (Agarwal et al., 1969) remains unsubstantiated following the negative results of the investigation into serological and cellular responses to immunisation with Strep. suis. The establishment and persistence, however, of the organism in the tonsillar crypts of older animals after oral infection does suggest the possibility that immunity may be stimulated and maintained by continued exposure to antigen from this source.

\*Bacteriocidal tests performed by Dr. G. H. K. Lawson, Department of Veterinary Pathology, Edinburgh University.

Inapparent infection of pigs and the carrier state in convalescent animals are important in an epidemiological context since reservoirs of Strep. suis will be created by tonsillar infection. Carrier sows are likely to transmit the organism to their litters or to other pigs, such horizontal spread being significant of course only in the breeding herd.

This work on the palatine tonsils and their role in the pathogenesis of Strep. suis infection provides merely an introduction to studies at the cellular level of the pathogenesis of bacterial disease following oral infection and a number of areas are suggested for investigation. The possibility that reservoirs of antigen form in the tonsillar crypts, maintaining stimulation of immunological activity, should be studied. The phenomenon of phagocytosis by the tonsillar crypt epithelium requires a quantitative approach to establish its significance as part of the tonsillar afferent route. A dynamic study of passage through the epithelium is indicated, in relation to the theory of antigen sampling and elimination. The nature of the tonsillar lymphoid tissue response merits examination employing antigens known to stimulate detectable and preferably measurable parameters of immunological reaction, for which Strep. suis proved an unsuitable model. In this area, the postulated production of lymphocytes "committed" to oral antigens reaching the lymphoid parenchyma is an aspect, perhaps the most important, worthy of detailed study. Indeed, should the immunological function of the palatine tonsils, and perhaps also of other lympho-epithelial tissues, be conclusively demonstrated, it would provide a theoretical basis for oral immunisation procedures.

In relation to Strep. suis infection, the nature of resistance

to overt disease remains obscure. It may prove that age resistance is a non-specific phenomenon: the bacteriocidal factor (Agarwal et al., 1969) therefore requires to be tested against other streptococci and unrelated bacteria. It would be interesting to determine whether this factor affects the distribution of Strep. suis. Finally, although the tonsils have been demonstrated as portals of bacterial entry, local environmental factors operating in the tonsils may be significant causes of variation in susceptibility to clinical disease and it is in this area that further studies may prove rewarding.

## APPENDIX I

The aim of the experiment briefly described here was to establish in piglets inapparent infection with Strep. suis and subsequently to examine for evidence of immunological response to the organism.

### Materials and Methods

Seven minimal disease piglets, 10 days of age, PM34/10 - 16, were infected orally (as in Chapters VII & VIII) with 1ml of a  $10^{-1}$  dilution in HSB of a 20 hour HSB culture of Strep. suis, giving  $25 \times 10^6$  colony-forming units per infective dose. Four piglets, PM34/17 - 20, litter-mates of the seven, were housed separately as non-infected control animals.

From day 1, all piglets were examined daily, rectal temperatures noted, tonsillar swabs taken on alternate days and blood samples daily for cultural examination.

### Results

Rectal temperatures and the recovery of Strep. suis from tonsillar swabs and blood samples are presented in Table A1 and Table A2.

All seven infected piglets yielded Strep. suis from the tonsillar swabs at the first sampling post-infection and by day 4 all had subsequently become bacteraemic and pyrexic. Sampling and work on existing samples was discontinued at the onset of bacteraemia, since this invalidated the primary objective which was to establish inapparent infection localised within the palatine tonsils and their

drainage lymph nodes.

The control piglets did not yield Strep. suis either from the tonsillar swabs or from the blood samples, but isolates were obtained which cross-reacted with Strep. suis in the capillary microprecipitin test. They produced, however, only a very weak and slow reaction with the PM34/A antiserum and were shown to be unrelated to Strep. suis as absorption removed cross-reacting antibody whilst retaining the specific activity against Strep. suis.

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